ROLE OF HEMODYNAMIC SHEAR STRESS ABNORMALITIES IN CALCIFIC AORTIC VALVE DISEASE

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by

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Abstract

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The aortic valve (AV) ensures unidirectional flow between the left ventricle and the aorta by opening during cardiac systole and closing during diastole. As compared to the normal tricuspid aortic valve (TAV) anatomy, which consists of three leaflets, the bicuspid aortic valve (BAV) is characterized by the presence of two leaflets, and is the most prevalent congenital cardiac anomaly. Regardless of the valve morphology, a common cause of valvular failure is calcific aortic valve disease (CAVD), a condition characterized by increased thickness and stiffness on the valve leaflets. Historically, CAVD has been considered a passive degenerative disease but is now recognized as an active pathology involving inflammation, remodeling and ossification and presumably triggered by atherogenic risk factors and hemodynamic cues. While TAV and BAV calcification seem to share common biological pathways, the calcification of the BAV is more rapid and severe than the TAV.

As evidenced by recent studies, the valve interacts closely with its surrounding hemodynamic environment to maintain valvular homeostasis by altering the biosynthetic
behavior of valve cells. Physiologic fluid shear stress (FSS), the frictional fluid force acting on the leaflet endothelium, contributes to valvular homeostasis by regulating valvular protein expression, glycosaminoglycan and DNA synthesis and endothelial cell alignment. In contrast, FSS abnormalities have been shown to promote endothelial activation and leaflet inflammation. Supported by those observations, the central hypothesis of this thesis is that hemodynamic FSS abnormalities contribute to CAVD development by regulating AV inflammation, extracellular matrix remodeling and valvular osteogenesis. Therefore, the overall goal of this work is to characterize the role of FSS abnormalities on early progression of CAVD in both TAVs and BAVs. Specifically, this thesis will address the following research questions: 1) What are the mechanisms by which FSS alterations are transduced into valvular pathological responses? 2) Are BMP-4 and TGF-β1 potential target molecules for the pharmacological treatment of CAVD? and 3) What are the reasons for the early development and severity of BAV calcification? These questions will be addressed via three specific aims. 1) To elucidate the mechanisms of CAVD secondary to FSS magnitude & frequency abnormalities; 2) To investigate the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling; and 3) To elucidate the mechanisms of CAVD secondary to BAV hemodynamic abnormalities. The approach integrated the implementations of a novel ex vivo device to condition porcine AV leaflets under physiological and pathologically FSS environments, standard biological techniques to assess valvular biological response and pharmacological inhibition/promotion to elucidate the mechanisms of FSS signal transduction.

As a first step, a FSS bioreactor was designed to replicate the native side-specific valvular FSS environment in the laboratory setting. The bioreactor was validated
mechanically using fluid structure interaction modeling and biologically in terms of sterility and tissue viability. The programming of the bioreactor with both physiological and pathological waveforms permitted to investigate the role of FSS in valvular pathogenesis.

Specific aim 1 sought to elucidate the modes of FSS mechanotransduction in AV tissue and the respective role of FSS magnitude and/or frequency abnormalities in CAVD. Porcine leaflets were subjected to 9 FSS environments defined by isolated/combined abnormalities in FSS magnitude and frequency. While cytokine expression was stimulated under elevated FSS magnitude at normal frequency, ECM degradation was stimulated under both elevated FSS magnitude at normal frequency and physiologic FSS magnitude at abnormal frequency. In contrast, combined FSS magnitude and frequency abnormalities essentially maintained valvular homeostasis. The results revealed the particular sensitivity of valvular tissue to FSS magnitude as opposed to FSS frequency.

In specific aim 2, the goal was to determine the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling. The methodology involved the *ex vivo* exposure of porcine aortic valve leaflets to side-specific physiological/pathological FSS and the use of pharmacological promoters/inhibitors of BMP-4 or TGF-β1. The results revealed the important role played by TGF-β1 in valvular remodeling through MMP-9 regulation.

Specific aim 3 investigated how BAV hemodynamic FSS abnormalities contribute to early progression of CAVD. Porcine leaflets were exposed *ex vivo* to the native TAV and type-1 BAV FSS and their acute pathological response was examined, including endothelial activation, pro-inflammatory paracrine signaling, VIC activation and
osteogenesis. The results revealed the pathogenic potential of the native BAV hemodynamic environment and the particular vulnerability of the fused BAV leaflet to calcification.

In conclusion, the contribution of FSS to the onset and early progression of CAVD has been demonstrated in this thesis work. The relationship between FSS magnitude/frequency and valvular responses can be used as input for prediction of CAVD progression. In addition, the findings from this dissertation can be used to further investigate the molecular signaling mechanisms in order to develop pharmacological treatments of CAVD.
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Chapter 1:  
INTRODUCTION

This chapter presents the background and rationale for this thesis research. It presents a literature review of the anatomy of the heart, the aortic valve, AV disease and current treatment options. Then there is an overview of AV hemodynamic, mechanobiology and current knowledge gaps.

1.1 Cardiac function and heart valves

The heart is a key component of the cardiovascular system whose function is to pump blood to the body. This organ consists of two pumps connected in series, each consisting of an atrium and a ventricle. The left and right atria are entry-points into the heart, while the left and right ventricles are responsible for blood ejection toward the systemic and pulmonary circulations. In the systemic circulation, blood leaves the heart through the aorta, goes to all the organs of the body through the systemic arteries and then returns to the heart through the systemic veins. In the pulmonary circulation, blood leaves the heart through the pulmonary arteries, goes to the lungs and returns to the heart through the pulmonary veins.

The heart has four valves (Figure 1.1) that maintain unidirectional flow [1]. These valves can be categorized into two types based on their anatomical location: atrioventricular valves (mitral and tricuspid valve) and semilunar valves (aortic and
pulmonary valve). The atrioventricular valves are anchored to the wall of the ventricles by chordae tendineae. The mitral valve allows the blood to flow from the left atrium into the left ventricle. The tricuspid valve regulates the passage of blood between the right atrium and the right ventricle. The semilunar valves do not have chordae tendineae and are more similar to venous valves. The AV lies between the left ventricle and the aorta and the pulmonary valve lies between the right ventricle and the pulmonary artery.

Figure 1.1: A schematic diagram of heart and heart valves: a) heart anatomy; b) valvular function during diastole and c) valvular function during systole [2].

The cardiac cycle consists of two phases, diastole and systole. During diastole, deoxygenated blood from the body returns to the heart through superior and inferior vena cava to fill right atrium. The blood then travels through the tricuspid valve into the right ventricle. In the other side, oxygenated blood from pulmonary veins returns to left atrium, and the blood flows through mitral valve into left ventricle. During systole, the right ventricular chamber pressure rises rapidly until it exceeds atrial pressure (7 mmHg) (Figure
1.2a) and the pulmonary valve opens allowing blood to flow through the pulmonary artery to the lungs. In the left ventricle during systole, the pressure must exceed the pressure in the aorta (80mmHg) which allows the AV to open. As the ventricle begins to relax the pressure falls rapidly, causing the AV to close (Figure 1.2b).

Figure 1.2: Pressure curves in the cardiac cycle: a) pressure in the right ventricle and pulmonary artery b) pressure in the left ventricle and aorta [3].

1.2 AV function and anatomy

The AV is located at the junction between the left ventricle and the aorta (Figure 1.1). The valve opens and closes in response to the pressure gradient generated between the left ventricle and the aorta to ensure unidirectional flow from the heart toward the systemic circulation [4,5]. During systole (ventricular contraction phase), the AV opens fully to ensure unobstructed and unidirectional blood flow from the left ventricle to the aorta while minimizing ventricular workload [6]. Conversely, during diastole (ventricular

3
relaxation phase), the valve closes completely to prevent backflow from the aorta toward the left ventricle.

The normal AV consists of three semi-lunar leaflets (Figure 1.3a) attached to an annulus [7], which consists of a ring of fibrous tissue located at the base of the leaflets that provides support and maintains the circular shape of the valve. The aortic root bulges outwards to form three sinuses. The right- and left-coronary aortic sinuses are connected to the main coronary arteries. The third sinus is referred to as the non-coronary aortic sinus [8]. The three valve leaflets are referenced according to their anatomic positions in relation to the sinus and are connected at commissures, which are the peripheral parts of the zones of apposition between the leaflets (Figure 1.3c). The normal AV is referred to as the tricuspid aortic valve (TAV).
The bicuspid aortic valve (BAV) is the most common congenital cardiac anomaly and is present in 2-3% of the general population [9]. While a normal TAV consists of three functional leaflets, a BAV is formed with only two (Figure 1.4a and b) and typically features a hypoplastic commissures (raphes) between the two partially fused leaflets. Three major types of BAV anatomies were identified by Sievers: type 0 (no raphe), type 1 (one raphe), and type 2 (two raphes), as shown in Figure 1.4c [10,11]. The type-1 BAV has three subtypes, with the most common morphology subtype (type 1A, 74-79%) resulting from
the fusion of the right- and left-coronary leaflets and featuring a central fibrous raphe at the site of leaflet fusion [11-13]. The two other subtypes are right- and non-coronary fusion (type-1B, 15-24%), left- and non-coronary fusion (type-1C, 2-3%) [14,15].

Figure 1.4: BAV anatomy and types: a) schematic diagram, b) picture of BAV and c) BAV types and the position of the raphe on the valve [10,11].

1.3 Leaflet structure

Regardless of the valve anatomy, AV leaflets consist of three distinct layers: 1) the fibrosa, which has a corrugated surface and faces the aorta; 2) the ventricularis, which is smooth and faces the left ventricle; and 3) the spongiosa, which is located between the fibrosa and the ventricularis (Figure 1.5). The fibrosa consists mainly of circumferentially
aligned collagen fibers including collagen I, III, and V [5]. The particular architecture of the fibrosa makes the valve stiffer in the circumferential direction than in the radial direction [16]. The ventricularis mainly consists of elastin fibers and some interspersed collagen, which makes the ventricularis more extendable than the fibrosa [17,18]. Lastly, the spongiosa consists of loose, watery connective tissue containing mainly glycosaminoglycans (GAGs). These long, multi-chain proteins bind water readily and give the spongiosa a gelatinous, watery consistency [19]. A recent study has suggested that GAGs may be important in providing a damping mechanism to reduce leaflet flutter when the leaflet is not under high tensile stress [20].

![Histological cross section of an AV leaflet (Hematoxylin and eosin stain).](image)

Valvular cells play an important role in the maintenance of valvular function [21]. The cells populating the leaflets are broadly categorized as: 1) valve endothelial cells (VECs); and 2) valve interstitial cells (VICs, Figure 1.5).
VECs are located on both sides of the leaflets and are responsible for maintaining a non-thrombogenic blood contact surface and for transmitting biochemical and mechanical signals to the VICs [22]. VECs express von Willebrand factor, endothelin-1 and nitric oxide, which play important roles in homeostasis [23,24]. Gene expression profiles have been shown to be different between the VECs on the fibrosa surface and those on the ventricularis surface. For example, multiple inhibitors of cardiovascular calcification (e.g., OPG, CNP, PTH and chordin) are significantly less expressed by the fibrosa endothelium than the ventricularis endothelium [25,26]. This may explain why calcific pathology predominantly develops on the fibrosa of the AV rather than on the ventricularis [27].

VICs populate the bulk of the leaflets and form an integral network along with the extracellular matrix (ECM). These cells play a key role in regulating the mechanical properties of the AV. Three cellular phenotypes have been identified in the VIC population: myofibroblasts, fibroblasts and smooth muscle cells (SMCs) [21,28-30]. The myofibroblasts account for a large percentage of VICs and have characteristics of both fibroblasts and SMCs. The myofibroblast phenotype is characterized by prominent stress fibers associated with α-smooth muscle actin (α-SMA) and is also involved in cell proliferation, migration and ECM remodeling [30]. Fibroblasts are responsible for synthesizing ECM components that are critical for the structural support of the tissue. Lastly, SMCs promote the contractile properties of the valve. In a more recent study, Liu et al suggested five phenotypes to better describe VIC function: embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), progenitor VICs (pVICs), and osteoblastic VICs (obVICs) (Figure 1.6) [31]. In fetal
development, embryonic progenitor endothelial/mesenchymal cells undergo endothelial-mesenchymal transformation to give rise to aVICs and/or qVICs in the normal AV. The aVIC phenotype has two other sources: one is activated from qVICs when the AV is subjected to an abnormal hemodynamic/mechanical stress or pathological injury, and the other is activated from pVICs including bone marrow-derived cells, circulating cells, and resident valvular progenitor cells are another source of aVICs. In promoting valve calcification, qVICs undergo osteoblastic differentiation into obVICs, which actively participate in the valve calcification process [31].

![Figure 1.6: VICs five phenotypes: embryonic progenitor endothelial/mesenchymal cells, qVICs, aVICs, pVICs, obVICs [31].](image)

1.4 AV disease

The majority of valvular diseases (63%) involve disorders of the AV [32]. AV disease covers all the spectrum of dysfunctions or abnormalities of the aortic valve. About five million Americans are diagnosed with AV disease each year. According to the American Heart Association Statistical Update, AV disease is responsible for nearly 20,000
deaths each year in the United States and is a contributing factor in about 42,000 deaths. The two types of AV disorders are stenosis and regurgitation.

1.4.1 AV stenosis

AV stenosis is a condition characterized by the narrowing, stiffening, thickening, fusion or blockage of the AV (Figure 1.7a). As a result, a stenotic AV generates increased resistance to blood flow. Many patients with moderate aortic stenosis do not present any clear symptoms and their conditions are frequently diagnosed as a heart murmur. When aortic stenosis becomes severe, symptoms such as shortness of breath, chest pain and dizziness may occur. The presence of significant AV stenosis results in left-ventricular pressure overload. Due to the increased pressure, the myocardium of the ventricle undergoes hypertrophy [33], which may lead ultimately to heart failure.

![Figure 1.7: AV disease: a) stenosis and b) regurgitation [34].](image-url)
1.4.2 AV regurgitation

AV regurgitation or incompetence is a condition in which blood leaks back to the ventricle at the end of systole because of incomplete AV closure. The nature and severity of the leakage may keep the heart from ejecting an adequate amount of blood through the AV (Figure 1.7b). AV regurgitation may result from rheumatic fever, congenital causes or aortic enlargement. Enlargement of the aorta can stretch the valve leaflets and produce AV regurgitation. The acute onset of AV regurgitation can also occur when there is an infection of the AV or a tear in the aorta [1]. AV regurgitation is characterized by a large increase in left ventricular pressure (160 mmHg) and left atrial pressure (25 mmHg) [35]. The backward flow of blood into the ventricular chamber during diastole results in a diastolic murmur.

1.4.3 Treatments for AV disease

Treatments for AV disease can be categorized into 1) valve replacement; 2) valve repair; and 3) Ross procedure.
Since the first successful heart valve replacement in 1952, over 4 million (300,000 annually) prosthetic replacement surgeries have been performed worldwide and over 50 artificial heart valve implants have been designed [36,37]. These designs are categorized into three types: mechanical (Figure 1.8a), bioprosthetic (Figure 1.8b-d) and transcatheter valves (Figure 1.8e-f) [38]. Bileaflet mechanical valves consist of two rigid leaflets pivoting about two hinges located on an artificial annulus. While they offer good durability, these implants are thrombogenic and require administration of lifelong anticoagulation therapies [39,40]. Bioprosthetic valves consist of three porcine or ovine tissue leaflets and
can be categorized into stented (Figure 1.8b and 1.8c) and stentless (Figure 1.8d) bioprostheses, both of which are associated with excellent clinical and hemodynamic outcomes [41]. While the deforming leaflets provide a non-thrombogenic alternative to the mechanical bileaflet valve, they progressively lose structural integrity, which dramatically limits their durability to 10-15 years [42-45]. Developed in the last decade, transcatheter valves consist of bioprosthetic valves mounted on an inflatable balloon and crimped in a catheter for implantation through the femoral artery. While they avoid the need for invasive open heart surgeries, they face the same durability problems as bioprostheses valves (10-15 years) and their implantation requires good blood vessel quality [46-49].

AV repair has been performed since the early days of open-heart surgery and aims at preserving normal leaflet mobility while restoring normal geometry to both the aortic annulus and the leaflets [50]. AV repair includes separating fused valve flaps, removing or reshaping tissue so the valve can close tighter and adding tissue to patch holes or tears or to increase support at the base of AV. Many AV repair surgeries have been developed specifically to treat AV regurgitation caused by root dilatation or dissection, which cannot easily be fixed by using a prosthetic replacement.

Lastly, the Ross procedure consists of replacing the patient’s diseased AV with his/her own healthy pulmonary valve and placing a valve prosthesis in the pulmonary position (Figure 1.9). This procedure offers several advantages over the more traditional aortic valve replacement with a manufactured prosthesis. For example, anticoagulation is not required as with mechanical valves, because prosthesis works under low pressure pulmonary circulation instead of the aortic high pressure system. Thus, individuals can lead an active life without the risks associated with anticoagulation therapy.
Figure 1.9: The Ross procedure: a) the pulmonary valve and a segment of the pulmonary artery are excised, b) diseased AV and proximal tissue is removed, leaving the right and left coronary arteries with only a button of tissue; and c) the pulmonary autograft is placed in the aortic position and the buttons of tissue on both the right and left coronary arteries are then sewn into that pulmonary segment and closed. A pulmonary valve and artery homograft is then replaced in the pulmonary position to replace the excised pulmonary segment [51].

1.4.4 Limitations of current treatment

Although tremendous progress has been made in the effectiveness of AV replacement, the current treatment strategies have their own limitations, such as vulnerability to blood clot formation, limited durability and post-operative complications (stroke, heart attack, infection and arrhythmias) associated with the need for invasive open-heart surgery or reoperation. AV repair is technically difficult and is only an option for leaky aortic valves. For both AV repair and the Ross procedure, not all patients are suitable candidates, especially those who are older and have other health conditions. Another major disadvantage of the Ross procedure is that it is a very technically demanding to replace two valves instead of one.
1.5 Calcific aortic valve disease (CAVD)

1.5.1 Disease presentation

CAVD accounts for all AV stenosis cases and is a less common cause for chronic regurgitation [52]. CAVD is characterized by structural changes such as increased thickness, stiffness and calcification of the leaflets. While 25% of individuals over the age of 65 and 48% of individuals over 85 are affected by CAVD, 2% of individuals over 65 and 4% of individuals over 85 develop severe forms of calcific lesions [53-55]. Although CAVD has been associated to different etiologies in the western world and developing countries [56], its pathophysiology is invariably characterized by the formation of calcific nodules on the surface of the leaflets that contributes to the progressive obstruction of the left ventricular outflow and leads ultimately to heart failure [54,57,58]. Risk factors associated with the presence of CAVD include older age, male sex, serum lipoprotein and LDL levels, height, hypertension, metabolic syndrome, and smoking [59].

The early stage of the disease is characterized by AV sclerosis (Figure 1.10a), which consists of cusp thickening without creating obstruction to the left ventricular outflow [60]. In the late stages of the disease, AV stenosis (Figure 1.10b) leads to an increased pressure gradient (>100 mmHg) between the left ventricle and the aorta. The left ventricle remodels in order to compensate for the pressure overload, which results in left ventricular hypertrophy and progressive decline in systolic function. Rapid progression of AV stenosis is defined as an increase of maximal transvalvular pressure across the valve of at least 10 mmHg per year [212].
1.5.2 CAVD pathogenesis

Historically, valvular calcification has been described as the result of two pathways: the dystrophic and osteogenic calcification pathways. The pathogenesis of dystrophic calcification involves apoptosis [61,62], necrosis [63] and propagation, all of which may be either intracellular or extracellular. The end-product is the formation of crystalline calcium phosphate. Initiation of intracellular calcification occurs in the mitochondria of dead or dying cells that are unable to regulate intracellular calcium, then propagation of crystal formation occurs [64,65]. In contrast, instead of deriving from degenerating cells, osteogenic calcification is considered as the progressive calcification of the valve follows an active process involving endothelial inflammation [5,66,67], ECM remodeling [68-70] and osteogenesis [57,71-74] (Figure 1.11). Valvular inflammation, which is the hallmark of the early stage of CAVD, has been linked to the activation of the leaflet endothelium via enhanced expression of vascular cell and intercellular adhesion molecules [75-78]. Elevated expressions of pro-inflammatory cytokines such as bone morphogenic proteins (BMPs) [79,80] and transforming growth factor-β1 (TGF-β1) [61,81] have also been
observed in early calcific lesions, demonstrating the key role played by paracrine signaling in CAVD development. Downstream of those events, the VICs switch from a quiescent fibroblastic phenotype to an activated myofibroblastic phenotype expressing α-SMA, desmin and vimentin or to an osteoblastic phenotype marked by runt-related transcription factor 2 (Runx2) [30,31,69,72]. Those activated phenotypes result in the progressive loss of valvular homeostasis caused by the upregulation of matrix metalloproteinases [82,83] and cathepsins [84-86], and in the formation of calcific lesions marked by the upregulation of bone matrix proteins [87-89]. In addition, the osteogenic Runx2/Cbfa1 pathway may be activated by specific genetic abnormalities (NOTCH1). Following their activation by Runx2, fibroblasts differentiate into valvular myofibroblasts with an osteoblast-like phenotype and express osteopontin, a protein involved in bone formation [90]. Osteopontin mRNA expression correlates with the degree of valvular calcification. Besides, alkaline phosphatase (Alk phos) activity is also involved in the development of calcific nodules and bone formation [91].
1.5.3 Potential key molecules

As described above, BMPs, TGF-β, MMPs and cathepsins mediate many of the molecular pathways involved in CAVD. Those molecules are described in details in the following section.

BMPs are pro-inflammatory cytokines, members of the TGF-β super family. BMP-4 is involved in shear-induced inflammation and capable of activating endothelial nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, and reactive oxygen species (ROS) production [93]. NADPH oxidase is a membrane-bound enzyme
complex, which can produce ROS. ROS production is known to activate the smad- and NFκB-pathways, which are responsible for increasing expression of inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which promotes monocyte adhesion to endothelial cells, a critical step in inflammation [93]. TGF-β1 is a polypeptide member of the TGF-β super family and is a secreted protein that controls many cellular functions, including ECM synthesis and controls cell growth, proliferation, differentiation and apoptosis [94]. TGF-β1 has been shown to promote ECM remodeling in porcine valve and VIC differentiation into active myofibroblasts in a dose-dependent manner, as determined by a significant increase in α-SMA [95]. Given their high binding affinities, noggin and SB-431542 are the ideal molecules to investigate the specific dependence of the FSS-induced inflammatory pathway on the cytokines BMPs and TGF-β1, respectively. Noggin is a well-known BMP antagonist that binds to BMPs with high affinity and prevents its downstream action [96-99]. The small molecule inhibitor SB-431542 inhibits specifically the TGF-β type-1 receptor and will block any signaling pathway triggered by the binding of TGF-β1 to its receptor [99,100].

ECM remodeling in CAVD consists not only of extensive fiber accumulation but also of the breakdown of matrix components through degradation and disorganization of collagen and elastin fibers. A perturbation of the equilibrium between fiber accumulation and degradation can lead to pathological ECM remodeling and compromised valvular function. The disorganization of collagen bundles and the fragmentation of elastin fibers contribute to valvular dysfunction and loss of leaflet elasticity, which promotes to the development of CAVD via the upregulation of MMPs, their tissue inhibitors (TIMPs) and cathepsins [68,101-103]. MMPs are a family of zinc- and calcium-dependent enzymes that
are mainly produced by inflammatory cells and can degrade collagen, elastin fibers and proteoglycans [104]. MMP activity is mediated mainly by binding with endogenous TIMPs. The local balance between MMP and TIMP expression and activity regulates the extent of tissue remodeling. MMP1, 2, 3, 9 and TIMP1, TIMP2 have been shown to be involved in the pathogenesis of CAVD [68,80,103]. In comparison to fresh valves, the expression levels of MMP1, 2, 3 and 9 are increased in calcified valves [68]. It has been demonstrated that cytokines such as TNF-α can contribute to cell proliferation and increased expression of MMPs and cathepsins in diseased valves [102].

Cathepsins L and S are potent elastolytic proteases that have been associated with atherosclerotic plaque progression [105] and dysfunctional heart valves [85]. They can efficiently degrade elastin and collagen fibers [106]. A recent study by Helske et al revealed that cathepsin S is the sub-type that is upregulated in stenotic AVs [84].

The areas of calcification co-localize with areas of oxidized LDL accumulation and inflammatory cell expression. As shown in vitro, oxidized LDL and cytokines produced by T-lymphocytes stimulate calcified nodule formation by valvular fibroblasts through pathways activated by BMPs [107]. BMPs are present in human AV lesions [82] and promote osteogenesis through the Wnt/Lrp5/β-catenin pathway [108] or Runx2/Cbfa1 pathway [109]. In the Wnt/Lrp5/β-catenin pathway, secretion of Wnt3a leads to the formation of the Lrp5/Wnt3a/Frizzled receptor complex, which increases β-catenin expression. When β-catenin enters into the nucleus, it activates transcription factors associated with bone formation.
1.6 Valvular hemodynamics

Under physiologic conditions, the flow through the valve is pulsatile and unidirectional with a mean cardiac output of 5 L/min. During diastole, the AV experiences a transvalvular pressure of 80 mmHg acting perpendicular to the leaflet (Figure 1.12a). During systole, the valve opens when the ventricular pressure becomes larger than the aortic pressure (transvalvular pressure ~4 mmHg) (Figure 1.12b).

Figure 1.12: Overview of AV hemodynamics: a) during diastole, the transvalvular pressure of 80 mmHg imposes bending and tensile stretch on the leaflets; and b) during systole, the blood forms a central jet and recirculation occurs behind the leaflets.
Flow studies in tricuspid polymeric valves have demonstrated the time-periodic nature of the flow, its transition to turbulence at peak systole and the presence of strong interactions between the blood and the leaflets [111,112]. As demonstrated by velocity fields (Figure 1.13), ventricular contraction results in the formation of a jet aligned along the long axis of the valve, and recirculation regions located in the aortic sinus behind each leaflet [110]. In type-1 BAV, the asymmetry of the valve generates a jet skewed toward the non-coronary leaflet, which is associated to increased vorticity both in the shear layers and in the non-coronary sinus, as revealed by flow visualization [113-115], radiography [116,117] and magnetic resonance imaging [118-120].

The AV functions in a complex mechanical environment, which includes pulsatile pressure, bending and tensile stresses, and FSS (Figure 1.12) [111,121]. The transvalvular...
pressure (80 mmHg) imposed on the leaflets during diastole generates large bending and
tensile stresses, and stretches the leaflets by up to 11% in the circumferential direction and
31% in the radial direction [5]. Resulting from the relative motion between the deforming
leaflets and the surrounding blood flow, FSS is the dominant component of the valvular
mechanical environment during systole. An interesting characteristic of the valvular FSS
is its side-specificity. Computational and experimental flow characterizations through the
TAV have revealed that the ventricularis experiences a high unidirectional pulsatile FSS
varying between 0 and 39 dyn/cm², while the fibrosa is exposed to a low bidirectional
oscillatory FSS ranging from 1.1 to 0.4 dyn/cm² (Figure 1.14a) [114,115,122]. In the BAV,
the FSS predicted in the base of the ventricularis of the fused and non-coronary leaflets
consist of pulsatile (i.e., positive) waveforms varying between 0 and 11 dyn/cm² and 0 and
78 dyn/cm², respectively. In contrast, the FSS predicted in the base of the fibrosa of the
same leaflets consist of oscillatory (i.e., alternatively positive and negative) waveforms
ranging from -4.5 to 1.1 dyn/cm² and -0.4 to 1.8 dyn/cm², respectively (Figure 1.14b) [114].

Figure 1.14: Predictions of the native side-specific FSS variations
on a)TAV leaflets and b) type-1 BAV leaflets (F: fibrosa; V:
ventricularis) [114].
1.7 AV mechanobiology

The AV functions in a complex mechanical environment including pulsatile pressure, bending and tensile stresses, and FSS. Recent studies indicate there may be an interplay between the hemodynamic environment and biological response of the valve.

1.7.1 Effects of pressure on AV biology

The pressure imposed on the AV leaflets varies throughout the cardiac cycle, thereby changing the bending and tensile stretch and the length of the leaflet. A significant increase in collagen synthesis was demonstrated under both steady and cyclic conditions at 140 mmHg and 170 mmHg, however this increase was not statistically significant at 100 mmHg [123]. In another study, the combination of high pressure magnitude (150–190 mmHg) and high frequency (2 Hz) resulted in proportional increases in collagen and GAG synthesis in porcine AV leaflets, while DNA synthesis remained unchanged [124]. A notable decline in α-SMA, a standard marker for VICs activation, was observed although no significant difference was observed between the hypertensive pressure and atmospheric control groups. These observations suggest the potential sensitivity of AV leaflets to abnormal pressure.

1.7.2 Effects of stretch on AV biology

Cyclic stretch is another mechanical stress experienced by AV leaflets. With increasing age, the valve tissue becomes less extensible. Multiple ex vivo studies have demonstrated that the level of stretch imposed on leaflet tissue could alter expression of cytokines, enzymatic activity, and protein biosynthesis in the AV [86,125-128]. It was reported that VICs respond to local tissue bending and tensile stretch by altering cellular
stiffness via collagen biosynthesis [129]. Balachandran et al. demonstrated that cyclic stretch was responsible for regulating the contractile phenotype of the valvular cells, with elevated stretch resulting in increased α-SMA expression and reduced sulfated glycosaminoglycan [86,126]. It was also reported that the presence of cytokines such as TGF-β1 in a cyclic stretch environment could result in altered matrix architecture and compromised AV function, underlining the importance of cyclic stretch in regulating valve structure, function, and disease progression [130].

1.7.3 Effects of FSS on AV biology

Previous studies indicate that FSS affects valvular biosynthetic activity, ECM remodeling and inflammation.

After exposure to steady or pulsatile FSS for 48 hours, protein, glycosaminoglycan and DNA synthesis in porcine leaflets increased during static incubation but remained at basal levels during continued exposure to flow [125]. The modulation of the synthetic activity was attributed to the presence of a FSS on the leaflet surface, which may have been transmitted to the VICs within the leaflet matrix through tensile forces.

FSS has also been shown to affect valvular ECM remodeling. Platt et al. demonstrated that exposure of leaflet tissue to a steady FSS of 25 dyn/cm² was able to induce ECM remodeling by increasing MMP-2 and MMP-9 activity and decreasing cathepsin L expression [131,132]. MMPs and cathepsins are known to play an important role in ECM remodeling and are considered early markers of CAVD. These results indicated the shear sensitivity of MMPs and cathepsins and their key role in flow-mediated valvular remodeling.
In addition, previous studies conducted in our laboratory have evidenced the existence of FSS-sensitive pro-inflammatory pathways and potential synergies between cytokines and cell adhesion molecules in the early valvular response to FSS alterations [133]. When fibrosa of the leaflet was exposed to native or ventricular FSS and the ventricularis was exposed to native or aortic FSS, there was an increased inflammatory response in terms of VCAM-1, ICAM-1, BMP-4 and TGF-β1 expressions on the fibrosa exposed to ventricular FSS (Figure 1.15).

As an extension of the previous study, the isolated effects of FSS magnitude on valvular inflammation were studied. As detected by immunohistochemistry, supra-physiologic FSS promoted endothelial activation (ICAM-1, VCAM-1) and increased cytokine expression (BMP-4, TGF-β1) on the fibrosa in a FSS magnitude-dependent manner (Figure 1.16). In contrast, neither physiologic nor sub-physiologic FSS elicited a
pro-inflammatory response. This study demonstrated the sensitivity of leaflet tissue to the surrounding FSS environment [134].

![Figure 1.16: Quantification of the pro-inflammatory and pro-osteogenic markers expressions after exposing the fibrosa to shear stress for 48 hours in standard culture medium (*p<0.05 vs. fresh; #p<0.05)](image)

1.8 Hemodynamic theory

While CAVD has been described historically as a passive degenerative process, it has now emerged as a highly regulated pathology presumably triggered by a combination of conventional cardiovascular risk factors [135], mechanical [136,137] and hemodynamic cues [26,138,139]. Recent evidence points to the existence of intimate interplays between the hemodynamic environment and valvular biology [53,135,140-142]. Physiologic FSS contributes to valvular homeostasis by regulating valvular biosynthetic activity [125,132] and endothelial cell alignment [143,144]. In contrast, FSS abnormalities have been shown to promote endothelial activation and leaflet inflammation [133,134], two precursor events to CAVD. Risk factors such as congenital valve defects, aging and hypertension...
[53,145,146] are also associated with hemodynamic alterations that may stimulate the development of calcific lesions. In fact, the BAV generates significant FSS abnormalities due to the presence of two functional leaflets instead of three [114] and stimulates the development of calcific lesions at a more rapid rate than in the TAV [147,148]. Aging and hypertension, which also promote CAVD pathogenesis, result in progressive alterations in leaflet matrix stiffness [149] and transvalvular flow rate [150], respectively [151].

The growing evidence for the existence of close relationships between the valve hemodynamic environment and valvular biology has motivated the formulation of a hemodynamic theory of CAVD (Figure 1.17) describing the hypothetical role played by the native valvular hemodynamics in the pathogenesis of CAVD [19,26,86,92,125,132-134,152-154]. According to this theory, CAVD would stem from valvular structural/hemodynamic alterations linked to aging mechanisms (e.g., leaflet stiffening) [55,91], congenital anatomical defects (e.g., BAV) [11,155] or flow abnormalities (e.g., hypertension) [156], which would lead in turn to the progressive alteration of the hemodynamic stress environment experienced by the valve leaflets. Abnormal stresses would be transduced by VECs and VICs into biological responses including inflammation, remodeling and ossification, which are precursor events to leaflet calcification [142,157-160]. Ultimately, the resulting stiffening of the leaflets caused by the presence of calcific lesions could cause further valvular structural abnormalities, which would amplify the pathological response.
1.9 Rationale for thesis research

The broad impact of CAVD justifies the need for advancing the science of this disease. Most valvular studies to date have focused on the exploration of palliative therapeutic strategies aimed at recovering valvular function without addressing the root cause of the disease. While tremendous progress has been made in the design of mechanical and bioprosthetic valves, valve replacements still face many challenges including surgical risks, implant durability and the need for lifelong anticoagulation treatments. Ideally, the next generation of therapeutic strategies should focus on non-invasive pharmacological modalities. The development of new medications requires the knowledge of the molecular pathways and biological mechanisms involved in AV pathogenesis, which unfortunately remains limited. The hemodynamic theory provides a new perspective on CAVD processes. The demonstrated sensitivity of valvular tissue to FSS and the potential role of
FSS in CAVD pathogenesis motivate the elucidation of the role played by native FSS abnormalities in CAVD pathogenesis. This dissertation will therefore focus on elucidating the effects of physiological and pathological FSS on the mechanisms of CAVD in TAV and BAV anatomies.
Chapter 2:

SPECIFIC AIMS

CAVD is the most common valvular heart disease. While tremendous progress has been made in the design and effectiveness of valvular implants, the replacement strategy is only palliative and does not address the root cause of the disease. The exploration of new therapeutic strategies focused on early detection and non-invasive intervention has been hampered by the limited understanding of CAVD pathogenesis. Preliminary studies have shown that endothelial activation may be triggered following exposure of the leaflet fibrosa to disturbed FSS and that the inflammatory mediators BMP-4 and TGF-β1 are key players in the FSS-induced response. The existence of FSS-sensitive pro-inflammatory pathways and synergies between cytokines and cell adhesion molecules have been evidenced in the early valvular response to high FSS magnitude. In addition, the BAV malformation, which causes high FSS magnitude on fused leaflet, has emerged as a major risk factor for CAVD [161-163]. However, some questions still remain unanswered: Q1) What are the mechanisms by which FSS alterations are transduced into valvular pathological responses? Q2) Are BMP-4 and TGF-β1 potential target molecules for the pharmacological treatment of CAVD? and Q3) What are the reasons for the early development and severity of BAV calcification? Supported by previous studies that have evidenced the sensitivity of leaflet tissue to the surrounding hemodynamics, the central hypothesis of this thesis is that hemodynamic FSS abnormalities regulate AV inflammation, extracellular matrix (ECM)
remodeling and valvular osteogenesis. This hypothesis was tested via the following three specific aims:

**Specific Aim 1:** To elucidate the mechanisms of CAVD secondary to FSS magnitude & frequency abnormalities.

**Specific Aim 2:** To investigate the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling.

**Specific Aim 3:** To elucidate the mechanisms of CAVD secondary to BAV hemodynamic abnormalities.

Specific aim 1 addressed Q1 by quantifying the pathological response of valvular tissue subjected to sub-physiologic/physiologic/supra-physiologic levels of FSS magnitude and/or frequency. Specific aim 2 addressed Q2 by characterizing the pathological response of AV leaflets subjected to physiological and pathological FSS following BMP-4 or TGF-β1 inhibition or promotion. Specific aim 3 addressed Q3 by comparing the biological response of valvular leaflets subjected to the native FSS experienced by TAV and type-1 BAV leaflets.

**Specific Aim 1: To elucidate the mechanisms of CAVD secondary to FSS magnitude & frequency abnormalities.**

Hemodynamic abnormalities are increasingly pointed as potential pathogenic contributors to CAVD onset and progression. *Ex vivo* studies conducted in our laboratory have evidenced the existence of FSS-sensitive pro-inflammatory pathways and potential synergies between cytokines and cell adhesion molecules in the early valvular response to FSS alterations. Despite the emerging evidence of the role played by FSS in valvular
pathology, the mode of FSS transduction into a pathological response remains poorly understood.

The hypothesis of this specific aim is that FSS magnitude and frequency abnormalities both contribute to early valvular CAVD pathogenesis. Porcine leaflets were subjected to sub-physiologic/physiologic/supra-physiologic FSS magnitude and frequency for 24, 48 and 72 hours bioreactor. Endothelial activation and paracrine signaling were investigated in terms of cell-adhesion molecules (ICAM-1, VCAM-1) and cytokines (BMP-4 and TGF-β1), respectively. Extracellular matrix (ECM) degradation was characterized by measuring the expression and activity of MMPs and cathepsin.

Specific Aim 2: To investigate the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling.

The effectiveness of the pharmacological approach depends on the ability to identify potential target molecules involved in the early stage of CAVD. Although some molecular pathways have been identified and mechanical forces are increasingly pointed as key players in CAVD development, the key molecules directly regulated by FSS are still largely unknown.

The hypothesis of the specific aim is that TGF-β1 and BMP-4 play important roles in FSS-induced endothelial activation and ECM remodeling under pathological FSS environment. The methodology involved the ex vivo exposure of porcine valvular leaflets to physiological and pathological FSS (supra-magnitude FSS or supra-frequency FSS) following the pharmacological promotion or inhibition of BMP-4 or TGF-β1. The biological assessment of the acute pathological response included inflammation and
remodeling via immunostaining and immunoblotting. The results identified potential molecular pathways for the future development of targeted cellular therapies.

**Specific Aim 3: To elucidate the mechanisms of CAVD secondary to BAV hemodynamic abnormalities.**

Although studies have demonstrated the sensitivity of leaflet tissue to FSS magnitude and the existence of flow abnormalities is BAVs, the elucidation of the role played by native BAV FSS abnormalities in CAVD pathogenesis has been hampered by the complexity of the native BAV hemodynamic environment and the challenge to replicate it in the laboratory setting. A recently published computational work on the comparison of TAV and type-1 BAV FSS [114] demonstrated that, while those hemodynamic abnormalities have little impact on the non-coronary BAV leaflet FSS, they significantly affect the pulsatility and magnitude of the FSS on the fused BAV leaflet, which is interestingly the leaflet most vulnerable to calcification [11].

The hypothesis of the specific aim is that BAV hemodynamic FSS abnormalities contribute to CAVD pathogenesis by promoting endothelial activation, pro-inflammatory paracrine signaling, VIC activation and osteogenesis. This hypothesis was tested *ex vivo* by exposing fresh porcine leaflets to the native TAV and type-1 BAV FSS. Immunostaining, immunoblotting and zymography were performed to compare the expressions and activities of biomarkers of valvular inflammation, ECM remodeling and osteogenic differentiation in response to both FSS environments.
Chapter 3:
MATERIALS AND METHODS

In this chapter, the design of the FSS bioreactor system and the methodology developed for its validation will be explained. The next section will compile mechanical treatment, the protocols for biological analyses and statistical analyses for each specific aim. The Matlab codes used for deriving the bioreactor input waveforms have been compiled in Appendix A. Detailed tissue and biological protocols have been compiled in Appendix B.

3.1 FSS bioreactor design

3.1.1 Native valvular FSS environment and previous FSS bioreactors

The characterization of the effects of FSS on valvular biology requires the replication of the native valvular FSS environment in the laboratory setting. As demonstrated in vivo [164], in vitro [111,112,165,166] and in silico [167-171], the valvular FSS is side-specific. The leaflet ventricularis is exposed to a high unidirectional pulsatile FSS, while the fibrosa experiences a low bidirectional oscillatory FSS. Two kinds of shear stress devices have been used to expose valvular cells to FSS: the parallel-plate flow chamber and the cone-and-plate viscometer. The former consists of two parallel plates between which fluid flow is established by an imposed pressure gradient. Due to the large
inertia of the driving components, such device is generally limited to the production of a steady FSS. The cone-and-plate apparatus which consists of an inverted cone rotating above a flat stationary plate [172] is more suitable for the production of a time-varying FSS. This device produces a flow essentially oriented along the circumferential direction.

Assuming steady and incompressible flow, low Reynolds number, small angle between the cone and the plate, the FSS distribution $\tau$ on the plate can be approximated as:

$$\tau = \mu \alpha \frac{r}{h + r \alpha}$$  \hspace{1cm} (3.1)

where $\mu$ is the dynamic viscosity of the working fluid, $\alpha$ is the angle between the cone and the plate, $h$ is the distance between the cone apex and the plate, and $r$ is the radial coordinate [172-177]. Cone-and-plate bioreactors have been used to expose endothelial cell monolayers to time-dependent FSS [178-181]. Although such devices are suitable for investigating cellular response to FSS, their implementation with whole pieces of tissue is more problematic due to the thickness of the tissue that results in local flow perturbations and secondary flow. In order to address this issue, Sucosky et al have described an improved cone-and-plate device to expose AV leaflet samples to controlled time-varying FSS [121]. The device consisted of a cylindrical base containing nine equi-angularly spaced wells, each accommodating a cylindrical tissue holder. A circular cover positioned on the base surface was used to clamp the periphery of the samples against the holders while exposing one sample surface to flow. Although this design permitted for the first time to expose one surface of leaflet tissue to in vivo-like hemodynamic conditions, its main limitation was its inability to expose simultaneously both leaflet surfaces to different
FSS, i.e., a requirement for the production of the native side-specific valvular hemodynamic environment.

3.1.2 Design description

The requirements of the new device were to expose valve leaflet samples to: 1) time-varying; 2) side-specific FSS. Those requirements were addressed by adopting a double cone-and-plate design consisting of two inverted cones rotating above one side of a plate mounted in the center of the bioreactor and separating the device into two compartments (Figure 3.1).

![Figure 3.1: Schematic of the double cone-and-plate device](image)

The complete design solution is shown in Figure 3.2a. The tissue-mounting plate consists of a thin circular plate (diameter: 115.4 mm, thickness: 0.5 mm) made of 300-series stainless steel and featuring six equi-angularly spaced holes (diameter: 7.0 mm)
located at 20 mm from the center of the plate. The plate thickness was chosen to match the measured average thickness of leaflets samples (i.e., $0.5 \pm 0.1 \text{ mm}$) in order to minimize the effects of tissue thickness on the surrounding flow. Located around each hole are ten suture holes (diameter: 0.76 mm) enabling the fixation of the tissue specimens to the plate. Side-specific FSS is produced by the rotation of two cones made of Delrin (diameter: 74 mm; angle: 179°), each fixed to a stainless-steel shaft (top diameter: 9.5 mm; bottom diameter: 38.1 mm; height: 54 mm) using three screws. Two cylindrical flanges made of polycarbonate (top flange: top outer diameter: 29.7 mm; bottom outer diameter: 115.4 mm; height: 41.5 mm; bottom flange: top outer diameter: 128.0 mm; bottom outer diameter: 27.4 mm; height: 56.9 mm) clamp the periphery of the plate via two o-rings (4464T222, McMaster-Carr, Aurora, OH) and close the device via six screws. Two vertical holes drilled through the top surface of the top flange ensure appropriate gas exchange between the culture medium located inside the device and the environment external to the bioreactor. Each flange contains one pair of sealed ball bearings (double sealed bearing 2780T53, McMaster Carr) to permit the rotation of the shafts relative to the flanges with minimal friction. In order to prevent leakage of culture medium through the bottom flange, a rotary shaft-ring seal (9562K42, McMaster-Carr) is mounted between the flange and the shaft above the top ball bearing. The distance between the top cone and the upper surface of the plate is adjusted via a cylindrical shaft collar sitting on the upper-most ball bearing and fixed on the shaft via a set screw. A second screw is threaded on top of the set screw to prevent it from backing out. The required distance of 200 µm between the apex of the cone and the plate is set prior to assembly by intercalating between the plate and the cone two cover slips (Model 48368-062, VWR Scientific Inc., West Chester, PA) whose
combined thickness was measured as 202±4 μm. A similar solution (shaft collar and set screws) is implemented to adjust the vertical distance between the bottom cone and the lower surface of the plate. After both cones height are adjusted and secured, the cover slips are removed and the apparatus assembled.

Figure 3.2: Design solution: a) exploded view of the cone-and-plate assembly; and b) mounting table.

A table was designed to maintain the cone-and-plate assembly in position during its operation (Figure 3.2b). The table made of polycarbonate consists of three horizontal platforms. The top and bottom platforms hold the servo motors while the middle platform accommodates the cone-and-plate assembly. The mount is fixed to a rectangular polycarbonate base supporting the perfusion system which consists of two sealed reservoirs containing culture medium and four peristaltic pumps (SP200FO, APT Instruments, Rochester, IL) perfusing medium at a constant flow rate of 1.8 mL/min. The total volume of culture medium used to run the device is 275 mL: each compartment of the double cone-and-plate bioreactor contains 30 mL of medium while the perfusion system (i.e., reservoirs
and Tygon tubing) accommodates 215 mL of medium (each reservoir: 100 mL; tubing: 15 mL). Two rotary servo motors (SM232AE-NPSN, Parker-Hannifin Corp., Cleveland, OH) are fixed to the top and bottom platforms and are coupled to the top and bottom shafts of the cone-and-plate assembly via two helical beam clamp-on coupling devices (2505K133, McMaster-Carr), allowing for both parallel and angular misalignments. The motors are controlled by two single-axis servo drives (Model GV6K Gemini GV-L3, Parker-Hannifin Corp., Figure 3.3). The two drives are linked by a 6 DB9 F/F null modem cable while one drive is connected to a laptop computer (T400 ThinkPad, Lenovo Inc., Morrisville, NC) running a proprietary servo drive software (Motion Planner, Parker-Hannifin Corp.) via a RJ 45 connector.

Figure 3.3: Servo drive connections
3.1.3 Input waveform derivation

The side-specific shear stress experienced by the aortic and ventricular surfaces of aortic valve leaflets was obtained from computational fluid dynamics simulations of the flow through a tri-leaflet valve [164,165,182]. The physiologic surface-averaged FSS experienced by the ventricular surface of the leaflet consisted of pulsatile waveform varying between 0 and 79 dyn/cm² over a cardiac period of 860 ms while the physiologic surface-averaged FSS experienced by the aortic surface consisted of a bidirectional oscillatory waveform ranging from -8 to +10 dyn/cm² (Figure 3.4). With the knowledge of the bioreactor geometry, the location of the tissue samples (r = 20 mm from the center of the plate), the target FSS variations on both sides of the tissue samples and the properties of the working fluid (Dulbecco’s Modified Eagle’s Medium - high glucose, Sigma-Aldrich Co., St Louis, MO, density: 1000 kg/m³; kinematic viscosity: 0.95 cSt). Equation 3.1 was used to determine the respective theoretical cone angular velocities (Figure 3.4). The Matlab codes used to derive the cone angular velocities are compiled in Appendix A.
Figure 3.4: Theoretical cone angular velocities calculated using Eq. 3.1 and producing the target native FSS experienced by: a) the ventricular; and b) the aortic surface of aortic valve leaflets.
3.2 Bioreactor validation

Figure 3.5: Outline of bioreactor validation

3.2.1 Mechanical validation

The production of two different flow environments in the top and bottom compartments of the bioreactor was expected to result in a pressure differential across the tissue samples which would lead in turn to tissue deformation. The deflection of the tissue samples under this time-varying pressure gradient could affect the validity of Equation 3.1 and the calculated cone velocities. Additionally, it was critical to verify that the maximum deflection of the samples remained smaller than the clearance between each cone and the plate. Lastly, another concern with the experimental design was the possible strain produced in the samples due to their vertical deflection. Given the particular mechano-sensitivity of aortic valve leaflets to stretch [183,184], it was important to verify that strain levels remained below thresholds known to result in a biological response and that FSS was the dominant mechanical stimulus experienced by the tissue samples mounted in the bioreactor. The mechanical validation was conducted computationally using the fluid...
structure interaction (FSI) capabilities of the commercial software ANSYS® CFX. Due to symmetry, only a 60° section of the bioreactor was modeled. The three-dimensional geometry consisted of a cylindrical tissue sample (diameter: 7 mm; thickness: 0.5 mm) and the fluid domains located in the top and bottom bioreactor compartments. The leaflet material was modeled as linear elastic and isotropic (density: 1056 kg/m³; Young’s modulus: 1.5 MPa; Poisson’s ratio: 0.45), an approximation employed in previous studies [167,185,186] and justified by the small size of the tissue samples (diameter: 7 mm). The properties of the culture medium (density: 1000 kg/m³; kinematic viscosity: 0.95 cSt) were assigned to the fluid domains. The lateral surface of the sample in contact with the plate was assigned a fixed-wall condition while the top and bottom surfaces in contact with the fluid domains were modeled as fluid-structure interfaces. The surfaces of the fluid domains interfacing with the top and bottom cones were modeled as moving walls rotating at the angular velocities calculated from Equation 3.1 and expected to produce the native ventricular and aortic FSS waveforms, respectively. The periodicity of the geometry was prescribed by assigning an interface condition to the two vertical surfaces bounding the fluid domain. All other surfaces were modeled as fixed walls. The flow and structure equations were solved using the arbitrary Lagrangian-Eulerian (ALE) approach [187] in which the fluid (culture medium) and structure (leaflet sample) domains are discretized using two deforming grids and the fluid mesh is updated at each time step to conform to the moving structure boundary. The structural domain was discretized using 2466 hexahedral elements while the fluid domain (i.e., top and bottom compartments) was meshed using 19164 hexahedral elements. The flow and leaflet equations were solved implicitly and iteratively until convergence was reached at each time step. A time-stepping
of 0.02s was chosen (i.e., 1/43 of a cardiac cycle). The simulation was initialized with both cones at rest and was run for five periods in order to eliminate transient effects. The data collected at each time point over the fifth period consisted of the maximum vertical deflection of the tissue sample, the minimum and maximum strains experienced by the sample in the tissue radial (i.e., direction of flow) and circumferential directions, the regional wall shear stress distributions on both sides of the tissue sample and the respective instantaneous surface-averaged wall shear stress. In addition, the dependence of those metrics on tissue material properties and thickness was investigated via two sensitivity analyses aimed at characterizing the impact of the inherent inter-sample variability on tissue mechanics. In the first sensitivity analysis, three elastic moduli (1.0, 1.5 and 6.5 MPa) selected in the physiologic range of leaflet radial elastic moduli [188] were implemented in the FSI model. The choice of the radial over the circumferential elastic modulus was justified by the particular orientation of the tissue samples in the bioreactor (i.e., radial direction of the samples aligned with the direction of the cone motion) and the expected maximum tissue deformation in that direction. In the second sensitivity analysis, three tissue thicknesses (400, 500 and 600 µm) were implemented in the FSI model while maintaining the thickness of the mounting plate at 500 µm. Those values correspond to the average leaflet thickness ± one standard deviation obtained from measurements performed in our laboratory.

3.2.2 Biological validation

The ability of the novel FSS bioreactor to expose biological tissue to native side-specific FSS while maintaining sterile conditions and preserving the tissue endothelium was tested experimentally. Porcine leaflets were excised and cultured in the bioreactor (see
The cones were programmed with the angular velocities (see Figure 3.4) required to expose each surface of the specimens to its specific native FSS for 96 hours. Endothelium integrity was characterized via von Willebrand factor immunostaining (anti-vWF, Sigma-Aldrich) and silver nitrate en-face staining (Sigma-Aldrich). Cellular apoptosis was assessed via TUNEL assay (In Situ cell death detection kit, Roche Diagnostics, Indianapolis, IN). Detections of apoptotic and vWF-positive cells were performed on tissue sections extracted every 500 µm throughout the depth of each tissue sample under a mercury lamp using a TR/FITC/DAPI filter. Cells with apoptotic fragments were detected under ultra-violet (UV) fluorescence using the FITC filter of the mercury lamp. The UV filter was used to image DAPI-stained cell nuclei. Given the rough topology of the fibrosa, silver nitrate staining was imaged only on the ventricularis (i.e., surface exposed to the most unfavorable FSS magnitudes and temporal variations) using a standard light microscope. Endothelial cell coverage on the ventricularis was quantified using Image J (NIH, Bethesda, MD) by calculating the ratio of the number of cells detected by silver nitrate staining to the surface area occupied by the tissue sample on each image. The apoptosis level was calculated as the ratio of the number of apoptotic cells to the total number of cells on each image. Statistical analyses refers to section 3.6.

3.3 Samples culture

3.3.1 Tissue harvest and preparation

Porcine valves (6-12 months) were obtained from a local abattoir (Martin’s Custom Butchering, Wakarusa, IN; permission was obtained from this slaughterhouse to use these animal parts), immediately rinsed in sterile Dubelcco’s phosphate buffered saline (PBS;
Sigma-Aldrich, St Louis, MO) and transported to the laboratory in ice-cold PBS. All subsequent procedures were conducted in a sterile flow hood. A circular section of 7 mm in diameter was excised from the base region of each leaflet (i.e., region most prone to calcification [67,147]). The samples were randomized, selected from different animals and different leaflets (i.e., left-coronary, right-coronary and non-coronary) and assigned to the different treatment groups outlined below. The samples were subjected to FSS for 48 hours (duration sufficient for initial biological changes secondary to mechanical stimulation to become evident [31,86,123,133,134,183,189]) using the bioreactor described in 3.1 [190]. For each experimental run, six tissue samples were stored in PBS then sutured to the mounting plate with 10 sutures for each sample. The native alignment of the tissue relative to blood flow was maintained by aligning the radial direction of the samples with the direction of the cone motion (Figure 3.6). The device was maintained in an incubator at 37°C and 5% CO₂ and was continuously perfused with standard culture medium (Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 3.7 g/L sodium bicarbonate, 0.05 g/L ascorbic acid, 10% non-essential amino acid solution and 1% penicillin-streptomycin; all from Sigma-Aldrich) at a rate of 108 mL/hour (i.e., two bioreactor volumes/hour). For each experiment, the perfusion system was totally drained and replenished with fresh medium after 24 hours. On completion of the FSS experiments, the 7-mm circular samples were harvested, washed three times in ice-cold sterile PBS and trimmed into smaller 5-mm disks in order to isolate the central region of the tissue that was exposed to the flow and discard the peripheral region that was sutured to the mounting plate. The resulting 5-mm samples were either frozen in optimum cutting temperature (OCT) medium (Electron Microscopy Sciences, Hatfield, PA), cut into 7-µm sections using
a Microm Cryo-Star HM 560MV Cryostat (Walldorf, Germany) and mounted on slides for histological and immunohistochemical analyses, or flash frozen in liquid nitrogen for immunoblotting or zymography analyses.

Figure 3.6: tissue mounting plate

3.3.2 Experimental conditions

3.3.2.1 Conditions for specific aim 1

The goal of this specific aim was to investigate the mechanisms of CAVD secondary to FSS abnormalities. As demonstrated in vitro [122] and computationally [114], the native valvular FSS environment is side-specific and can be idealized as a unidirectional pulsatile FSS varying between 0 and 80 dyn/cm² on the ventricularis and a bidirectional oscillatory FSS varying between -8 and +10 dyn/cm² on the fibrosa. In addition to the physiologic FSS signal (group 1), eight abnormal FSS conditions were generated by decreasing/increasing the magnitude and/or frequency of the physiologic signal (groups 2-9; Figure 3.5). This analysis resulted in nine pairs of FSS waveforms
(isolated effects of FSS magnitude: groups 1, 2, 3; isolated effects of FSS frequency: groups 1, 4, 5; combined effects of FSS magnitude and frequency: conditions 1, 6, 7, 8, 9), each pair corresponding to the temporal FSS variations experienced by the fibrosa and ventricularis.

Leaflets were cultured for 48 hours under standard culture medium (DMEM supplemented with 10% fetal bovine serum, 3.7 g/L sodium bicarbonate, 0.05 g/L ascorbic acid, 10% non-essential amino acid solution and 1% penicillin-streptomycin; all from Sigma-Aldrich). Control group is fresh sample. Following the biological assessment of all tissue groups, the mechanical treatment producing the most significant response was identified and two additional experiments were performed over 24 and 72 hours to characterize the time-dependence of the FSS mediated biological response.
<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
<th>Group 7</th>
<th>Group 8</th>
<th>Group 9</th>
</tr>
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<tr>
<td>FSS magnitude</td>
<td>100%</td>
<td>50%</td>
<td>200%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td>50%</td>
<td>200%</td>
<td>200%</td>
</tr>
<tr>
<td>FSS frequency</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td>200%</td>
<td>50%</td>
<td>200%</td>
<td>50%</td>
<td>200%</td>
</tr>
</tbody>
</table>

Figure 3.7: Side-specific FSS conditions considered in specific aim 1 (V_FSS: ventricularis FSS, F_FSS: fibrosa FSS).
3.3.2.2 Conditions for specific aim 2

The goal of this specific aim was to investigate the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling. The native valvular FSS (group 1, from SA1), which is a unidirectional pulsatile FSS varying between 0 and 80 dyn/cm$^2$ on the ventricularis and a bidirectional oscillatory FSS varying between -8 and +10 dyn/cm$^2$ on the fibrosa, was exposed to both surface of the leaflets. Besides native FSS, to investigate the role of BMP-4 and TGF-β1 in pathological ECM remodeling environment, supra-physiologic FSS magnitude at normal frequency (group 3, from SA1) and supra-physiologic FSS frequency normal magnitude (group 5, from SA1) were also applied on valvular leaflets.

Five variations of the culture medium composition were used to characterize the role played by BMP-4 and TGF-β1 in the FSS-induced inflammatory response and ECM remodeling (Table 3.1). Those treatments included standard culture medium (group 1); pro-BMP-4 medium (group 2); anti-BMP-4 medium (group 3); pro-TGF-β1 medium (group 4) and anti-TGF-β1 medium (group 5). The pro- BMP-4 and pro- TGF-β1 culture media were obtained by supplementing the normal culture medium with BMP-4 (R&D Systems) and TGF-β1 (R&D Systems), respectively. The anti-BMP-4 and anti-TGF-β1 media were prepared by supplementing the normal culture medium with the BMP antagonist noggin (R&D Systems) and the TGF-β1 inhibitor SB-55431542 (Sigma-Aldrich), respectively. The conditioning of tissue samples from groups 1 - 5 to FSS was conducted in the bioreactor for 48 hours.
TABLE 3.1
CULTURE MEDIUM FORMULATIONS TO INVESTIGATE THE ROLES AND SYNERGY OF BMP-4 AND TGF-β1

<table>
<thead>
<tr>
<th>No.</th>
<th>Culture medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal medium</td>
<td>DMEM with 10% FBS, 3.7 g/L sodium bicarbonate, 0.05 g/L ascorbic acid, 10% non-essential amino acid solution and 1% penicillin-streptomycin</td>
</tr>
<tr>
<td>2</td>
<td>Pro-BMP-4 medium</td>
<td>Normal +10 ng/mL BMP-4</td>
</tr>
<tr>
<td>3</td>
<td>Anti-BMP-4 medium</td>
<td>Normal +100 ng/mL noggin</td>
</tr>
<tr>
<td>4</td>
<td>Pro-TGF-β1 medium</td>
<td>Normal +10 ng/mL TGF-β1</td>
</tr>
<tr>
<td>5</td>
<td>Anti-TGF-β1 medium</td>
<td>Normal +1 μM SB-431542</td>
</tr>
</tbody>
</table>

3.3.2.3 Conditions for specific aim 3

The goal of this specific aim was to investigate the role played by BAV FSS abnormalities in CAVD pathogenesis. One control group (fresh tissue) and three mechanical treatment groups were considered in this study: 1) fresh porcine leaflet tissue (control); 2) porcine leaflet tissue exposed to TAV leaflet FSS; 3) porcine leaflet tissue exposed to fused BAV (F-BAV) leaflet FSS; and 4) porcine leaflet tissue exposed to non-coronary BAV (NC-BAV) leaflet FSS. The native FSS waveforms experienced by the fibrosa and ventricularis in the base of each leaflet (i.e., TAV, F-BAV and NC-BAV) were obtained from FSI simulations in TAV and type-1 BAV anatomies [114]. The average FSS predicted in the base of the ventricularis of TAV, F-BAV and NC-BAV leaflets consisted of pulsatile (i.e., positive) waveforms varying between 0 and 39 dyn/cm², 0 and 11 dyn/cm² and 0 and 78 dyn/cm², respectively, over a cardiac cycle of 0.86 s. In contrast, the average FSS predicted in the base of the fibrosa of the same leaflets consisted of oscillatory (i.e.,
alternatively positive and negative) waveforms ranging from -1.1 to 0.4 dyn/cm$^2$, -4.5 to 1.1 dyn/cm$^2$ and -0.4 to 1.8 dyn/cm$^2$, respectively (Figure 3.8). Leaflets were cultured for 48 hours in standard culture medium.

![Figure 3.8: FSS experienced by the fibrosa and ventricularis: a) TAV, b) NC-BAV and c) F-BAV.](image)

3.4 Biological analyses

The general procedures for H&E staining, TUNEL assay, silver nitrate staining, immunostaining, immunoblotting and gelatin zymography are briefly introduced below. Detailed protocols for all experimental steps are compiled in Appendix B.

3.4.1 H&E staining

H&E staining was used to examine tissue histology. Frozen tissue sections were thawed for 10 minutes at room temperature, rinsed in PBS for 10 minutes and incubated in activated hematoxylin (Electron Microscopy Sciences) for 2 minutes. Slides were washed for 5 minutes in tap water, stained with 0.2% ammonia in water for 1 minute, rinsed in tap water, counterstained for 5 minutes in eosin (Electron Microscopy Sciences), rinsed in water, then dehydrated. A resinous mounting agent was applied and the slides were
coverslipped and allowed to dry overnight before viewing. H&E images were recorded using the normal white light of a Nikon E600 imaging microscope.

3.4.2 TUNEL assay

Detection of cell apoptosis was performed by using a TUNEL assay kit (Roche Applied Science, Indianapolis, IN). Cryopreserved tissue sections were fixed with 4% paraformaldehyde in PBS for 20 minutes, washed with PBS for 30 minutes, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS (2 minutes on ice) and rinsed twice with PBS. Staining was performed by incubating tissue sections for 1 hour at 37°C in a humidified chamber in the dark in 50 µl of TUNEL reaction mixture. DAPI counterstaining was used to improve image contrast. Negative controls were prepared by incubating fixed and permeabilized tissue sections in 50 µl of TUNEL label solution (without terminal transferase). Positive controls were prepared by incubating fixed and permeabilized tissue sections with DNase I Recombinant (2,500 U/ml in 50mM Tris-HCl, pH 7.4, 1 mg/ml BSA, Roche Applied Science) for 10 minutes prior to labeling. Fluorescent images were acquired on a Nikon E600 imaging microscope.

3.4.3 Silver nitrate staining

Silver nitrate staining was used to examine endothelium integrity. Cryopreserved tissue sections were rinsed with PBS, then incubated in 0.25-0.5% silver nitrate dissolved in distilled water for approximately 30sec. Finally, tissue sections were fixed with 4% paraformaldehyde for 10min. Silver nitrate staining images were recorded using the normal white light of a Nikon E600 imaging microscope.
3.4.4 Immunostaining

Frozen tissue sections were flash frozen in optimum cutting temperature media. The slides were first thawed to room temperature and then blocked using 10% animal serum in PBS (Sigma-Aldrich), 0.2% Trixon-100 (Sigma-Aldrich) and 1% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific) in 1x PBS for 1 hour at room temperature. Following the blocking step, the slides were then incubated overnight at 4°C in primary antibody in 2 – 10% blocker at the dilutions shown in Table 3.2. Following primary antibody incubation, sections were washed 3 times in 1x PBS and incubated with anti-rabbit or anti-mouse (all from Santa Cruz) secondary antibodies at 1:100 dilution for 2 hours at room temperature. The tissue sections were then washed 3 times in 1x PBS, counterstained with 1 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich), mounted with fluorescence mounting medium (Dako), cover slipped and stored at 4°C. Slides were subsequently imaged under the mercury lamp of a Nikon E600 imaging microscope using a TR/FITC/DAPI filter.
TABLE 3.2

ANTIBODY CONCENTRATIONS FOR IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>1:50</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1:25</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>BMP-4</td>
<td>1:150</td>
<td>Abcam</td>
</tr>
<tr>
<td>cathepsin L</td>
<td>1:25</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>cathepsin S</td>
<td>125</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>MMP-2</td>
<td>1:100</td>
<td>EMD Millipore</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1:100</td>
<td>EMD Millipore</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>1:50</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>1:150</td>
<td>EMD Millipore</td>
</tr>
</tbody>
</table>

3.4.5 Immunoblotting

Following each mechanical treatment, the specimens were pulverized using a mortar and pestle in liquid nitrogen, homogenized in ice-cold RIPA buffer (Santa-Cruz) and centrifuged at 7,000g to pellet extracellular matrix debris for 8 minutes at 4°C. The supernatant was assayed for protein concentration using a bicinchoninic acid (BCA) protein assay (Pierce). Equal amounts of tissue lysates were resolved by reducing SDS-PAGE. After transfer to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore) using a mini trans-blot cell (Bio-Rad), the blots were blocked with 5% non-fat drymilk and probed with a primary antibody at the dilutions shown in Table 3.3. Depending on the primary antibody, appropriate anti-rabbit and anti-mouse HRP secondary antibody (1:2000, Santa Cruz) was then used. The membranes were finally incubated in horseradish peroxidase-conjugated streptavidin. Immunopositive bands were then detected using a
luminol-based chemiluminescence reagent (Pierce) against standard radiography film in a
darkroom. The films were then analyzed by densitometry using ImageJ (NIH).

**TABLE 3.3**

ANTIBODY CONCENTRATIONS FOR IMMUNOBLOTTING

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentrations</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>BMP-4</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>α-SMA</td>
<td>1:200</td>
<td>Dako</td>
</tr>
<tr>
<td>Runx2</td>
<td>1:1500</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>1:500</td>
<td>EMD Millipore</td>
</tr>
</tbody>
</table>

3.4.6 Gelatin zymography

Zymography was performed to quantify the activity of the proteolytic enzymes MMP-2 and MMP-9. Equal amounts of tissue lysates assayed by BCA were resolved by sample buffer (Bio-Rad) and loaded in the gel (Bio-Rad). After running, the gels were washed in renaturation buffer (Bio-Rad) and developing buffer (Bio-Rad). The gels were stained by adding stain solution (Sigma-Aldrich), de-stained in deionized water and scanned. The images were then analyzed by densitometry using ImageJ.

3.5 Image analysis and quantification

Semi-quantification of immunostained images: the semi-quantitative assessment of immunostained images was carried out using ImageJ. Briefly, the intensities of positively stained regions were estimated and normalized by the number of cells visible in the images.
to yield a quantity consistent to a biomarker expression per cell. Distinction was not made between endothelial and interstitial cells during the cell counting.

Densitometric assessment of immunoblots and zymograms: the dedicated macro of ImageJ was used to plot the histogram of individual lanes/bands in blots/gels. These histograms were then integrated to obtain the mean intensity of each immune-positive band. For the blots, these intensities for a particular protein were then normalized by the intensity values for β-actin (Santa Cruz), which was used as a housekeeping protein.

3.6 Statistical analyses

Each experimental group consisted of six leaflet samples. All quantitative data were expressed as means ± standard error and then normalized with respect to the values measured in fresh tissue (control). Data from all experiments were first tested for normality by the Anderson-Darling method, then analyzed using ANOVA to determine if there was significant contribution by a particular mechanical treatment on the measured parameters, followed by the Bonferroni post-hoc test. A p-value of less than 0.05 was used as a measure of statistical significance. All statistical analyses were performed using SPSS (IBM).

3.7 Pro-inflammatory and remodeling state indices

In specific aim 1, the net effect of a particular FSS environment on the pro-inflammatory state of the tissue after 48 hours of conditioning was estimated by calculating a pro-inflammatory state index defined as the product of the fold change in expression of each pro-inflammatory mediator (ICAM-1, VCAM-1, BMP-4, TGF-β1) with respect to fresh tissue:
\[ I = f(\text{ICAM-1}) \times f(\text{VCAM-1}) \times f(\text{BMP-4}) \times f(\text{TGF-β1}) \]  

(3.2)

Similarly, the contributions of FSS abnormalities to valvular remodeling were quantified via a tissue remodeling index defined as the product of the fold change in expression of each ECM remodeling marker (MMP-2, MMP-9, cathepsin-S and cathepsin-L) relative to fresh tissue:

\[ R = f(\text{MMP-2}) \times f(\text{MMP-9}) \times f(\text{cathepsin-S}) \times f(\text{cathepsin-L}) \]  

(3.3)

An index smaller than one indicates a net decrease in pro-inflammatory/remodeling response, while an index larger than one reflected a net increase in tissue pro-inflammatory/remodeling response. In specific aim 1, the nine pro-inflammatory and remodeling state indices resulting from the nine FSS magnitude-frequency treatments were interpolated using a biharmonic spline in order to predict the dependence of the tissue pathologic state on FSS magnitude and frequency.
Chapter 4:
RESULTS

This chapter compiles all the results from this dissertation. The first section contains the results of the mechanical validation and biological validation of the FSS bioreactor. Sections 4.2-4.4 describe the detailed results for each of the three specific aims. Specific aim 1 sought to investigate the mechanisms of CAVD secondary to FSS abnormalities. Specific aim 2 studied the role played by BAV FSS abnormalities in CAVD pathogenesis. Specific aim 3 investigated the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling.

4.1 Bioreactor validation

4.1.1 Mechanical validation

4.1.1.1 Sample deformation

The FSI model of the bioreactor was run to predict the deformation and FSS distributions on both surfaces of a leaflet sample. The sample deformation and the variations of the FSS distributions at eight time points on the upper (ventricular) and lower (aortic) surface of the leaflet sample are shown in Figure 4.1a and Figure 4.1b, respectively. The maximum vertical deflection of the sample predicted by the model is 47.7 μm and occurs near peak systole (t = 0.2s). This deformation is only 8.7% of the total clearance.
(0.55 mm) between the surface of the cone and the surface of the plate at the location where the sample is mounted. Therefore, the time-dependent pressure gradient across the tissue samples resulting from the production of different flows in the top and bottom compartments of the bioreactor does not cause the tissue to contact the cone and does not impair the normal operation of the device.

Figure 4.1: FSI predictions of the deformations and FSS distributions on: (a) the ventricular (top); and (b) the aortic (bottom) surface of a tissue sample mounted in the bioreactor during one period.

4.1.1.2 FSS distribution

The observation of the FSS distributions on the lower (aortic) and upper (ventricular) surface of the leaflet sample suggests the non-uniformity of the FSS on each
surface. This non-uniformity can be explained by the deformation of the sample in response to the time-dependent pressure gradient established by the differential rotation of the two cones, and the existence of a secondary flow resulting from the finite dimensions of the bioreactor and the existence of a finite gap between the apex of each cone and the plate. However, the surface-averaged FSS variations predicted by the FSI model on the top (ventricular) and bottom (aortic) surfaces of the tissue sample are in good agreement with the respective target (native) FSS both qualitatively and quantitatively (Figure 4.2). The maximum surface-averaged FSS on the ventricular surface is 76.4 dyn/cm$^2$, which is 3.3% lower than the native maximum FSS of 79 dyn/cm$^2$ on that surface. Similarly, the maximum surface-averaged FSS on the aortic surface is 9.4 dyn/cm$^2$, which is 6% lower than the native maximum FSS of 10 dyn/cm$^2$ on that surface. In addition, the root-mean square errors between the computed and targeted FSS waveforms are 5.5% and 6.7% of the peak FSS value on the ventricular and aortic surface, respectively. Therefore, the results demonstrate the ability of the novel FSS device to expose valvular leaflet tissue to side-specific FSS.
Figure 4.2: Comparison between the target (native) FSS and the surface-averaged FSS predicted by the FSI model over a tissue sample on: a) the ventricular; and b) the aortic side of a tissue sample. The error bars indicate the maximum and minimum FSS values computed over the surface of the tissue sample.
4.1.1.3 Effect of tissue elastic modulus

The results of the dependence of sample deflection, strain and FSS distributions on tissue elastic properties are summarized in Table 4.1. As expected, there is a nearly linear inverse relationship between tissue elastic modulus and sample deflection. The maximum tissue deflection obtained with the smallest elastic modulus considered in this sensitivity study (i.e., 1.0 MPa) is only 13% of the total available clearance between the cone and the plate. Strains were computed in both radial and circumferential directions. As expected, an increase in elastic modulus results in a decrease in strain magnitude in both directions. Interestingly, similar strain magnitudes are obtained in both directions, regardless of the elastic modulus. This can be explained by the isotropic and incompressible material formulation implemented in the FSI model and the location of the maximum tissue deflection near the center of the sample. More importantly, the maximum tissue radial and circumferential strains (i.e., 1.1%) obtained with the lowest elastic modulus are negligible as compared to the average strains experienced by aortic valve leaflets in vivo (i.e., radial strain: 40%; circumferential strain: 10%) [109,191]. Those results demonstrate that FSS is the dominant mechanical stimulus experienced by the samples mounted in the bioreactor. Finally, there is a weaker correlation between tissue elastic modulus and FSS. The results suggest 8% decrease and 13% increase in peak surface-averaged wall-FSS on the ventricular and aortic surface, respectively, in response to a 6.5-fold increase in elastic modulus (from 1.0 to 6.5 MPa).
TABLE 4.1

EFFECT OF TISSUE ELASTIC MODULUS ON SAMPLE DEFLECTION, STRAIN
AND FSS.

<table>
<thead>
<tr>
<th>Elastic modulus (MPa)</th>
<th>Maximum tissue deflection (µm)</th>
<th>Radial strain (%)</th>
<th>Circumferential strain (%)</th>
<th>Surface-averaged ventricular FSS (dyn/cm²)</th>
<th>Surface-averaged aortic FSS (dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>72</td>
<td>-0.5</td>
<td>1.1</td>
<td>0.6</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-8.5</td>
<td>8.9</td>
</tr>
<tr>
<td>1.5</td>
<td>48</td>
<td>-0.4</td>
<td>0.7</td>
<td>0.6</td>
<td>76.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-8.5</td>
<td>9.4</td>
</tr>
<tr>
<td>6.5</td>
<td>11</td>
<td>-0.1</td>
<td>0.2</td>
<td>0.6</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-8.5</td>
<td>10.1</td>
</tr>
</tbody>
</table>

4.1.1.4 Effect of tissue thickness

The effects of tissue thickness on sample deflection, strain and FSS distributions are summarized in Table 4.2. Sample deflection is weakly correlated with tissue thickness. The mounting of a tissue sample 20% thicker and 20% thinner than the plate results in a significant 56% and 85% increase in peak tissue deflection, respectively. Interestingly, while variability in elastic modulus was shown to impact only the maximum strain experienced by the sample (see Table 4.1), the introduction of a discrepancy between the tissue thickness and the plate thickness affects both the minimum and maximum strain levels. However, the maximum strain level (i.e., 1.5%) resulting from the mounting of a tissue sample 20% thicker than the plate is still negligible as compared to the physiologic leaflet strain levels measured in vivo. Lastly, the FSI predictions suggest that variability in sample thickness is associated with variability in FSS on each sample surface. The mounting of a sample 20% thinner than the plate results in 2% increase and 11% decrease
in the range of FSS experienced by the ventricularis and fibrosa, respectively. Conversely, the mounting of a sample 20% thicker than the plate results in 30% increase and 8% increase in the range of FSS experienced by the ventricularis and fibrosa, respectively.

**TABLE 4.2**

**EFFECT OF TISSUE THICKNESS ON SAMPLE DEFLECTION, STRAIN AND FSS**

<table>
<thead>
<tr>
<th>Tissue thickness (μm)</th>
<th>Maximum tissue deflection (μm)</th>
<th>Radial strain (%)</th>
<th>Circumferential strain (%)</th>
<th>Surface-averaged ventricular FSS (dyn/cm²)</th>
<th>Surface-averaged aortic FSS (dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>max</td>
<td>min</td>
<td>max</td>
<td>min</td>
</tr>
<tr>
<td>400</td>
<td>75</td>
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<td>1.2</td>
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<td>1.0</td>
</tr>
<tr>
<td>500</td>
<td>48</td>
<td>-0.4</td>
<td>0.7</td>
<td>-0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>600</td>
<td>89</td>
<td>-1.4</td>
<td>1.5</td>
<td>-1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

4.1.2 Biological validation

4.1.2.1 Endothelium integrity

The validated cone angular velocities producing the native side-specific FSS on the tissue samples was programmed in the servo drives and following conditioning for 96 hours, six porcine aortic valve leaflet specimens were analyzed in terms of endothelial integrity, cellular morphology, and cellular apoptosis. First, it was critical to verify that the FSS conditions imposed by the culture system did not affect the integrity of the endothelial layer of the tissue samples. The results obtained from vWF immunostaining demonstrate the preservation of the endothelial layer on each side of the tissue sample exposed to native FSS conditions (Figure 4.3). Silver nitrate en-face staining performed on the ventricularis
of fresh tissue and tissue exposed to physiologic FSS for 96 hours suggests no qualitative difference in endothelial cell morphology and alignment (Figure 4.3). Although the quantitative data suggest a 18% decrease in endothelial cell density on the ventricularis of conditioned tissue relative to that in fresh tissue, this difference was not statistically significant (p>0.05).

Figure 4.3: Comparison of endothelium integrities in fresh tissue and tissue exposed to physiologic FSS for 96 h: vWF and DAPI staining (A: aortic surface; V: ventricular surface; green: vWF-positive cells; blue: DAPI-positive cells); silver nitrate en-face staining on the ventricularis; and quantification of endothelial cell coverage (error bars indicate standard deviation).
4.1.2.2 Cell apoptosis

DAPI staining on fresh tissue and tissue conditioned under physiologic FSS shows that normal cell morphology and viability were maintained during culture in the novel cone-and-plate device (Figure 4.4). No cell fragments or apoptotic bodies were detected. In addition, minor cellular apoptosis was detected via TUNEL assay throughout the tissue in both tissue groups (Figure 4.4). Cellular apoptosis, where observed, was not localized to either side of the leaflet and was distributed in the endothelial, sub-endothelial and interstitial layers of the tissue. Specimens exposed to native side-specific FSS in the bioreactor were characterized by a percentage of apoptotic cells (0.057±0.018%) not statistically different (p>0.05; n=6) from that calculated on fresh tissue (0.064±0.029%). Although no quantitative assessments of tissue necrosis and medium sterility were performed, the regular inspection of the culture medium during each experimental run did not evidence any trace of contamination or loss of sterility. This biological validation demonstrates the ability of the bioreactor to maintain leaflet structure, cell viability and cell proliferation for the intended culture duration of 96 hours.
Figure 4.4: TUNEL and DAPI stainings on fresh tissue and tissue exposed to native side-specific FSS for 96 h (A: aortic surface; V: ventricular surface; green: apoptotic cells; blue: DAPI-positive cells); and quantitative results (error bars indicate standard deviation).

4.1.3 Bioreactor validation summary

The bioreactor validation results demonstrate the capability of the bioreactor to expose tissue samples to isolated FSS. In addition, the FSS distribution on each side of the samples is minimally impacted by physiologic variations in elastic modulus. Therefore, provided that inter-sample variability can be maintained within an acceptable range, the characterization of the tissue mechanical properties prior to an experiment is not necessary. As compared to our previous single-sided FSS bioreactor, this novel bioreactor can provide
a better understanding of the cell- and tissue-level responses, as well as the cell-cell and cell-ECM communications.

The native valvular hemodynamic stress environment includes stretch, pressure and flow. Ex vivo and in vitro studies have shown that each mechanical signal results in a specific response in terms of cellular alignment, proliferation, biosynthetic activity, tissue remodeling and tissue pathogenesis. While the response of leaflet tissue to isolated FSS is important, the effects of combined mechanical forces need to be considered to obtain a complete understanding of valvular biology. Therefore, the new FSS device could be complemented by a flexible plate exposing the samples to cyclic strain and a pressure chamber \[118\] enclosing the cone-and-plate assembly and achieving desired levels of cyclic pressure.

4.2 Specific Aim 1

Specific aim 1 is to investigate the mechanisms of CAVD secondary to FSS abnormalities by subjecting valvular leaflets to sub-physiologic/physiologic/supra-physiologic FSS magnitude and frequency.

4.2.1 Effects of FSS abnormalities on endothelial activation

Following tissue exposure to physiologic and non-physiologic levels of FSS magnitude and frequency, immunostaining was performed to examine endothelial activation. Positive staining for ICAM-1 (Figure 4.5a) and VCAM-1 (Figure 4.5b) was only detected on the endothelial lining of the fibrosa in tissue subjected to supra-physiologic FSS magnitude and physiologic frequency (group 3). Although the results are supported by the semi-quantitative analysis that suggests a 6.4-fold and 8.6-fold increase
in ICAM-1 and VCAM-1 expression, respectively, relative to the fresh controls, and a 4.5-fold and 6.5-fold, respectively, relative to the physiologic FSS treatment (Figure 4.5c), those changes were not statistically significant (p>0.05).

![Figure 4.5](image)

Figure 4.5: Effects of FSS abnormalities on endothelial activation:
a) Immunostaining for ICAM-1 and b) VCAM-1 after FSS conditioning for 48 hours and c) semi-quantitative results (F: fibrosa, blue: cell nuclei; green: positively stained cells).

4.2.2 Effects of FSS abnormalities on paracrine signaling

BMP-4 and TGF-β1 immunostaining and immunoblotting were performed to investigate the effects of FSS abnormalities on paracrine signaling. Similar to the cell-adhesion molecules, BMP-4 and TGF-β1 expression (Figure 4.6a and 4.6b, respectively)
was detected in the sub-endothelial layer of tissue exposed to supra-physiologic FSS magnitude at normal frequency (group 3). Those observations are supported by the Western blot results (Figure 4.6c) which suggest that supra-physiologic FSS magnitude (group 3) resulted in a significant 5-fold increase in BMP-4 and TGF-β1 expression relative to fresh controls. Tissue exposure to physiologic FSS (group 1) essentially maintained the BMP-4 and TGF-β1 expression levels measured in fresh controls. In addition, tissue exposure to abnormal frequencies of elevated FSS (groups 8 and 9) significantly decreased the expression of the cytokines with respect to the fresh controls (group 8: 82% and 80% decrease in BMP-4 and TGF-β1 expression, respectively; group 9: 79% and 83% decrease in BMP-4 and TGF-β1 expression, respectively) and the tissue conditioned under physiologic FSS (group 8: 89% and 50% decrease in BMP-4 and TGF-β1 expression, respectively; group 9: 87% and 58% decrease in BMP-4 and TGF-β1 expression, respectively).
4.2.3 Effects of FSS abnormalities on valvular remodeling

The effects of FSS abnormalities on valvular remodeling were investigated by comparing the expression of the proteolytic enzymes MMP-2 and MMP-9 and the elastolytic proteases cathepsin L and cathepsin S. MMP-2 and MMP-9 immunostaining...
(Figure 4.7a and 4.7b, respectively) suggest the maintenance of normal enzymatic expression in response to physiologic FSS (group 1) and combined alterations in FSS magnitude and frequency (groups 6-9). In contrast, elevated FSS magnitude (group 3) and abnormalities in FSS frequency (groups 4 and 5) resulted in increased proteolytic enzyme expression relative to fresh controls. The semi-quantitative results (Figure 4.7c) support those observations. Tissue from groups 3, 4 and 5 exhibited a significant increase in MMP-2 expression relative to both fresh tissue (7.6-fold, 10.5-fold and 10.0-fold, respectively) and tissue subjected to physiologic FSS (7.2-fold, 9.9-fold and 9.4-fold, respectively). MMP-9 expression was significantly increased in response to supra-physiologic FSS magnitude at normal frequency (group 3) and isolated abnormalities in FSS frequency (groups 4 and 5) relative to fresh tissue (9.4-fold, 9.7-fold and 10.0-fold, respectively) and tissue conditioned under physiologic FSS (15.1-fold, 15.6-fold and 16.1-fold, respectively).
Figure 4.7: Effects of FSS abnormalities on proteolytic enzyme expression and activity: a) immunostaining for MMP-2 and b) MMP-9 after FSS conditioning for 48 hours, c) semi-quantitative results and d) zymography results with densitometric assessment (F: fibrosa, blue: cell nuclei; green: positively stained cells, * p<0.05 vs. fresh control, # p<0.05 vs. physiologic FSS).

Gelatin zymography was carried out to quantify MMP-2 and MMP-9 activity in the different tissue groups (Figure 4.8). The densitometric analysis of the zymograms revealed that physiologic FSS (group 1) maintained MMP-2 activity but elicited a significant 78% decrease in MMP-9 activity with respect to fresh control. Tissue from group 4 exhibited a 28.6-fold increase in MMP-2 activity as compared to fresh tissue (p<0.05) and a 18.5-fold
increase relative to tissue exposed to physiologic FSS (p<0.05). There was also a significant increase in MMP-9 activity in tissue from groups 3 and 5 as compared to fresh tissue (1.4-fold and 2.0-fold, respectively) and tissue conditioned under physiologic FSS (6.2-fold and 8.9-fold, respectively). In contrast, combined alterations in FSS magnitude and frequency (groups 6-9) were associated with the maintenance of MMP-2 activity but a significant decrease in MMP-9 activity (0.4-fold, 0.1-fold, 0.1-fold and 0.1-fold change, respectively) relative to fresh tissue.

![Figure 4.8: Effects of FSS abnormalities on proteolytic enzyme activity, zymography results with densitometric assessment (* p<0.05 vs. fresh control, # p<0.05 vs. physiologic FSS).](image)

While cathepsin L and cathepsin S expression was not detected in response to physiologic FSS (Figure 4.9a and b, respectively), widespread positive staining was observed under supra-physiologic FSS magnitude at normal frequency (group 3) and supra-physiologic FSS frequency at normal magnitude (group 5). The semi-quantitative results
(Figure 4.9c) indicate a significant 11.1-fold and 8.5-fold increase in cathepsin L expression in tissue exposed to supra-physiologic FSS (group 3) relative to fresh controls and tissue subjected to physiologic FSS, respectively. Although cathepsin L was upregulated in response to combined abnormalities in FSS magnitude and frequency (groups 6-9), the response was not statistically different from that in fresh controls. Cathepsin S expression measured in tissue from groups 3 and 5 was significantly increased relative to fresh controls (4.5-fold and 3.9-fold, respectively) and tissue exposed to physiologic FSS (5.2-fold and 4.5-fold, respectively).
Figure 4.9: Effects of FSS abnormalities on elastolytic protease expression: a) immunostaining for cathepsin-L and b) cathepsin-S after FSS conditioning for 48 hours and c) semi-quantitative results (F: fibrosa, blue: cell nuclei; green: positively stained cells, * p<0.05 vs. fresh control, # p<0.05 vs. physiologic FSS).

4.2.4 Pro-inflammatory and remodeling indices

With most pro-inflammatory state index variations confined in the supra-physiologic FSS magnitude range, the results suggest the particular vulnerability of leaflet inflammation to elevated FSS levels (Figure 4.10a). The symmetry of the pro-inflammatory state index distribution also suggests that, at a given FSS magnitude, pro-inflammatory pathways are equally sensitive to an increase or a decrease in FSS frequency. The FSS environment maximizing the pro-inflammatory state index consists of a supra-physiologic
FSS magnitude (i.e., 200% physiologic level) at a relatively moderate FSS frequency (i.e., 112% physiologic level). Similarly, the analysis of the remodeling state index distribution reveals the strong dependence of valvular remodeling processes on elevated FSS magnitude (Figure 4.10b). However, the skewness of the distribution toward the upper left quadrant suggests the higher sensitivity of those processes to supra-physiologic than to sub-physiologic FSS frequencies. The FSS environment maximizing the remodeling state index consists of a supra-physiologic FSS magnitude (i.e., 194% physiologic level) at a relatively moderate FSS frequency (i.e., 115% physiologic level).

Figure 4.10: Correlation between FSS abnormalities and leaflet pathological state: a) pro-inflammatory state index and b) remodeling state index after 48 hours of FSS conditioning.

4.2.5 Time-dependence of the FSS mediated biological response

The time-dependence of the leaflet pathological response following conditioning to supra-physiologic FSS under normal frequency (i.e., FSS environment shown to be most
conducive to early CAVD mechanisms) was studied by assessing the biological response of tissue exposed to this environment for 24 and 72 hours and by comparing it to those obtained in the fresh controls and tissue conditioned for 48 hours (Figure 4.11a). The semi-quantitative analysis of the stained images (Figure 4.11b) revealed no significant difference in the expression of all biomarkers between the fresh controls (i.e., 0-hour time point) and the 24-hour time point. In contrast, while ICAM-1 and VCAM-1 expressions did not increase significantly between 24 and 48 hours of FSS conditioning, all other biomarkers were significantly upregulated between those two time points (5.2-fold, 5.1-fold, 5.9-fold, 6.9-fold, 9.6-fold and 2.6-fold increase in BMP-4, TGF-β1, MMP-2, MMP-9, cathepsin L and cathepsin S, respectively). Interestingly, the expression of all biomarkers was essentially maintained beyond the 48-hour time point as no further significant change occurred between 48 and 72 hours of conditioning.
Figure 4.11: Time-dependence of pro-inflammatory and ECM remodeling biomarker expression following tissue exposure to supra-physiologic FSS magnitude under normal frequency for 24, 48 and 72 hours: a) immunostaining and b) semi-quantitative results (F: fibrosa, V: ventricularis, blue: cell nuclei; green: positively stained cells, * p<0.05 vs. fresh control, # p<0.05 vs. 24 h).
4.3 Specific Aim 2

Specific aim 2 is to investigate the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling by culturing valvular leaflets under physiological and pathological FSS by promoting/inhibiting BMP-4 or TGF-β1 in the culture medium.

4.3.1 Effects of BMP-4 and TGF-β1 on endothelial activation

Porcine leaflets were exposed to physiological FSS for 48 hours in normal culture medium, or pro-BMP-4 culture medium supplemented with recombinant BMP-4 (BMP-4 treatment group), anti-BMP-4 culture medium supplemented with the specific BMP antagonist noggin (noggin treatment group), pro-TGF-β1 culture medium supplemented with recombinant TGF-β1 (TGF-β1 treatment group), and anti-TGF-β1 culture medium supplemented with the specific TGF-β1 inhibitor SB-431542 (SB treatment group). Immunostaining was performed to examine endothelial activation. Little positive staining for ICAM-1 and VCAM-1 was detected on the endothelial lining of the fibrosa in tissue samples. The results are supported by the semi-quantitative analysis that suggests no statistically significant change between control group (fresh tissue) and all five culture medium groups (Figure 4.12). Similar results were also observed following tissue exposure to supra-physiologic FSS magnitude and exposure to supra-physiologic FSS frequency.
Figure 4.12: Effects of BMP-4 or TGF-β1 on ICAM-1 and VCAM-1 expression under physiological FSS (F: fibrosa; green: positively stained cells; blue: cell nuclei.)
4.3.2 Synergistic effects of BMP-4 and TGF-β1

Porcine tissue exposure to physiologic FSS under normal culture medium group, BMP-4 treatment group, noggin treatment group, TGF-β1 treatment group or SB treatment group. Immunoblotting results suggest no significant changes in TGF-β1 expression in BMP-4 treatment group or noggin treatment group with respect to the fresh control (Figure 4.13a and 4.13b). The results also indicate no significant changes in BMP-4 expression in TGF-β1 treatment group and SB treatment group with respect to the fresh controls (Figure 4.13a and 4.13c).

![Immunoblotting results](image)

**Figure 4.13:** BMP-4 and TGF-β1 expressions after exposing to physiologic FSS: a) immunoblotting, b) densitometric results of medium supplemented with BMP-4 or Noggin, and c) densitometric results of medium supplemented with TGF-β1 or SB-431542).

Under supra-physiological FSS magnitude, porcine leaflets were cultured in a normal culture medium group, BMP-4 treatment group, noggin treatment group, TGF-β1
treatment group or SB treatment group. Immunoblotting results suggest significant (p<0.05) increases in TGF-β1 expression in the normal group, BMP-4 treatment group and noggin treatment group with respect to the fresh controls (4.7-fold, 6.2-fold and 4.1-fold, respectively, Figure 4.14a and 4.14b). Figure 4.14c suggests significant (p<0.05) increases in BMP-4 expression in the normal group, TGF-β1 group and SB treatment group with respect to the fresh controls (2.6-fold, 2.2-fold and 2.1-fold, respectively).

![Immunoblotting results showing different treatments](image)

**Figure 4.14:** BMP-4 and TGF-β1 expressions after exposing to supra-physiologic FSS magnitude: a) immunoblotting, b) densitometric results of medium supplemented with BMP-4 or Noggin, and c) densitometric results of medium supplemented with TGF-β1 or SB-431542 (*: p<0.05 vs. fresh control).

Porcine leaflets were conditioned under supra-physiological FSS frequency using each of the five culture media. Immunoblotting results suggest no significant change in TGF-β1 expression in the BMP-4 treatment group or noggin treatment group with respect to the fresh control (Figure 4.15a and 4.15b). Also, the results suggest no significant change
in BMP-4 expression in the TGF-β1 treatment group and SB treatment group with respect to the fresh controls (Figure 4.15a and 4.15c).

![Figure 4.15: BMP-4 and TGF-β1 expressions after exposing to supra-physiologic FSS frequency: a) immunoblotting, b) densitometric results of medium supplemented with BMP-4 or Noggin, and c) densitometric results of medium supplemented with TGF-β1 or SB-431542.]

4.3.3 Effects of BMP-4 and TGF-β1 on valvular remodeling

Immunostaining was performed to examine MMP-2, MMP-9, cathepsin L and cathepsin S expressions in the leaflet tissue samples exposed to physiological FSS. No statistically significant (p>0.05) differences in MMP and cathepsin expression was detected with respect to the fresh controls.

Under a supra-physiological FSS magnitude, the semi-quantitative assessment indicates a significant (p<0.05) increase in MMP-2 expression in the normal group, BMP-4 treatment group and TGF-β1 treatment group with respect to the fresh controls (9.4-fold,
8.4-fold and 11.1-fold, respectively, Figure 4.16). Significant (p<0.05) increases were also observed in MMP-9 expression in the normal culture medium group, noggin treatment group and TGF-β1 treatment group with respect to the fresh controls (12.2-fold, 9.3-fold and 16.2-fold, respectively, Figure 4.16). The most significant finding is that SB treatment significantly decreases MMP-9 expression with respect to the normal culture medium group and the TGF-β1 treatment group. In addition, the results in Figure 4.17 show that there are significant (p<0.05) increases in cathepsin L expression in the normal group and the TGF-β1 treatment group with respect to the fresh controls (11.9-fold and 15.5-fold, respectively). Significant (p<0.05) increases were also observed in cathepsin S expression in the normal culture medium group, BMP-4 treatment group, noggin treatment group, TGF-β1 treatment group, and SB treatment group with respect to the fresh controls (6.0-fold, 6.0-fold, 5.5-fold, 5.4-fold and 3.2-fold, respectively).
Figure 4.16: Effects of BMP-4 and TGF-β1 on MMP-2, MMP-9 expression under supra-physiological FSS magnitude: a) immunostaining, b) semi-quantitative results (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control; #: p<0.05 vs. Normal culture medium group; +: p<0.05 vs. TGF-β1 treatment group)
Figure 4.17: Effects of BMP-4 and TGF-β1 on cathepsin L and cathepsin S expression under supra-physiological FSS magnitude: a) immunostaining, b) semi-quantitative results (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control)
The semi-quantitative assessment of immunoblotting from tissue conditioned with a supra-physiological FSS frequency, indicates significant (p<0.05) increases in MMP-2 expression in the normal group, BMP-4 treatment group, TGF-β1 treatment group and SB treatment group with respect to the fresh controls (12.6-fold, 13.2-fold, 14.5-fold and 13.4-fold, respectively, Figure 4.18). The results also suggest a significant (p<0.05) increase in MMP-9 expression in the normal culture medium group, Noggin treatment group, and TGF-β1 treatment group with respect to the fresh controls (15.7-fold, 14.9-fold and 17.3-fold, respectively, Figure 4.18). Similarly with tissue cultured under supra-physiological FSS magnitude, SB treatment significantly decreases MMP-9 expression with respect to normal culture medium group and TGF-β1 treatment group. In addition, the results in Figure 4.19 shows there are non-significant (p<0.05) increases in cathepsin L expression in all experimental groups with respect to the fresh controls. Significant (p<0.05) increases are observed in cathepsin S expression in BMP-4 treatment group, Noggin treatment group and TGF-β1 treatment group with respect to the fresh controls (7.4-fold, 6.3-fold, 6.2-fold and 7.4-fold, respectively).
Figure 4.18: Effects of BMP-4 and TGF-β1 on MMP-2, MMP-9 expression under supra-physiological FSS frequency: a) immunostaining, b) semi-quantitative results (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control; #: p<0.05 vs. Normal culture medium group; +: p<0.05 vs. TGF-β1 treatment group)
Figure 4.19: Effects of BMP-4 and TGF-β1 on cathepsin L and cathepsin S expression under supra-physiological FSS frequency: a) immunostaining, b) semi-quantitative results (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control
4.4 Specific Aim 3

Specific aim 3 is to investigate the role played by BAV FSS abnormalities in CAVD pathogenesis by subjecting valvular leaflets to the native FSS experienced by TAV and type-1 BAV leaflets.

4.4.1 Leaflet structure and cell viability

Following tissue exposure to TAV and BAV FSS (groups 2, 3 and 4), the specimens were harvested and processed for standard H&E staining. Regardless of the mechanical treatment, no visible difference in tissue structure, cell density and leaflet thickness was detected between samples exposed to FSS and fresh controls (Figure 4.20a). Based on H&E staining alone, specimens conditioned under TAV and BAV FSS exhibited the same three-layered leaflet structure as that observed in fresh tissue. The quantification of the leaflet thickness with ImageJ in tissue from groups 2, 3 and 4 yielded average values of 328 ± 29, 352 ± 40, and 282 ± 26 μm, respectively, which were not statistically different (p>0.06) from the thickness measured in fresh controls (302 ± 25 μm) (Figure 4.20b). Lastly, the maintenance of cellular viability was verified by TUNEL assay. In all groups, apoptotic cell fragments were detected mainly in the sub-endothelial layer of the fibrosa (Figure 4.20c). Specimens exposed to TAV, F-BAV and NC-BAV FSS exhibited percentages of apoptotic cells (1.0 ± 0.4%, 1.1 ± 0.6% and 0.8 ± 0.2%, respectively) statistically similar (p>0.90) to that measured in fresh tissue (0.7 ± 0.2%) (Figure 4.20d). These results suggest that leaflet structure and cell viability are not affected by TAV and BAV FSS.
4.4.2 Effects of FSS abnormalities on endothelial activation

Immunostaining was performed to characterize the effects of TAV and BAV FSS on valvular endothelial activation in terms of ICAM-1 and VCAM-1 expression (Figure 4.21). Positive staining for ICAM-1 was only detected on the endothelial lining of the fibrosa in tissue subjected to F-BAV FSS conditions. Similarly, specimens exposed to F-BAV FSS exhibited a side-specific expression of VCAM-1, preferentially on the fibrosa. All other treatments did not result in any detectable change in cell-adhesion molecule expression relative to the fresh controls.
4.4.3 Effects of FSS abnormalities on paracrine signaling

BMP-4 and TGF-β1 immunostaining was performed to investigate the potential of BAV FSS abnormalities to stimulate paracrine signaling pathways characteristic of the early stage of CAVD. While BMP-4- and TGF-β1-positive staining was detected near the endothelium in tissue exposed to TAV and NC-BAV FSS, expression levels were not qualitatively different from the baseline levels measured in fresh tissue (Figure 4.22a). In contrast, exposure of leaflet tissue to F-BAV FSS resulted in widespread BMP-4 and TGF-β1 expressions localized to the sub-endothelial layer of the fibrosa. Those results are supported by the Western blot results (Figure 4.22b and c) that suggest significant (p<0.05) increases in BMP-4 and TGF-β1 expression in tissue exposed to F-BAV FSS with respect to the fresh controls (2.4-fold and 3.7-fold increase, respectively). In contrast, BMP-4 and TGF-β1 levels measured in response to tissue conditioning under TAV and NC-BAV FSS were not statistically different from those in fresh tissue. Those results demonstrate that the
specific FSS environment experienced by the F-BAV leaflet is able to stimulate paracrine signaling via BMP-4- and TGF-β1-dependent pathways.

Figure 4.22: Effects of TAV and BAV FSS on paracrine signaling: a) BMP-4 and TGF-β1 immunostaining, b) immunoblotting and c) densitometric results in porcine aortic valve leaflets subjected to TAV and BAV FSS (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control; #: p<0.05 vs. TAV FSS; +: p<0.05 vs. NC-BAV FSS).

4.4.4 Effects of FSS abnormalities on α-SMA and Runx2 expressions

The effects of BAV FSS abnormalities on VIC differentiation mechanisms were investigated by measuring α-SMA and Runx2 expressions following tissue exposure to TAV and BAV FSS. α-SMA is a non-specific indicator of the myofibroblast phenotype, while Runx2 is a key transcription factor associated with osteoblast differentiation and is also a downstream transcription factor for BMP-4. While the trends emerging from the
immunoblotting data suggested increased $\alpha$-SMA expression in response to F-BAV and NC-BAV FSS (2-fold, 1.2-fold increase, respectively) relative to the level measured in fresh controls, the absence of statistical significance suggested that TAV and BAV FSS essentially maintained $\alpha$-SMA expression level (Figure 4.23). Similarly, while Runx2 expression exhibited a similar trend (1.3-fold and 1.2-fold increase in response to F-BAV and NC-BAV FSS, respectively), the upregulation was not statistically significant. While gene expression studies are required to confirm this observation, those results suggest that BAV FSS abnormalities did not trigger molecular mechanisms associated with VIC myofibroblastic and osteoblastic differentiation over the short duration of our experiments.

Figure 4.23: Effects of TAV and BAV FSS on $\alpha$-SMA and Runx2 expressions: a) $\alpha$-SMA and Runx2 immunoblotting and b) densitometric results in porcine aortic valve leaflets subjected to TAV and BAV FSS.
4.4.5 Effects of FSS abnormalities on ECM remodeling

The downstream effects of BAV hemodynamic abnormalities on valvular remodeling were investigated by comparing the secretion of the proteolytic enzymes MMP-2, MMP-9, their tissue inhibitor TIMP-2, and the elastolytic proteases cathepsin L and cathepsin S in the different tissue groups. Immunostaining results (Figure 4.24a) suggested increased enzymatic expressions in tissue exposed to BAV FSS as compared to those measured in tissue exposed to TAV FSS and in fresh controls. In addition, those molecules were primarily detected in the sub-endothelial layer of the fibrosa. Tissue exposed to TAV and BAV FSS consistently exhibited more TIMP-2-positive cells than fresh tissue and, when detected, TIMP-2 expression was localized in the leaflet sub-endothelial layers. The semi-quantitative analysis of the immunostaining results supported those observations (Figure 4.24b). F-BAV FSS abnormalities resulted in a significant increase in MMP-2, MMP-9, cathepsin L, cathepsin S and TIMP-2 expression relative to fresh controls (6.3-fold, 16.8-fold, 11.7-fold, 16.7-fold and 5.5-fold respectively; p<0.05).

While the exposure of leaflet tissue to NC-BAV FSS also resulted in the increased expression of each biomarker relative to the fresh controls, only MMP-9 and TIMP-2 were significantly upregulated (14.2-fold and 3.8-fold increase, respectively; p<0.05). In contrast, TAV FSS maintained MMP and cathepsin expressions at the same levels as those in fresh tissue. The impact of the different FSS environments on the remodeling state of the tissue was also investigated by examining TIMP-2-to-MMP-2 ratio (Figure 4.24c). Interestingly, while no statistical difference was found between the groups, the overall trend indicated higher ECM remodeling potential (i.e., smaller TIMP/MMP ratio) in response to F-BAV FSS (TIMP/MMP=109%) and NC-BAV FSS (TIMP/MMP=118%).
than in response to TAV FSS (TIMP/MMP=497%) or in fresh tissue (TIMP/MMP=156%). Gelatin zymography was carried out to quantify the active forms of MMP-2 and MMP-9 in the different tissue groups (Figure 4.25). The densitometric analysis of the zymograms revealed a significant increase in proteolytic enzyme activity in tissue exposed to F-BAV FSS as compared to fresh tissue (1.7-fold and 2.4-fold increase in MMP-2 and MMP-9 activity, respectively; p<0.05). Those results suggest that BAV FSS abnormalities tend to break valvular homeostasis by upregulating catabolic enzyme expression and activity, and creating an imbalance between catabolic enzymes and their tissue inhibitors.
Figure 4.24: Effects of TAV and BAV FSS on valvular remodeling: a) MMP-2, MMP-9, TIMP-2, cathepsin L and cathepsin S immunostaining, b) semi-quantitative results and c) TIMP-2/MMP-2 ratio in porcine aortic valve leaflets subjected to TAV and BAV FSS (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control; #: p<0.05 vs. TAV FSS).
4.4.6 Effects of FSS abnormalities on osteocalcin expression

The investigation of bone matrix synthesis following VIC osteoblastic differentiation was performed via the detection of osteocalcin, a glycosylated phosphoprotein important in skeletal bone mineralization. Immunostaining results (Figure 4.26a) indicate abundant osteocalcin expression preferentially localized to the fibrosa and spongiosa of tissue exposed to F-BAV FSS. In contrast, fresh tissue and tissue exposed to TAV and NC-BAV FSS expressed little amount of osteocalcin. Consistent with those observations, the Western blot results suggest a 5-fold increase (p<0.05) in osteocalcin expression between tissue exposed to F-BAV FSS and fresh controls (Figure 4.26b). In contrast, similar osteocalcin levels were detected in fresh tissue, tissue exposed to TAV
FSS and tissue exposed to NC-BAV FSS. These data suggest the higher osteogenic susceptibility of the F-BAV leaflet to secrete bone-like matrix in response to its abnormal FSS environment.

Figure 4.26: Effects of TAV and BAV FSS on valvular osteogenesis: a) Osteocalcin immunostaining, b) immunoblotting and c) densitometric results in porcine aortic valve leaflets subjected to TAV and BAV FSS (F: fibrosa; green: positively stained cells; blue: cell nuclei; *: p<0.05 vs. fresh control; #: p<0.05 vs. TAV FSS; +: p<0.05 vs. NC-BAV FSS).
Chapter 5:
DISCUSSION

In this chapter, first of all, the contribution of FSS magnitude and frequency abnormalities on early CAVD pathogenesis will be discussed. The following section will be a discussion on the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling. The next section will discuss the effects of BAV FSS abnormalities on AV inflammation and remodeling. Lastly, limitation of this study will be discussed.

5.1 Effects of FSS magnitude and frequency abnormalities on early CAVD pathogenesis

The objective of specific aim 1 was to elucidate the respective and combined effects of abnormalities in FSS magnitude and frequency on the early mechanisms of CAVD in porcine aortic valve leaflets. The key results of specific aim 1 can be summarized as follows: 1) leaflet tissue is selectively more sensitive to alterations in FSS magnitude than frequency; 2) FSS abnormalities promote paracrine signaling via BMP-4 and TGF-β1-dependent pathways and ECM degradation via MMP- and cathepsin-dependent pathways; and 3) the FSS mechanisms of early CAVD development are time-dependent. Therefore, the results confirm the key contribution of FSS alterations to valvular pathogenesis and suggest the capability of FSS abnormalities to trigger early CAVD events in the absence of any other risk factor.
5.1.1 *Ex vivo* experimental model compared with *in vivo* and *in vitro* models

Experimental *in vivo* and *in vitro* models of CAVD proposed to date have relied on two leading hypotheses. The cholesterol hypothesis focuses on the traditional risk factors to study the biological events leading to CAVD [108,192,193]. In contrast, the hemodynamic hypothesis, which is foundation of the present study, assumes that mechanical stresses have the ability to activate these disease pathways. These divergent hypotheses have been reviewed in two theories on the development of CAVD: the LDL-density-radius theory [142] and the LDL-density-pressure theory [194], which account for the potential differences in timing and initiation events in the development of CAVD. While these theories have pointed out the important differences between the signaling of mechanical stresses and hypercholesterolemia, they have also suggested their intricate synergies. Therefore, the elucidation of CAVD pathogenesis requires the exploration of both the lipid and mechanical stress mechanisms of CAVD.

It is important to note that an abnormal FSS frequency per se is not an alteration likely to occur *in vivo*. Tachycardia conditions, which may result in acute FSS frequency alterations, are not chronic and therefore unlikely to contribute to a long-term pathological response. However, the rationale for varying the frequency of the FSS signal was to investigate the effects of changes in the harmonics of the FSS waveform experienced locally by the leaflets. Such phenomenon has been evidenced on BAV leaflets [114,195] and is also expected to occur with hypertension and aging, i.e., two known CAVD risk factors that affect leaflet dynamics and are likely to subject the leaflets to local alterations in the harmonics of the FSS signal.
5.1.2 Pathogenic nature of elevated FSS magnitudes

The results demonstrated the particular sensitivity of valvular pro-inflammatory pathways to elevated FSS levels as illustrated by the increased expressions of the cytokines BMP-4 and TGF-β1. The ability of supra-physiologic mechanical stress environments to trigger pro-inflammatory processes has been observed ex vivo in the contexts of elevated pressure, stretch and FSS. Exposure of porcine aortic valve leaflets to hypertensive levels in a pressure chamber was shown to upregulate pro-inflammatory gene networks in interstitial cells [196]. In another study, the long-term (14 days) ex vivo exposure of porcine valve leaflets to elevated stretch with an osteogenic culture medium demonstrated stretch magnitude-dependent tissue mineralization mediated by BMP-4 and TGF-β1 pathways [189]. Similarly to elevated stretch and pressure, the present results indicate that supra-physiologic FSS magnitudes increase the vulnerability of valvular tissue to calcification via BMP-4- and TGF-β1-dependent pathways. Therefore, the findings suggest the pathogenic nature of elevated FSS magnitudes and their potential role as a trigger of mechanosensitive events leading to CAVD initiation and development. Those results are also consistent with previous in vivo evidence of the role played by BMP-4 in CAVD pathogenesis [197-199].

5.1.3 Role of proteolytic enzymes

The study also indicated increases in elastolytic enzyme expression and proteolytic enzyme expression and activity under elevated FSS magnitude (at normal frequency) and abnormal FSS frequency (at normal magnitude), suggesting the progressive loss of valvular homeostasis and the alteration of valvular biosynthetic activity. The ability of supra-physiologic mechanical stress environments to break the delicate balance between ECM
synthesis and degradation has been observed previously in the contexts of elevated pressure, stretch or a combination of these. Hypertensive levels have been shown \textit{ex vivo} to promote collagen and sulfated glycosaminoglycan (sGAG) production but to decrease cathepsin L, MMP-2 and MMP-9 expression and activity in porcine leaflets [132,200]. Elevated strain levels have been shown to reduce sGAG content but to promote cellular proliferation and apoptosis, collagen content and proteolytic enzyme expression and activity in porcine leaflets [86]. Lastly, valve leaflets subjected \textit{ex vivo} to combined pathological levels of pressure and stretch exhibit increased ECM synthesis and a progressive switch of the valve interstitial cells from a contractile to a more synthetic phenotype [201]. Consistent with those observations, the present study suggests that the expression and biosynthetic activity of some proteolytic enzymes may be mechanosensitive and play a key role in the progression of CAVD under altered mechanical loading.

An interesting result is the apparent synergistic effects of FSS magnitude and frequency abnormalities on ECM degradation and paracrine signaling. In fact, leaflets exposed to physiologic FSS or combined abnormalities in FSS magnitude and frequency (i.e., four corners of the maps shown in Figure 4.10) exhibited low pro-inflammatory and remodeling state index values. Interestingly, while leaflet exposure to elevated FSS magnitude (at normal frequency) produced the most significant pathological response, the combination of elevated FSS magnitude and abnormal frequency returned the expressions of cell-adhesion molecules, pro-inflammatory cytokines and proteases to their physiologic baseline levels. The same attenuating effect between mechanical stress magnitude and frequency has been observed in valvular response to cyclic pressure, for which collagen and sGAG syntheses were increased in response to elevated pressure magnitude and
frequency, but to a lesser extent than in response to elevated pressure at normal frequency [123]. Those results highlight the complexity of valvular mechanobiology and the sensitivity of valvular tissue to both the magnitude and frequency of mechanical stress signals.

5.1.4 Side-specific biological responses

It is important to note that the immunofluorescence images included in the paper focused on the fibrosa side due to the complete absence of positive stain on the ventricularis. Depending on the mechanical treatment, protein and enzyme expressions were distributed either in the subendothelial layer of the fibrosa or deeper in the tissue. Those results may correlate with the previously established phenotypic difference between the VECs lining the leaflet fibrosa and ventricularis [53].

The implementation of a double cone-and-plate bioreactor in this study enabled the simultaneous exposure of the leaflet fibrosa and ventricularis to their respective native FSS directionality. Therefore, our study is the first one to investigate the effects of side-specific FSS on valvular biology. However, the isolated effects of FSS magnitude on valvular cytokine expression and endothelial activation have been studied previously using a single-sided FSS bioreactor subjecting the fibrosa to mild and severe supra-physiologic FSS levels [134]. The exposure of the fibrosa to increasing FSS levels while maintaining the ventricularis under a static flow condition resulted in the same FSS magnitude-dependent increase in cytokine expression as that observed in the present study. In contrast, the non-significant increase in VCAM-1 and ICAM-1 expressions suggested by the results in response to elevated FSS contrasts with the significant endothelial activation response observed in that study following the exposure of the fibrosa to mild and severe supra-
physiologic FSS magnitudes. We speculate that the absence of significant endothelial dysfunction in the present study may be due to the implementation of a more physiologic FSS bioreactor, which subjected simultaneously both leaflet surfaces to side-specific FSS.

5.1.5 Time-dependence of the FSS mediated biological response

Another contribution of specific aim 1 is the elucidation of the timescale required for the transduction of valvular FSS into a biological response. While the results indicate that the FSS-mediated pathological response after 24 hours of conditioning was essentially similar to that obtained in fresh tissue (i.e., initial time point), cytokine and protease expressions peaked after 48 hours of exposure to FSS and then remained relatively constant until the 72-hour mark. Similar temporal responses have been observed previously in the contexts of valvular mechano-response to cyclic FSS [133,134,167,202] and stretch [86,183]. Those results in specific aim 1 support the previous evidence that 48 hours of conditioning are necessary and sufficient for the initial transduction of mechanical abnormalities into a pathological response [31]. Interestingly, the temporal response also indicates that the activation of the leaflet endothelium, which also occurred at 48 hours, did not precede the upregulation of cytokines and proteases. This result is consistent with the earlier ex vivo study on the single-sided effects of FSS magnitude on valvular biology, which indicated that the pharmacological supplementation or inhibition of TGF-β1 had a dramatic effect on adhesion molecule and BMP-4 expressions but BMP-4 supplementation and inhibition resulted in a milder effect on the overall pathological response [134]. Those results and the observations described in the present study support the upstream role of paracrine signaling in the FSS-mediated response relative to endothelial activation mechanisms.
The comparison of the results obtained in the fresh control group and in the group exposed to physiologic FSS for 48 hours (group 1) revealed no statistical difference in the expression of the different biomarkers considered in the study. Only MMP-9 activity was significantly downregulated under physiologic FSS as compared to the control group (78% decrease). To understand the significance of this result, it is important to note that the valve functions in a complex mechanical environment consisting of cyclic stretch, pulsatile pressure and FSS [154]. Therefore, specific aim 1 suggests that, in the absence of any other mechanical stimulus, FSS might be able to maintain relatively normal valvular homeostasis over 48 hours. This result is consistent with the previous demonstration of the constant renewal of valvular tissue in response to mechanical stimulation [152] and indicates that, while the full spectrum of mechanical signals may be needed to maintain valvular homeostasis, FSS may play a dominant role over other forces in this process.

5.2 Role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling

5.2.1 Role played by BMP-4 and TGF-β1

Some studies indicated BMP-4 is involved in activating the smad- and NFκB-pathways, which are responsible for increasing expression of ICAM-1 and VCAM-1 [88]. TGF-β1 has been shown to promote endothelial activation and ECM remodeling in porcine valve and VIC differentiation into active myofibroblasts in a dose-dependent manner [95,203]. However, the results in specific aim 3 indicated that BMP-4 or TGF-β1 supplementation didn’t result in significant increase in ICAM-1, VCAM-1, MMP-2, cathepsin L and cathepsin S. An interesting finding is that, under both supra-physiological
FSS magnitude and supra-physiological FSS frequency, inhibition of TGF-β1 significantly decreased the expression of MMP-9 compared with normal culture medium group. This indicates that TGF-β1 plays an important role in valvular remodeling through regulating MMP-9 expression. This finding is supported by several studies, which indicate MMP-9 expression is modulated by several growth factors and inflammatory cytokines such as TGF-β1 in sheep VICs [61,204].

5.2.2 Synergistic effect of BMP-4 and TGF-β1

Hoehn et al proposed a hypothetical model of the interactions between TGF-β1 and BMP-4 in response to pathologic alterations in valvular shear stress magnitude: an increase in shear stress leads to activation of TGF-β1 and the binding of TGF-β1 to its receptor. The primary pathway leads to an increase in endothelial ICAM-1 and VCAM-1 and a secondary pathway leads to the activation of BMP-4. BMP-4 binds to its receptor and signals ICAM-1 and VCAM-1 as well as a positive feedback signal that leads to an increase in TGF-β1 [134]. However, the results in specific aim 2 did not seem to support this pathway. BMP-4 treatment didn’t result in any increase of TGF-β1 and adhesion molecule expressions in any FSS conditions, while promotion of TGF-β1 binding didn’t result in any upregulation of all biomarkers tested.

5.2.3 Possible reasons for the inconsistency

Possible reasons that no significant expression of ICAM-1, VCAM-1, MMP-2, cathepsin L and cathepsin S was observed in BMP-4 or TGF-β1 treatment group may be explained by the following aspects. One explanation is short duration of the experiments. Since Noggin and SB-431542 are preventing the binding of BMP-4 and TGF-β1 with their receptors, the expression of ICAM-1, VCAM-1, MMP and cathepsin, which may be
downstream in the BMP-4 and TGF-β1 related pathway, will occur relatively late resulting in the absence of significant changes in the expression of these molecules in the acute response. Longer duration experiments, such as 7 days or 14 days, may address different roles played by BMP-4 and TGF-β1. Another explanation is that, due to the crosstalk between TGF-β1/ BMP-4 and other signaling pathways, recombinant TGF-β1 alone or recombinant BMP-4 alone may not promote significant endothelial activation and valvular remodeling. For example, one study demonstrated that combining TGF-β1 with Wnt3A induced greater myofibroblast differentiation and α-SMA transcription than TGF-β1 treatment alone in VICs [205]. To determine whether Noggin and SB-431542 efficiently prevent the binding of BMP-4 and TGF-β1 with their receptors, expression of SMAD signaling can examined.

5.3 Effects of BAV FSS abnormalities on initiation and progression of CAVD

Despite the compelling evidence for the involvement of hemodynamic stress abnormalities in valvular disease, the elucidation of the role played by the native BAV hemodynamics in CAVD development has been hampered by its complexity and the challenge to replicate it in the laboratory setting. Using an ex vivo approach enabling the exposure of aortic valve leaflets to in vivo-like TAV and BAV FSS, we demonstrated the potential of BAV hemodynamic stresses to trigger biological events marking the early stage of CAVD, namely endothelial activation, pro-inflammatory paracrine signaling, ECM degradation and possibly osteogenesis. The differential tissue responses following exposure to TAV, F-BAV and NC-BAV FSS confirm the sensitivity of aortic valve leaflets to their surrounding FSS environment and indicate the pathogenic potential of the native
BAV hemodynamic environment as well as the particular vulnerability of the F-BAV leaflet to calcification.

5.3.1 Role of BAV FSS abnormalities

The main contribution of specific aim 2 is the demonstration of the key role played by BAV hemodynamic stress abnormalities in the initiation and progression of CAVD. The use of normal AV leaflet tissue in the experiments permitted to eliminate the possible involvement of any intrinsic genetically programmed biology in the biological responses. Therefore, the only factor that could possibly produce the differential biological outcomes described in this study is the mechanical treatment to which each tissue group was subjected. Another important contribution is the apparent dependence of the severity of the pathological response on the imposed degree of FSS abnormality. While leaflets conditioned under NC-BAV FSS essentially exhibited the same biological signature as fresh tissue or leaflets conditioned under TAV FSS, leaflets exposed to F-BAV FSS demonstrated upregulation in all CAVD biomarkers except those associated with VIC differentiation mechanisms (i.e., α-SMA and Runx2). Interestingly, the previous characterization of TAV and BAV hemodynamics had revealed the existence of drastic differences in FSS magnitude, which attain a maximum in the base of the fibrosa (600% and 240% increase in FSS magnitude on the F-BAV and NC-BAV leaflet, respectively, relative to the TAV leaflet) and become increasingly significant during valvular sclerosis [114]. Clinical studies have also shown that the F-BAV leaflet is the most common site of calcific lesion formation in type-1 BAV [147]. This parallel provides compelling support to the hemodynamic theory of CAVD in the BAV and suggests that BAV hemodynamics
may contribute to the rapid and severe development of calcific lesions by imposing abnormally elevated FSS in the base of the F-BAV leaflet.

Another interesting point is the apparent contrast between the low sensitivity of α-SMA and Runx2, as suggested by the absence of statistical significance between the different tissue groups, and the global increase in pro-calcific potential observed in response to BAV FSS. One explanation for this apparent inconsistency may be related to the short duration of the experiments. While α-SMA and Runx2 are not specific markers of VIC myofibroblastic and osteoblastic differentiations, the maintenance of their expression levels may indicate the non-active state of those processes. Since VIC activation is an event occurring relatively late in the calcification pathway, the absence of significant changes in the expression of these molecules in the acute response described in this study was expected. Complementary studies focusing on gene and ED-A fibronectin expression (i.e., cellular fibronectin expressed by fibroblasts at very early stages of differentiation into myofibroblasts) and calcium assay are needed to examine this hypothesis.

5.3.2 Leaflet-specific biological responses

In addition to this side-specificity, the results indicate that FSS-induced endothelial activation is leaflet-specific in the BAV and occurs primarily on the fused leaflet. This observation can be explained by the leaflet-specific FSS environment present in the BAV, which is characterized by highly elevated and mildly elevated FSS magnitudes on the fibrosa of the fused and non-coronary leaflets, respectively, relative to TAV leaflets (6-fold and 2.4-fold increase, respectively) [114] and the previously established ability of supra-physiologic FSS levels to trigger endothelial activation in a FSS magnitude-dependent manner [134].
5.3.3 Accelerated pathogenesis of CAVD

A striking observation is the magnitude of the pathological response following the acute exposure of normal valve leaflets to BAV hemodynamic abnormalities. While this response does not seem consistent with the relatively slow progression of CAVD, which occurs over years in vivo [206,207], this accelerated pathogenesis may be explained by the particular ex vivo environment produced by the FSS device, which purposely eliminated all mechanical cues normally experienced in vivo but FSS. In vivo, the difference of pressure across the valve generates a compression force normal to the leaflet fibrosa, which varies cyclically during the cardiac cycle and attains a peak during diastole [5]. The deformation of the leaflets in response to this cyclic pressure in turn generates an axial stretch, which affects the length of the leaflet [5]. While pressure and stretch are critical to proper valvular function and coaptation, they also play critical roles in valvular homeostasis and disease by regulating ECM biosynthesis and degradation, cellular proliferation and differentiation, and inflammatory processes. Collagen, sulfated glycosaminoglycan (sGAG) and DNA syntheses have been shown to be modulated in pressure and stretch magnitude- and frequency-dependent manners [123,183]. While pulsatile pressure tends to downregulate VIC differentiation into myofibroblasts [123,208], cyclic stretch was shown to upregulate this contractile phenotype [183]. In addition, the expression and activity of proteolytic enzymes such as cathepsins and MMPs, which play important roles in CAVD pathogenesis, were shown to be mechanosensitive to stretch [86]. Lastly, elevated pressure and stretch levels have been shown to modulate valvular inflammation via BMP-4- and TGF-β1-dependent pathways [136,189,200,209]. Those studies suggest that valvular homeostasis is maintained, at least partially, by synergies
between the mechanical signals received by the leaflets *in vivo*. Therefore, the absence of the compensatory mechanisms regulated by stretch and pressure may have compromised valvular homeostasis and amplified the pathological response observed in the experiments.

5.3.4 VECs and VICs in FSS mechanotransduction

In addition, the results in specific aim 2 provide further insights into the role played by VECs and VICs in FSS mechanotransduction. As compared to stretch and pressure, which propagate throughout the leaflet and stimulate both VECs and VICs, FSS is an interfacial stress sensed primarily by VECs. Interestingly, while BAV FSS abnormalities upregulated cell-adhesion molecule expression in VECs lining the leaflet fibrosa, all other downstream effects (i.e., cytokine expression, ECM remodeling, osteogenesis) are VIC-specific processes. This observation suggests that VECs are able not only to sense FSS abnormalities but also to transduce those signals deeper in the tissue by altering VIC function. This is consistent with recent studies that have evidenced the existence of an intricate communication network between the two cell types, as illustrated by the difference in collagen and sGAG synthesis between intact and endothelium-denuded leaflet tissue subjected to FSS [154], the change in interstitial cell phenotype and matrix synthesis between intact and endothelium-deprived VIC-VEC co-culture models exposed to steady FSS [27], and the modulation of valvular ECM remodeling by FSS magnitude [132]. Although the mode of communication between the two cell types remains largely unknown, paracrine expression has been suggested as a potential mechanism. *Ex vivo* studies have shown the upregulation of pro-inflammatory cytokines in the leaflet interstitium following endothelial exposure to abnormal FSS magnitude or pulsatility [133,134]. Therefore, the increased BMP-4 and TGF-β1 expressions detected in response
to BAV FSS abnormalities provides further evidence of the involvement of paracrine signaling in VEC-VIC communication and its role in the transduction of FSS abnormalities into a pathological response.

5.3.5 Local FSS investigation

Lastly, although experimental and analytical techniques have been used to estimate TAV and BAV leaflet FSS [115,122,165,195], the choice to consider predicted rather than measured FSS values was based on the requirement to expose the circular leaflet specimens to their local FSS. As compared to the experimental techniques, which provide FSS estimates at a point or along a line, the use of a computational model [114] permitted to generate FSS data over the exact leaflet region from which the circular tissue samples were extracted. In addition, while the experimental approach should always be considered the gold standard for flow characterization, it may not be the most suitable for the calculation of spatially dependent quantities such as FSS, especially on moving valve leaflets. In fact, experimental uncertainties in the estimation of the distance between the location of the velocity measurement and the moving wall and in the measurement of near-wall flow velocities may compromise the calculation of the spatial velocity gradient near the leaflet surface and, in turn, the estimation of the FSS. Those considerations justify the need for a better characterization of valvular FSS.
5.4 Limitations

5.4.1 Experimental duration

The experimental duration considered in this study was limited to 48 hours. While leaflet calcification in both the TAV and the BAV typically occurs over a much longer timescale than that considered in this study, the present results suggest acute differences in the biological responses of leaflet tissue exposed to TAV and BAV FSS. Those acute effects are necessary for the characterization of the contribution of BAV hemodynamic abnormalities to valvular calcification since they may shape the longer-term mechanisms involved in the progression of valvular disease. Nevertheless, the ability to extend the experimental duration would provide a more complete map of the processes involved in the formation of BAV calcific lesions. Moreover, the leaflet tissue, cultured first under 48 hour non-physiological FSS and then 48 hour physiological FSS, can be used to examine whether these acute effects are reversible or not.

5.4.2 Experimental *ex vivo* model

The *ex vivo* approach adopted in this study enabled the characterization of the valvular mechano-sensitive response to TAV and BAV WSS while preserving the native architecture of the leaflets (i.e., VICs and VECs embedded in their extracellular matrix). Although this approach provides insights into the global tissue response resulting from the native cell-cell and cell-ECM interactions, it is not capable of providing a mechanistic description of those processes or isolating the response of each cell type. Gene and cell studies are needed to better characterize the signaling pathways triggered by FSS abnormalities.
5.4.3 Physiological FSS waveform

*In vivo*, FSS experienced by AV leaflet is side-specific. In the current work, FSS applied in the bioreactor is based on Ge’s computational fluid dynamics simulations. Studies on the characterization of more physiological side-specific FSS experienced by valve leaflets under such conditions are currently under way in our laboratory. Specifically, we are developing computational and experimental methodologies based on FSI modeling and particle-image velocimetry, respectively, to capture the native valvular FSS state under such conditions. The programming of the new FSS apparatus with those pathological waveforms will permit to investigate the effects of pathologically relevant FSS on valvular pathogenesis.

5.4.4 Generalization to human CAVD

Lastly, it is important to recognize that although the biological results described in this paper might translate to adult human valves, one should account for the intricate differences between porcine and human valve tissues and their respective biology. In this context, while this study suggests a link between BAV hemodynamic abnormalities and early calcification mechanisms, the generalization to human valvular disease pathogenesis requires complementary experiments with human tissue.
Chapter 6:
CONCLUSION

This dissertation investigates the effects of normal and abnormal FSS on early progression of CAVD. These experiments were performed on porcine leaflets using an *ex vivo* FSS bioreactor. The answers provided by this thesis to the initial research questions can be outlined as follows.

*What are the mechanisms by which FSS alterations are transduced into valvular pathological responses?*

In specific aim 1, this study investigated the mechanisms of adaptation of valve leaflets to FSS abnormalities and the potential of supra-physiologic FSS magnitude or sub-/supra-physiologic FSS frequency to upregulate key CAVD mechanisms such as paracrine signaling and ECM degradation. The results revealed the sensitivity of valve leaflets to FSS abnormalities and the ability of supra-physiologic FSS magnitude or sub-/supra-physiologic FSS frequency to upregulate key CAVD mechanisms such as paracrine signaling and ECM degradation (Figure 6.1). Therefore, this work confirms the key contribution of FSS alterations to valvular pathogenesis and suggests the capability of FSS abnormalities to trigger early CAVD events even in the absence of any other risk factor.
Figure 6.1: Upregulation and downregulation of early markers of CAVD in response to FSS abnormalities (↓↓: 50%–75% expression vs. fresh tissue; ↓: 75%–100% expression; ↑: 100%–200% expression; ↑↑: 200%–500% expression; ↑↑↑: 500% expression; p,0.05 for all results).

Are BMP-4 and TGF-β1 potential target molecules for the pharmacological treatment of CAVD?

In specific aim 2, the role of BMP-4 and TGF-β1 in FSS-induced valvular endothelial activation and ECM remodeling was investigated by promoting/inhibiting BMP-4 or TGF-β1 in the culture medium. The results indicated that TGF-β1 inhibition directly regulated valvular remodeling through MMP-9 downregulation (Figure 6.2). In contrast, BMP-4 or TGF-β1 supplementation or inhibition did not result in any significant changes in endothelial activation (ICAM-1, VCAM-1) and ECM remodeling (MMP-2, cathepsin L and cathepsin S). Promotion of BMP-4 did not lead to an increase in TGF-β1. Therefore, this specific aim provides evidence that SB-431542 is a potential candidate molecule for regulating ECM remodeling. In addition, while BMP-4 and TGF-β1 are mechanosensitive
to FSS (see specific aim 1), their inhibition is not sufficient to completely block the FSS-induced pathological response.

![Diagram of Abnormal FSS](image)

**Figure 6.2:** A model of TGF-β1 in response to pathologic alterations in FSS

What are the reasons for the early development and severity of BAV calcification?

Specific aim 3 clearly demonstrated the pro-calcific potential of the hemodynamic abnormalities produced by the BAV anatomy. The data indicated that the native FSS abnormalities present on the F-BAV leaflet promote endothelial activation on the fibrosa, pro-inflammatory paracrine signaling and catabolic enzyme secretion, and bone matrix synthesis. Although the results seem to point to the existence of correlations between the local degree of FSS abnormality on BAV leaflets and leaflet vulnerability to CAVD, further investigations are required to demonstrate causality. The results of this thesis also suggest that calcific BAV disease may not solely result from a particular genetic predisposition to calcification but from complex synergies between the intrinsic genetically
programmed biology of BAV tissue and the hemodynamic abnormalities experienced by BAV leaflets.

Figure 6.3: Hemodynamic theory of valvular calcification. The red arrows describe the relationships established by this thesis.

In conclusion, the importance of FSS in valvular inflammation, ECM remodeling has been demonstrated in this dissertation. The ex vivo provide further support to the hemodynamic theory of valvular calcification. Elevated FSS magnitudes and BAV FSS are triggers of mechanosensitive events (inflammation and ECM remodeling) leading to CAVD initiation and development (Figure 6.3). The findings can be used to further elucidate the molecular signaling pathways mediated by the FSS environment in order to develop non-invasive drug-based therapies for CAVD.
Chapter 7:
SUGGESTED FUTURE WORK

The current study instigated the effects of FSS on early progression of CAVD. Based on the results, there are exciting possibilities for future work.

7.1 Effect of Combined Mechanical Forces

This study has focused on understanding the effects of isolated mechanical stimulation, FSS, on CAVD. The next step is to investigate the effects of combined loadings on AV using more complicated bioreactors. The loadings will be two or more mechanical forces. Van Dyke WS et al has developed a mechanical bioreactor for concomitant FSS and substrate strain that can be applied in cellular-level (Van Dyke WS 2012). Or an organ culture system, which can mimic combined mechanical forces on valve leaflets, can help to investigate the early progression of CAVD in more efficient way.

7.2 Understanding the signaling pathways of CAVD

This work was focused on understanding the “big picture” identifying key molecules involved in FSS-induced valve calcification, such as ANGII, TWIST1 and IL-β1 and identifying key gene mutation, like NOTCH1, HEY1 and HEY2. Further research may focus on potential pathways, such as Lrp5/wnt/β-catenin signaling, angiotensin
converting enzyme pathway, which may play an important role in the future potential medical therapy.
A.1 Convert shear stress to angular velocity

\% Angular Velocity calculator (rps)

clear all

rho=1056; \%kg/m^3
mu=0.00095; \%kg/(m*s)
r=0.02; \%m
h=0.0002; \%m
a=0.5*2*pi/360; \%rad

v=mu/rho;
x=load('ss.txt');
t = x(:,1);
ss = x(:,2); \%shear stress
av = ss/10*(h+r*tan(a))/mu/r/(2*pi); \%angular velocity

plot(t,av,'b')

A.2 Code generator for aortic side

\%servo_code.m
\%Motion profile code generator for the GEMINI GV6K servo drive
\%This program generates four lines of code: time, acceleration, velocity \%
\%and displacement of the motor shaft.
clear all;

time_interval = 86;
delta_t=0.01;
\%load the velocity (rps)
x=load('ss_av.txt');
av = x(:,1);
disp('MC0')
disp(' ')
for i=1:time_interval  
% i is the number of points at which the vel and disp info are calculated

vel_rps = av(i);
%if vel_rps<0
%  vel_rps=0;
%  flag=1;
%end;
% calculate the angular displacement over the time step (radian)
% disp_rad = vel_rads*delta_t;
disp_rad = vel_rps*(2*pi)*delta_t;
% convert displacement in motor unit (4000 motor units = 2 pi radians)
disp_mu = disp_rad*4000/(2*pi);
% following loop avoids having a disp_mu of zero.
if disp_mu < 1 && disp_mu > 0;
  disp_mu=1;
elseif disp_mu < 0 && disp_mu > -1;
  disp_mu=-1;
else
  disp_mu=round(disp_mu);
end
% generate the lines of code
line1=['V',num2str(abs(vel_rps))];
line2=['D',num2str(disp_mu)];
line3=['GOBUF1'];
disp(line1)
disp(line2)
disp(line3)
% disp(i)
% generate VF0.000
if (av(i) > 0 && av(i+1) < 0) || (av(i) < 0 && av(i+1) > 0)
  line4=['VF0.000'];
  line5=['GOBUF1'];
disp(line4)
disp(line5)
end
end;
disp(' ')
disp('END')
A.3 Code generator for ventricular side

%servo_code.m
%Motion profile code generator for the GEMINI GV6K servo drive
%This program generates four lines of code: time, acceleration, velocity
%and displacement of the motor shaft.
clear all;

time_interval = 86;
delta_t=0.01;
%load the velocity (rps)
x = load('ss_av.txt');
av = x(:,1);
disp('MC01')
disp('A400.000')
disp(' ')
for i=1:time_interval
    %i is the number of points at which the vel and disp info are calculated
    vel_rps = av(i);
    if vel_rps<0
        vel_rps=0;
        flag=1;
    end;
    disp_rad=vel_rps*(2*pi)*delta_t;
    disp_rad=vel_rps*(2*pi)*delta_t;
    disp_mu=disp_rad*4000/(2*pi);
    if disp_mu < 1 && disp_mu > 0;
        disp_mu=1;
    elseif disp_mu < 0 && disp_mu > -1;
        disp_mu=-1;
    else
        disp_mu=round(disp_mu);
    end
    line1=['V',num2str(abs(vel_rps))];
    line2=['D',num2str(disp_mu)];
    line3=['GOBUF1'];
    disp(line1)
    disp(line2)
    disp(line3)
    disp(i)
end
gerenate AD400.000
if i >= 2 && i < time_interval
    if ( av(i) > av(i+1) && av(i) > av(i-1))
        line4=[''];
        line5=['AD400.000'];
        line6=[''];
        disp(line4)
        disp(line5)
        disp(line6)
    end
end
end;
disp(' ')
disp(' END')

A.4 Bioreactor connection

- Turn on both servo drives. (Light should be red)

- Prepare Drive 2.
  - Connect null modem cable (Labeled Drive2) from computer to the RS 232/485 port on Drive 2.
  - Type the following commands in the terminal on computer:
    ADDR2 (Sets the address of the drive as 2)
    ERRVL0 (Sets the error level to 0, so that prompts do not continuously pop up.)
  - Disconnect cable from the computer end.

- Prepare Drive 1.
  - Connect other null modem cable (Labeled Drive1) from computer to the RS 232 port on Drive 1.
  - Type the following commands in the terminal on computer:
    ADDR1 (Sets the address of the drive as 1)
    ERRVL0 (Sets the error level to 0, so that prompts do not continuously pop up)
  - Disconnect cable from the computer end.
  - Connect null modem cable (Labeled Drive2) to RS 232/485 port on Drive1.

- Connect the cable (Labeled Drive1) to the computer. (from RS 232 Drive 1 to computer)
- Current Connections Summary
  o One null modem cable should connect the RS 232/485 ports of Drive 1 and Drive 2.
  o One null modem cable should connect the RS 232 port of Drive 1 to the computer.
  o The RS 232 port on Drive 2 should be open.
- Type the following commands into the terminal on the computer. These lines allow the servo drives to communicate with each other.
  ERLVL0
  1_PORT1
  1_ECHO2
  1_PORT2
  1_ECHO3

- From this point on, anything typed in the terminal should appear double (eg. LAB would appear as LLAABB)

- Set up User Buttons
  o Right click one of the User Buttons on the right hand side of the terminal
  o Type: 1_raortp : 2_rventp (there are two “space” before and after the “:”)
  o Click Enter

- Type the following in the terminal:
  DRIVE1 (Will appear as DDRRIIVVEE11)
  LH0 (Will appear as LLHH00)

- Click on the User button you set up. This should run both motors simultaneously. If only one starts, stop the program and click the user button again. You may have to try this up to three times.
APPENDIX B:

EXPERIMENT PROTOCOLS

B.1 Autoclave

Autoclave

Preparation:
- Turn on the autoclave and the Boiler (big switch on wall)
  - Wait ~1 hour until the jacket pressure reaches set value

Fluid

Preparation:
- Place a small piece of autoclave tape on the lid
- Fill the autoclave tub with ~1" of water
- Loosen the caps of the PBS bottles and set in tub
- Place tub in Autoclave and close the door (display should read ‘door closed’)

Operation:
- Press (1) for cycle selection
- Press (3) for liquid
  - Adjust the parameters accordingly
    - Jacket Pressure $\rightarrow$ 20.0 psi
    - Chamber Temp $\rightarrow$ 250.0 °F
    - Sterilizing Time $\rightarrow$ 20 min
    - Positive Pulses $\rightarrow$ OFF
- Press (9) to exit
- Press (9) to exit
- Press ‘Run’
- Press ‘Enter’

Post Operation: (careful it will be very hot!)
- Wear Gloves
- Remove Tub and close lids

Non-liquid Items

Preparation:
- Seal items in Autoclave bags, date and initial
- Place items in Autoclave Tub

Operation:
- Press (1) for cycle selection
- Press (2) for dry
  - Adjust the parameters accordingly
    - Jacket Pressure $\rightarrow$ 20.0 psi
    - Chamber Temp $\rightarrow$ 250.0 °F
    - Sterilizing Time $\rightarrow$ 10 min
    - Positive Pulses $\rightarrow$ OFF
- Press (9) to exit
- Press (9) to exit
- Press ‘Run’
B.2 Slaughterhouse trip

**Slaughterhouse trip**

**Preparation:**

The day before:
- notify the slaughterhouse (Martin's Custom Butchering, 574-862-2982)
- prepare 2L of 1X PBS in a glass container and autoclave
- autoclave the container filled with 1X PBS
- autoclave 1 pair of large scissors
- autoclave 1 pair of large scissors, 1 pair of curved scissors and 1 pair tweezers all in the same bag
- place the sterile 1X PBS in the sterile hood
- place the plastic containers in the sterile hood
- place the bag with 2 pairs of scissors and tweezers in the sterile hood
- run the decontamination cycle (UV light on)

Before leaving to the slaughterhouse:
- fill the cooler with ice
- put 0.5L of sterile 1X PBS in each plastic container (work in the sterile hood) and close the containers
- put the containers, the surgical pad and the scissors in the cooler

At the slaughterhouse:
- keep the plastic containers closed until tissue is ready to be placed
- cut the heart keeping a few inches of aorta attached
- place the heart in the container and close
B.3 Culture medium

**Culture medium preparation**

**Recipe (1L of DMEM):**

1. Combine all following chemicals

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Substance</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.36 g</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
<td>Fridge</td>
</tr>
<tr>
<td>0.05 g</td>
<td>ascorbic acid</td>
<td>Fridge/acid cabinet</td>
</tr>
<tr>
<td>1 ml</td>
<td>100X non-essential amino-acids</td>
<td>Fridge</td>
</tr>
<tr>
<td>3.7 g</td>
<td>sodium bicarbonate (NaHCO₃)</td>
<td>Cupboard</td>
</tr>
<tr>
<td>1000 mL</td>
<td>NanoPure H₂O</td>
<td>Acid cabinet</td>
</tr>
<tr>
<td>1 M HCl</td>
<td></td>
<td>Acid cabinet</td>
</tr>
<tr>
<td>1 M NaOH</td>
<td></td>
<td>Base cabinet</td>
</tr>
</tbody>
</table>

2. Adjust pH to 7.4 using 1M HCl or 1M NaOH or CO₂

3. Sterile Filter under the hood

4. Store in 4°C fridge.

5. Add 5% antibiotics before using this culture medium
B.4 H&E Immunohistochemistry

H&E Immunohistochemistry on Frozen Sections

Materials:

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin</td>
<td>Harris' Hematoxylin w/o Mercury (EMS: 26041-20)</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Eosin Y, 1% Aqueous (EMS: 26051-10)</td>
</tr>
<tr>
<td>Ammonia</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>Ethanol, 200 proof (Fisher: 04-355-451)</td>
</tr>
<tr>
<td>Xylene</td>
<td>SafeClear II Xylene Substitute (Fisher: 23-044-192)</td>
</tr>
<tr>
<td>Permament</td>
<td>Permament Mounting Medium (Fisher: SP15-100)</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>Acetic Acid, Glacial (Fisher: BP2401-500)</td>
</tr>
</tbody>
</table>

Protocol:

1. Allow samples to warm to room temperature (RT) for ~10min on paper towels.
2. Hydrate slides in 1xPBS.
3. Stain with activated* Hematoxylin for 2min (read bottle to activate).
4. Rinse slides for 5min with tap (not DI) water.
5. Intensify stain with 0.2% Ammonia in H2O for 1min.
6. Rinse slides 2 x 5min with tap water.
7. Stain with Eosin Y for 5min.
8. Rinse slides 2 x 5min with tap water.
9. Dehydrate slides (2min each)
   a. 95% Ethanol
   b. 95% Ethanol
   c. 100% Ethanol
   d. Xylene
   e. Xylene
10. Mount with 1:1 xylene permount
B.5 TUNEL assay

Apoptosis (cell death) on Frozen Sections

Reagents:

<table>
<thead>
<tr>
<th>Fixation Solution:</th>
<th>4% Paraformaldehyde (0.04g/ml) in 1xPBS, pH 7.4, freshly prepared</th>
<th>Cabinet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeabilisation Solution:</td>
<td>0.1% Triton X-100, 0.1% sodium citrate (0.001g/ml), freshly prepared in H2O</td>
<td>Cabinet</td>
</tr>
<tr>
<td>Positive Control:</td>
<td>DNase I Recombinant @ 3000 – 3 U/ml in 50mM Tris-HCl, pH 7.5, 1 mg/ml BSA (Roche 04536282001)</td>
<td>Fridge/Lab 206</td>
</tr>
</tbody>
</table>

Protocol:

**Note:** Fix and permeabilise two additional samples for the negative and positive labeling controls.

1. Allow sections to warm to room temperature (RT) for ~10min on paper towels.
2. Fix for 5 min in ice-cold acetone in -20°C freezer.
3. Dry slides on towels for ~10min at RT. Once dry, circle sections on the back of the slides with fine sharpie.
4. Rehydrate slides 2 x 5min in 1xPBS at RT.
5. Fix tissue section with **Fixation Solution** for 20 min at 15 - 25°C
6. Wash for 30 minutes with 1xPBS  
   **Note:** For storage, dehydrate fixed tissue sections for 2 minutes in absolute ethanol and store at -15 to -25°C.
7. Incubate slides in **Permeabilisation Solution** for 2 minutes on ice (2 - 8°C).

**Preparation of TUNEL reaction mixture**

One pair of tubes (vial 1: Enzyme Solution, and vial 2: Label Solution) is sufficient for staining 10 samples by using 50µl TUNEL reaction mixture per sample and 2 negative controls by using 50µl Label Solution per control.

**Note:** The TUNEL reaction mixture should be prepared immediately before use and should not be stored. Keep TUNEL reaction mixture on ice until use.

1. Remove 100µl **Label Solution** (purple, vial 2) for two negative controls.
2. Add total volume (50µl) of **Enzyme Solution** (blue, vial 1) to the remaining 450µl of **Label Solution** in vial 2 to obtain 500µl TUNEL Reaction Mixture.
3. Mix well to equilibrate components.
### Controls

| Negative Control: | Incubate fixed and permeabilized cells with 50μl of **Label Solution** (without terminal transferase) instead of **Tunnel reaction mixture** |
| Positive Control: | Incubate fixed and permeabilized cells with **DNase I Recombinant** (3000 U/ml – 3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 minutes at 15 - 25°C to induce DNA strand breaks, prior to labeling procedures |

### Labeling Protocol for Adherent Tissue

1. Rinse slides 2 x 5 minutes with 1xPBS.

2. Dry area around sample.

3. Add 50μl of **TUNEL reaction mixture** on sample. *protect from light*
   
   **Note:** For the negative control add 50μl **Label Solution** each.
   
   Pipette 10 μL of TUNEL reaction mixture onto each piece of tissue, be careful not to disrupt the tissue, and place slides in a slide book.

4. Incubate slide in a humidified atmosphere for 50 minutes at 37°C in the dark.

5. Rinse slide 3 x 5 minutes with 1xPBS.

6. Counterstain for 8 min with ~98μL of DAPI solution (2.5μL in 10mL PBS)

7. Rinse 2 x 5min in 1xPBS at RT

8. Rinse briefly in dH₂O and mount with DAKO fluorescent mounting medium

9. Store slides at 4°C, protected from light.

10. Samples can directly be analyzed under a fluorescence microscope or embedded with antifade prior to analysis (use an excitation wavelength in the range of 450 – 500nm and detection in the range of 515-565nm, ie. green).
B.6 Silver nitrate staining

**Silver Nitrate Staining**

**Reagents:**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Description</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>silver nitrate solution, Sigma-Aldrich (85193-100ml)</td>
<td>Fridge</td>
<td></td>
</tr>
<tr>
<td>4% paraformaldehyde</td>
<td>Fridge</td>
<td></td>
</tr>
<tr>
<td>1XPBS</td>
<td>Fridge</td>
<td></td>
</tr>
</tbody>
</table>

**Equipment:**

- standard light microscope

**Protocol:**

1. Cut the leaflets out and rinse well (3 quick washes) with PBS.
2. Incubate AV with 0.25-0.5% silver nitrate dissolved in distilled water for approximately 30 sec/50 sec.
3. Rinse with PBS (3 quick washes).
4. Fix AV with 4% paraformaldehyde for 10 min.
5. Rinse with PBS (3 quick washes).
6. Place AV on slide and photograph using a standard light microscope. (We did not mount but placing a large coverslip over the tissue might help with focusing.)
B.7 BrdU staining

### BrdU stain on Frozen Sections

**Reagents:**

<table>
<thead>
<tr>
<th>Blocker</th>
<th>2.5% Normal Goat Serum (GS) (sc-2044), 0.3% Tolit X-100, 1% DMSO in 1xPBS</th>
<th>Freezer &amp; Cupboard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Antibody:</td>
<td>Anti-BrdU, mouse monoclonal @ 1:200 in 2% GS - Vortex, Santa Cruz Biotech, (Dako M0744)</td>
<td>Fridge</td>
</tr>
<tr>
<td>Secondary Antibody:</td>
<td>Goat Anti-mouse IgG FITC, @ 1:200 in 1xPBS Santa Cruz Biotech, (sc-2010)</td>
<td>Fridge</td>
</tr>
<tr>
<td>Counter Stain :</td>
<td>DAPI, Sigma-Aldrich (D8417)</td>
<td>Fridge</td>
</tr>
<tr>
<td>Mounting :</td>
<td>DAKO Fluorescent Mounting Medium, (# S3023)</td>
<td>Fridge</td>
</tr>
</tbody>
</table>

**Protocol:**

1. Allow sections to warm to room temperature (RT) for ~10 min on paper towels.
2. Fix for 5 min in ice-cold acetone in -20°C freezer.
3. Dry slides on towels for ~5 min at RT, rinse in PBS 5 min.
4. Pretreat sections with proteinase K for 10 min at RT in normal slide holder (Dilute 7.5 μl of 20 mg/ml stock pKot. K in 150 ml of 1x PBS)
5. Rehydrate slides 2 x 5 min in 1x PBS at RT
6. Incubate sections in 4N(4 mol/L) HCl for 30-45 min at 37°C (prewarm HCl)
7. Rinse 10 min in 0.1 M sodium borate (pH 8.5) (or tris-buffered EDTA (sigma 93302))
8. Rinse in H2O 2 x 10 min
9. Rinse in PBS 2 x 10 min
10. Block for 1 hour with 90 μL of 10% DS at RT in H chamber. **Vortex before use.**
11. Incubate for 2 hours at with ~98 μL 1° Ab per section in H chamber (or overnight at RT).
12. Rinse 3 x 10 min in 1x PBS at RT.
13. Incubate for one hour at RT in humid chamber with ~98 μL 2° Ab per section, protect from light from this point on.
14. Rinse 2 x 5 min in 1x PBS at RT.
15. Counterstain for 8 min with ~98 μL of TOPRO-3 solution (1:750)
16. Rinse 3 x 10min in 1xPBS at RT

17. Rinse briefly in dH2O. Once dry, circle sections on the back of the slides with fine sharpie and mount with DAKO fluorescent mounting medium

18. Store slides at 4°C, protected from light.

19. Image stain within one week.
B.8 Immunohistochemistry

**Immunohistochemistry on Frozen Sections**

1. Allow sections to warm to room temperature (RT) for ~10 min on paper towels.
2. Fix for 5 min in ice-cold acetone in -20°C freezer.
3. Dry slides on towels for ~5 min at RT.
4. Rehydrate slides 2 x 5 min in 1xPBS at RT.
5. Block for 1 hour with 98 μL of 10% DS at RT. **Vortex before use.**
6. Incubate for 2 hours at 4°C with ~98 μL 1° Ab per section.
7. Rinse 2 x 5 min in 1xPBS at RT.
8. Incubate for one hour at RT in humid chamber with ~98 μL 2° Ab per section, protect from light from this point on.
9. Rinse 2 x 5 min in 1xPBS at RT.
10. Counterstain for 8 min with ~98 μL of DAPI solution (2.5 μL in 10 mL PBS)
11. Rinse 2 x 5 min in 1xPBS at RT
12. Rinse briefly in dH2O. Once dry, circle sections on the back of the slides with fine sharpie and mount with DAKO fluorescent mounting medium
13. Store slides at 4°C, protected from light.
14. Image stain within one week.
B.9 Immunohistochemistry (humidity box)

**Immunohistochemistry Staining (Version 2)**

**Protocol:**

1. Allow sections to warm on heater (37°C) for ~20min.
2. Circle the sample by using Fluid Block pen.
3. Rinse the samples in PBS for ~20min
4. Block for 1 hour with 200μL of blocker in PBS (10% D/S/GS, 0.2% TritonX-100, 1%DMSO) at RT. **Vortex before adding serum.**
5. Incubate for 2 hours (or overnight) at RT in humid chamber with ~200μL 1° Ab | per section. (1° Ab is diluted in blocker)
6. Rinse 3 x 5min in 1xPBS at RT.
7. Circle sections on the back of the slides with fine sharpie
8. Incubate for one hour at RT in humid chamber with ~200μL 2° Ab per section. **protect from light from this point on.**
9. Rinse 3 x 5min in 1xPBS at RT.
10. Counterstain for 8 min with ~200μL of DAPI solution (2.5μL in 10mL PBS)
11. Rinse 2 x 5min in 1xPBS at RT
12. Rinse briefly in dH₂O. Once dry, and mount with DAKO fluorescent mounting medium
13. Store slides at 4°C, protected from light.
14. Image stain within one week.
B.10 BCA Assay

BCA Assay (Bicinchoninic Acid)

Reagents:

<table>
<thead>
<tr>
<th>Chemicals:</th>
<th>BCA protein assay (Thermo Fisher)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equipment:</td>
<td>1.5ml microtube</td>
</tr>
<tr>
<td></td>
<td>96-well microplate</td>
</tr>
</tbody>
</table>

Protocol:

1. Prepare standards

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Water (uL)</th>
<th>Volume &amp; Source of BSA</th>
<th>Final BSA Conc (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 uL of Stock</td>
<td>2000</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 uL of Stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 uL of Stock</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 uL of B</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 uL of C</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 uL of E</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 uL of F</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 uL of G</td>
<td>25</td>
</tr>
<tr>
<td>Blank</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Working range for the standard BCA assay is 20-2,000 µg/mL (0.02-2 µg/µL) protein
*Keep the tubes containing the standards in a box in the 4°C for future use


*Total volume for each well is 200uL

3. Prepare the 9 standards in the table above in 1.5mL tubes and prepare the unknown samples

4. Pipette 25µL of each standard or unknown sample replicate into a microplate well.

5. Add 200µL of the WR to each well and shake plate vigorously on a plate shaker (30 sec.).

6. Incubate covered plate for 30min at 37°C (solution will turn purple in incubator)

7. Measure the absorbance at 562nm on the plate reader.
B.11 Sample preparation

Sample homogenization and tissue lysate preparation

Reagents:

<table>
<thead>
<tr>
<th>Chemicals:</th>
<th>RIPO Lysis Buffer</th>
<th>Fridge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equipment:</td>
<td>Microcentrifuge tubes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rotor-stator homogenizer</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Styrofoam container with ice</td>
<td></td>
</tr>
</tbody>
</table>

Protocol:

1. Prepare RIPO lysis buffer according to its instructions. *Immediately prior to lysing cells, combine 10 μl PMSF solution, 10 μl sodium orthovanadate solution and 10-20 μl protease inhibitor cocktail solution per ml of 1X RIPO Lysis buffer to prepare complete RIPO.*

2. Add appropriate volume of ice-cold lysis buffer per sample tube. In general, the guideline is to use 3 mL lysis buffer per gram of tissue. (i.e. for a 5 x 5 mm aortic valve leaflet sample, use 200 μL lysis buffer)

3. Disrupt and homogenize tissue with electric homogenizer at lowest setting (‘1’) for about 30 – 60 s (make sure foam does not spill out of sample tube). Ramp up the homogenizer speed to “6” for about 15 s. Clean the homogenizer tip between samples.

4. Incubate on ice for ~20 minutes to let the foam subside. Repeat step 4 (at least 3 times) until tissue is completely homogenized.

5. Centrifuge tubes at 5000 - 7000 g for 5 minutes at 4°C. Remove supernatant and centrifuge again if necessary. The supernatant fluid is the total cell lysate.

6. Conduct DC protein assay (if protein assay is not being conducted immediately, store tubes in -80°C freezer. Store unused lysis buffer in fridge for up to a week).
B.12 Weston blotting

Western blot

Protocol:

Transfer

1. After electrophoresis, we need to transfer proteins from gel to a membrane that is suitable for antibody probing. Prepare transfer buffer. (700ml DI water, 200ml methanol, 100ml tris-glycine)
2. For each gel to transfer to a membrane:
   a. Get rectangular tub, transfer buffer from cold room, one black and white transfer case per gel, two pieces of filter paper, two sponges, container of ice.
   b. Cut out rectangular piece of PVDF membrane and notch top right hand corner in order to distinguish between different membranes.
   c. Soak membrane in methanol for 2-5 mins.
   d. Pre-soak components (4 pads, 2 filter papers & membrane) in 1X transfer buffer (200 mL is enough) in shallow container
   e. Pour transfer buffer into rectangular tub. Open black/white transfer case into tub so that the white side is downside and black is upside.
   f. Assemble “sandwich” in apparatus tray
      Upper electrode (red anode+) -white
      Sponge Pad
      Filter paper
      PVDF Membrane  "the cutting notch is on the top left hand now
      Gel
      Filter paper
      Sponge Pad
      Lower electrode (black cathode -) –black
      ("Removing any air bubbles which may have formed is very important for good results)
3. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.

4. Add the frozen blue cooling unit. Place in tank and till to the transfer buffer in the tank.

5. Put on the lid, plug the cables into the power supply, and run the blot.

   Standard Field Overnight Transfer: 30V constant, 90mA, 10W
   High Intensity Field 1 Hour Transfer: 100V constant 350mA, 50W

6. After transfer ends, remove membrane and wash with dH2O or TBST for 5 min.

7. Next soak in Ponceau Red solution for 5 min. Then rinse with dH2O until red bands of protein show up. This step is to verify that proteins have indeed transferred from gel to membrane and that there was equal protein loading in each lane. Ponceau stain as it can be used several times and rinse membrane with either DI water.

8. At this point, the membrane can be cut with a razor blade horizontally so that more than one antibody can be probed for. This should only be done if a protocol for that particular antibody has been established and it is known at what molecular weight it will appear.

9. Wash membrane 2 – 3x in DI water to remove Ponceau stain.

**Antibody Staining:**

1. Block membrane in blocker (either 5% nonfat dry milk or 3% BSA in TBST) for 1 hour at RT

2. Wash membrane for 5 min 1 – 2x in fresh blocker at RT.

3. Incubate in appropriate concentration of primary antibody diluted in blocker overnight at 4°C or 2 hours at RT.

4. Wash membrane for 5 min 3x in fresh blocker at RT.

5. Incubate in appropriate concentration of HRP-conjugated secondary antibody diluted in blocker for 1 – 2 hours at RT.

6. Wash membrane for 5 min 3x in fresh blocker at RT. Keep in 4°C if not image at once.

**Film and Developing**

1. Use Pierce Western Blotting substrate (1:1) to visualize bands for 10 mins.
2. Bring all of the following items with you in the dark room: your membrane w/marker, film, scissors, autoradiography cassette, developer and fixer and trays for each chemical, timer, white coat
3. Cut film to size of membrane plus marker. Place membrane in cassette face up, set film on top and close cassette tightly/
4. Expose film. Initial exposure of 1-3 minute can give a good indication of whether immunoblotting was successful and if longer film exposure is needed.
5. Place film in developer until bands are obvious on film (about 30-60s)
6. Rinse film with water to remove developer and place in fixer. Film should be completely clear, minus your bands, before removing.
7. Wash film with water again to remove fixer
8. Allow film to dry (hanging is the best)
9. Developer and Fixer and reusable so pour back into bottle for future use. Developer should be changed when solution is becoming brown and fixer should be changed when film is taking longer to become clear or not becoming clear
B.13 Zymography

**MMP GELATIN ZYMOGRAPHY**

**Protocol:**

2. Prepare gel and load 1XTris/Glycine/SDS running buffer, check leaking.
3. Load gels with 8-10 μg of protein and markers.
4. Run gels at 125V at 90min.
5. Gels washed 30 min in 150ml renaturation buffer (2.5% Triton-X 100) at RT with gentle agitation.
6. Remove the renaturing buffer and add 150 mL of developing buffer to the gel. Incubate for 30 minutes at RT with gentle agitation.
7. Remove the developing buffer and add 150 mL more of developing buffer to the gel. Incubate overnight (16-18 hours) at 37°C.
8. Remove the developing buffer and rinse three times (5 minutes each) with deionized water at RT with gentle agitation.
9. Stain the gel by adding 20 mL of stain solution to the gel. Incubate for 1 hour at RT with gentle agitation.
10. Rinse the stained gel in a large volume of deionized water, 2-3 times for 5mins each.
11. Rinse gel in 100 mL or more Coomassie Brilliant Blue De-staining Solution until desired resolution is attained.
12. For better results, store gel in deionized water and incubate for another hour or more at room temperature.
13. Carefully move the gel from the water and place in a plastic sheet protector.
14. Scan the gel with a resolution of 300 dpi or higher. Save the image.


150


152


155


[174] Pelech I., A.H. Shapiro, Flexible disk rotating on a gas film next to a wall, J Appl Mech. 31 (1964) 577-84.


