RICE UNIVERSITY

Remodeling of the Extracellular Matrix Components of the Mitral Valve Due to Alterations in the Mechanical and Chemical Environments of the Tissue

by

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ABSTRACT

Remodeling of the Extracellular Matrix Components of the Mitral Valve Due to Alterations in the Mechanical and Chemical Environments of the Tissue

by

Janet Christine Elliott Barzilla

This body of research explored mitral valve remodeling due to changes in the mechanical and chemical environments of the tissue in order to better predict patient response to medical device and drug therapies. This work was novel both in its characterization of the mitral valve and in the consideration of the impacts of the biological environment on the multilayered valve structure. Left ventricular dilation due to congestive heart failure (CHF) changes the mechanical forces experienced by the mitral valve. Collagen, cellular, glycosaminoglycan, and proteoglycan compositions of valve tissues were therefore compared between CHF patients treated with and without the left ventricular assist device (LVAD). Mechanical properties of these tissues were also tested to assess effects of matrix changes on valve function. Since mechanical stimulation was determined to be required to maintain valve structure and function, a novel splashing bioreactor was then designed to maintain the valve mechanical environment in vitro in preparation for studies altering the chemical environment of the tissue. After validation, this bioreactor was used to study initial changes to
valve structure after two weeks of organ culture with media containing the serotonin receptor agonists serotonin and norfenfluramine (fen-phen) as well as a receptor antagonist. In the mechanical stimulation study, CHF valve tissue exhibited fibrotic remodeling compared to normal tissue, impeding normal mitral valve function. LVAD treatment, while it tended to restore functionality of the mitral valve chordae, encouraged compensatory remodeling that did not improve leaflet structure or mechanical behavior. Conversely, the gentle stretching provided by bioreactor was deemed sufficient to maintain matrix composition after two weeks of culture. Serotonin and norfenfluramine exposure appeared to upregulate different proteoglycans within the mitral valve structure, and exposure to a serotonin receptor antagonist had varying levels in success in blocking these drug effects. The results from these studies showed that changes to the mechanical (CHF and LVAD) and chemical (serotonin and norfenfluramine) environments altered mitral valve tissue structure and function. These effects of device use and drug therapies should be considered when assessing treatment for pathologies, such as CHF and obesity, which may not appear to directly impact the mitral valve.
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On a more personal note, I’d like to thank my Mom and Dad and Sandra and Frank. Your help made this all possible. I want to acknowledge Caitlin and Alix – you’ll be here soon enough – and Jane O for just being like a sister to me. Finally, I want to thank my husband, Scott, and my little Annie (save the best for last and all that). I love you both more than anything else in life! - Janet
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Chapter 1. Introduction

The overall goal of this research project was to explore the mechanisms behind remodeling of the extracellular matrix (ECM) of the mitral valve. Two categories of potential stimuli were studied: mechanical and chemical. For the purposes of this research, mechanical stimulation of the valve was represented by changes in the forces imparted upon the tissue due to dilation of the left ventricle with congestive heart failure (CHF) as well as any reversal of this dilation after left ventricular assist device (LVAD) implantation. Chemical stimulation of the valve was represented by changes to the milieu due to the introduction of the serotonergic agents serotonin and fen-phen. Both types of stimuli have been noted clinically to induce changes to the valve structure that impede normal valve function.

To fully comprehend the consequences of altering the mechanical or chemical environment of mitral valve tissue, investigation of the early mechanisms of these diseases is required. Understanding these disease mechanisms can give researchers the tools needed to treat valve disease early or even to prevent it altogether. If LVAD use is determined to cause pathological changes to the valve tissue, changes should be made in the device protocol. For example, the physician could recommend that the device be turned off periodically or that the mitral valve be replaced prior to device implantation. If a concern exists that a new pharmaceutical may cause serotonergic valve disease,
the specific receptor activity of the drug can be altered, or the receptor itself can be blocked.

Dilation of the left ventricle caused by CHF instigates pathological changes to the myocardium as well as to aortic and mitral valve tissue. While the LVAD improves left ventricular function and occasionally reverses CHF effects on the cardiac muscle, its use can also lead to pathological remodeling of the aortic valve. Although the effects of LVAD use on the aortic valve have been well-characterized, its impact on the mitral valve has not been studied. If the LVAD is to be used as a destination therapy for CHF, as opposed to a bridge to heart transplantation, the goal should be to restore tissue structure and function of both the cardiac and the valve tissue. Otherwise, a workaround for these patients should be developed. The unknown LVAD influence on mitral valve remodeling led to the first specific aim of this project:

**Aim 1:** Determine how LVAD treatment, when compared to current oral medical management therapies, affects the ECM composition and the resulting material behavior of the mitral valve of CHF patients.

The study of tissue damage in CHF patients involved the use of human valve tissue exposed to CHF conditions and LVAD treatment in vivo. When attempting to induce valve damage to study the early mechanism of disease-induced remodeling, however, an in vitro system was desired. The first specific
aim assumed that pathological changes to valve tissue were due to changes in
the mechanical environment. Since a further goal of this project was to study the
effects of changes of the chemical environment of the mitral valve, a bioreactor
was designed to mimic the mechanical forces imparted upon the valve in its
biological environment to ensure that observed structural changes were due to
the chemical stimulus. The bioreactor design and validation studies were the
subject of the second specific aim:

Aim 2: Develop an inexpensive, easily reproducible bioreactor to
maintain the mechanical environment of the mitral valve while
investigating the impact of an altered chemical environment on the
tissue.

The formation of superficial plaques on patients taking Fen-Phen and
other serotonergic drugs has been extensively documented in the past decade.
These plaques are similar in composition to those observed in patients with
carcinoid syndrome, which leads to an excess of serotonin in the circulatory
system. Knowledge of the early mechanism of this plaque formation, as well as
confirming the specific receptor activated, can assist in the treatment for these
patients and provide researchers with information needed to prevent the release
of similar drugs to the public in the future. The study of the activity of these
serotonin receptor agonists formed the final specific aim:
Aim 3: Determine receptor effects and early remodeling of mitral valve ECM composition due to the introduction of two serotonin receptor agonists, serotonin and norfenfluramine.

The completion of these three specific aims gave a complete picture of remodeling of the mitral valve ECM due to changes in the mechanical and chemical environments of the tissue. This structural remodeling had consequences to the normal function of the valve. A thorough understanding of structural and functional changes to the mitral valve tissue will lead to improved treatment of afflicted patients.

The next two chapters in this document focus present the current state of the literature. Since this project focused on ECM remodeling, the second chapter will begin with an overview of the different ECM components and turnover mediators and their contribution to the anatomy of the normal mitral valve. The background will then review the known impacts of CHF and LVAD treatment on the structures of the myocardium and left heart valves. The pathologies and proposed mechanisms of serotonergic valve disease will also be presented. In preparation for the design and validation of the splashing bioreactor in the later chapters of this work, current bioreactor designs used in the organ culture and tissue engineering of heart valves will also be discussed. Chapter three is a review article previously published in the American Journal of Geriatric Cardiology\(^1\). This article discusses the tissue engineering of heart valves in the elderly, a population that is frequently affected by CHF as well as valvulopathy.
The use of donor hearts is uncommon in the elderly; therefore, characterizing the effects of the LVAD on the diseased heart is of particular importance in this population. The splashing bioreactor designed and used in Aims 2 and 3 could also be applied to tissue engineering of the valve, and a selection of the bioreactors currently used in this application is also discussed in chapter three.

Chapters four, five, and six each discuss one of the three specific aims that comprise this research project. Chapter four will describe the remodeling of the mitral valve to its pathological CHF state and any recovery of the tissue structure due to LVAD treatment (Aim 1). Chapter five will discuss the design, construction, and validation of the splashing bioreactor (Aim 2). The use of this bioreactor to expose the mitral valve to serotonergic drugs will be contained in chapter six (Aim 3). The final chapter of this work will review the results obtained for each aim, discuss the significance of this body of research to the current literature, and indicate possible future directions for this work.
Chapter 2. Background

This chapter will review the literature pertaining to heart valve pathology and subsequent extracellular matrix (ECM) remodeling. The major ECM components and turnover mediators will be discussed first, as will their role in normal heart valve tissue structure. The anatomy of the normal mitral valve will be presented in order to give the reader a basis of comparison to the abnormal valves examined throughout this research project. The relationship between normal valve structure and function, i.e. the valve mechanical properties, will also be discussed. Next, the review will introduce the heart pathologies to be studied in this report, congestive heart failure and serotonergic valve disease, and their known effects on the heart and valves. After examining these pathological valve environments, current heart valve bioreactor models influencing the design of the splashing bioreactor in Chapter 5 will be presented. This chapter concludes with a brief synopsis of the unresolved questions in the literature that this body of research has examined in greater detail in Chapters 4 through 6.

2.1. Extracellular Matrix Components

The ECM is made of three major components: collagen, elastin, and proteoglycans/glycosaminoglycans (PGs/GAGs). A main function of ECM is to provide structure and the material properties to a tissue. The ECM also acts as the cell environment and plays a role in signal transduction to the cell. Finally, the ECM sequesters biological molecules; for example, the core protein of the
PG decorin will bind and sequester transforming growth factor-β (TGF-β), inhibiting its effect. All of these classes of ECM components, which are described below in greater detail, are found in heart valves.

2.1.1. Collagen

Collagen is the protein that provides mechanical integrity and strength to tissue and is the main load-carrying component of soft tissues. Multiple types of collagen exist; fibrillar types I, III, and V are of greatest interest in studying the heart valves\(^2\). The basic molecular structures of these three types, described below, are the same.

*Figure 2.1: Collagen triple helix. Common amino acids in the α-chain are noted\(^3\)*

The primary structure of collagen is a peptide string of amino acid triplets with the form glycine-X-Y, where X and Y are frequently either proline or hydroxyproline (Figure 2.1). Each α-chain contains a sequence of about 300
triplets in addition to shorter terminal domains. All collagens are immediately modified after synthesis by hydroxylation of the proline and lysine residues into hydroxyproline and hydroxylysine. Hydroxyproline, which is found only in collagen and elastin, is often used as an indicator of the amount of collagen in tissue\textsuperscript{4,5}.

The secondary structure is a tight, right-handed \(\alpha\)-helix with a regular residue spacing of 0.286 nm and angular separation of 108°. This structure satisfies the geometric constraints placed on the \(\alpha\)-helix by the large ring group on the proline residues and the small hydrogen side group of the glycine residues\textsuperscript{4} (Figure 2.1).

The tertiary structure is a triple helix comprised of three individual \(\alpha\)-helices coiled around each other. Collagen type I is a heterotrimer of two \(\alpha1\) chains and one \(\alpha2\) chain. Collagen type III is a homotrimer of \(\alpha1\)\textsubscript{III}, and collagen type V is a heterotrimer of \(\alpha1\)\textsubscript{V}, \(\alpha2\)\textsubscript{V}, and \(\alpha3\)\textsubscript{V}\textsuperscript{4}. The triple helix is a chemically stable structural unit, also called procollagen, that is both stiff and flexible. The procollagen molecule is about 1.5 nm wide and 300 nm long, with a pitch of 10.4 nm\textsuperscript{4}. The terminal domains are cleaved after secretion into the extracellular space to form tropocollagen, also called collagen. These collagen molecules are arranged in a quarter-staggered assembly to form fibrils, which bundle to form collagen fibers.

A number of molecules assist collagen fibrillogenesis and can be used as an indication of collagen turnover within the tissue. Heat shock protein 47 (HSP47) is a molecular chaperone that appears to prevent denaturation of
unstable regions (areas lacking hydroxyproline) of the collagen triple helix while in the cell. Prolyl-4-hydroxylase (P4H) also has a role in collagen production, but shows a higher affinity for non-helical intermediates. Lysyl oxidase (LOX) catalyses the cross-links in collagen and elastin fibers; studies with knockout mice have indicated that a deficiency of LOX leads to thinner elastic fibers and loosely-packed collagen.

Normal mitral valves contain 74% type I, 24% type III, and 2% type V collagen. Types I and III are often found in association with each other, and type V is found in the pericellular space. Previous studies have found that certain types of diseased valves (i.e., myxomatous, or floppy valve, disease) contain increased concentrations of all three types of collagen, but in different proportions. For example, the ratio of collagen type I to collagen type III is higher in a normal valve than in a myxomatous valve; it is presumed that type III collagen production increases as a result of the body’s repair processes. Additionally, the type V collagen increase may signal an increase in the DNA content, and therefore cell number, of the valves.

2.1.2. Glycosaminoglycans

Four classes of GAGs exist: hyaluronan (HA); chondroitin sulfate (CS)/dermatan sulfate (DS); heparin/heparan sulfate (HS); and keratan sulfate (KS). GAGs are unbranched polysaccharides of varying length made of repeating disaccharide units, usually an amino sugar and uronic acid (Figure 2.2). GAGs are frequently associated with PGs and play a role in tissue compressibility. Negatively charged sulfate and carboxyl groups create a highly
anionic domain for the GAG disaccharide. Cations are then attracted to the GAG, and the system becomes osmotically unbalanced. Water is then drawn toward the molecule to restore osmotic equilibrium, resulting in a highly hydrated domain.

![Diagram of repeating disaccharide structure]

**Figure 2.2: Basic repeating disaccharide structure of GAG. The glucuronic acid and N-acetyl-glucosamine are characteristic of HA.**

The differences between the four types of GAGs lay in the two disaccharide components. Glucuronic acid (Figure 2.2) and iduronic acid, the two types of uronic acid found in the GAG disaccharide, vary by a rotation of the carboxyl group (highlighted in Figure 2.2). Most GAGs also contain either N-acetyl-glucosamine or N-acetyl-galactosamine as the amino sugar. HA, the only GAG that is not part of a PG (Section 2.1.3), consists of glucuronic acid and N-acetyl-glucosamine. HA is often found in the synovial fluid and loose connective tissues and plays a role in introducing water into the ECM. CS and DS are very similar in structure, with both GAGs containing N-acetyl-galactosamine as the amino sugar. Their difference lies in the uronic acid epimer present; CS contains glucuronic acid, while DS contains iduronic acid. CS is found in cartilage and
some connective tissue\textsuperscript{8,10,11} and, along with HA, contributes to the compressibility of a tissue\textsuperscript{11}. DS, on the other hand, has a general distribution within the animal tissues. Heparin and HS both contain glucosamine, but heparin usually contains iduronic acid while HS contains glucuronic acid. Heparin is found in the granules of mast cells, and HS is present within the PGs on many different cell surfaces. KS is made of repeating units of galactose and N-acetyl-glucosamine; galactose replaces the uronic acid present in the other GAG classes. Two types of KS exist: type I is present in the cornea, and type II is found in loose connective tissue\textsuperscript{8,10,11}. Common alterations to the GAGs include the introduction of sulfate groups at the 4 and 6 carbon of the amino sugar, as in C4S or C6S\textsuperscript{11}.

The primary GAGs of interest in the mitral valve are HA and CS/DS. CS/DS are affiliated with the structural PGs in the ECM that are abundant either in areas experiencing tension and compression. A previous study documenting levels of GAGs and PGs in normal mitral valves has shown that compressive regions of the valve tend to contain high levels of HA and water as well as a high ratio of C6S to C4S. Regions exposed to tensile force have a high overall concentration of DS and a high ratio of 4-sulfated CS/DS to 6-sulfated CS/DS\textsuperscript{12}. Changes in GAG concentrations can indicate valve matrix remodeling.

2.1.3. \textbf{Proteoglycans}

PGs are comprised of at least one GAG covalently linked to a core protein (Figure 2.3). Although some PGs are present at cell surfaces, PGs are also abundant in the ECM and can interact with collagen and elastin to direct the
matrix structure. The negatively-charged GAGs within the PGs allow the ECM to absorb water and resist compression on the tissue, as described in Section 2.1.2. Since the GAG polysaccharide chains tend to be long (30-250 disaccharides\(^{13}\)), the PGs can also act as a barrier to the flow of molecules within the matrix. Additionally, PGs occupy a large portion of the ECM relative to collagen and elastin, allowing the PG to act as space fillers\(^{11}\).

![Aggrecan](image1)

![PG-M/Versican](image2)

**Figure 2.3: Schematics of the proteoglycans aggrecan and versican. Versican, a large chondroitin sulfate proteoglycan, is commonly found in the mitral valve. HABR=hyaluronan-binding region, GAG=glycosaminoglycan attachment region, EGF=epidermal growth factor repeat, LEC=Type C lectin, C=complementary protein\(^{14}\).**

The main PGs of interest in the mitral valve are the CS/DS PGs versican, decorin, and biglycan. Versican is a large CS PG\(^{10}\) that is predominantly found in regions of compression of the mitral valve. Versican can also aggregate with HA to act as a lubricant within the tissue and to prevent certain receptor-ligand interactions within the ECM\(^{10,12}\). The tensile regions of the mitral valve
predominantly contain the small leucine-rich PGs (SLRPs) biglycan and decorin. Decorin is known to regulate collagen fibrillogenesis *in vitro*\(^{10} \), and it is abundant in tissues containing type I collagen. Decorin, therefore, likely provides strength to load-bearing tissues. Biglycan is not generally found in association with decorin, but it is also known to play roles in collagen fibril diameter and tissue strength\(^{12} \). The ability of versican to bind water makes this PG a compressive load bearing element, while the close associations of biglycan and decorin with collagen make these PGs indirectly responsible for bearing tensile loads within tissues.

2.1.4. **Elastin**

Elastic fibers in adult tissue are comprised of elastin with a sparse covering of microfibrils\(^{15} \). Elastin, the predominant protein in the fiber, provides extensibility and gives tissue the ability to recover after placement of a load\(^{15,16} \). Tropoelastin, the soluble form of elastin secreted by a cell, is made mostly of the hydrophobic amino acids. Glycine and proline, respectively, comprise about 1/3 and 1/9 of these residues. Each tropoelastin molecule contains approximately 40 lysine residues that form covalent cross-links between the monomers\(^{15} \). Microfibrils are made mostly of the proteins fibrillin and microfibril-associated glycoprotein. Microfibrils act as a scaffold to align tropoelastin, and the structure can be cross-linked by LOX\(^{15,16} \). Previous studies have also documented a co-localization between versican and elastin fibers\(^{17,18} \).
2.1.5. **ECM Turnover Mediators**

2.1.5.1. **Matrix Metalloproteinases**

Matrix metalloproteinases (MMPs) influence many cellular activities by their ability to degrade ECM proteins when activated. MMPs can influence normal and pathologic biologic processes such as wound repair and cancer development\(^{19}\). More than 25 MMPs have been characterized thus far; the primary MMPs of interest in the current research are MMP1, MMP2, MMP9, and MMP13.

Each individual MMP will degrade multiple ECM proteins; the proteins of interest to this project are listed below. MMP1, also called collagenase-1, digests collagen types I and III. MMP2, or gelatinase A, digests collagen types I, III, and V in addition to decorin, elastin, and fibrillin. MMP9, or gelatinase B, digests collagen type V, decorin, elastin, and fibrillin. MMP13, also called collagenase-3, digests collagen types I and III.

The basic MMP structure includes a “pre” domain, a “pro” domain, and a catalytic domain. The “pre” domain, an N-terminal signal sequence, is generally removed prior to secretion. The propeptide “pro” domain keeps the enzyme inactive until removed. The catalytic domain contains the zinc-binding region of the enzyme\(^{19}\). Most MMPs are therefore initially secreted aszymogens and can be activated via cleavage by serine proteases or other MMPs. MMP9, for example, can be activated by MMP3, MMP10, and MMP13\(^{20}\).
2.1.5.2. Tissue Inhibitors of Metalloproteinases

As the name suggests, tissue inhibitors of metalloproteinases (TIMPs) bind to and inhibit the activity of the different MMPs. At least four TIMPs are known; TIMP1 and TIMP2 are of primary interest in this project. MMP1, MMP2, MMP9, and MMP13 are all inhibited by TIMP1 and TIMP2\textsuperscript{20, 21}.

2.2. Valve Anatomy

The heart contains four valves to direct forward blood flow and prevent reverse flow through the heart and circulatory system. The two valves of the left heart, the mitral and aortic, are the focus of this project, because the pathological conditions examined herein are usually presented by the left heart valves.

2.2.1. Mitral Valve

The mitral valve is located between the left atrium and the left ventricle (Figure 2.4).

![Figure 2.4: Mitral valve location between the left atrium and left ventricle\textsuperscript{22}.](image_url)
The mitral valve attaches to the heart wall at the top part of the valve, the annulus. When the valve closes, the free edges of the valve leaflets meet in coaptation. The valve opens to allow the blood to flow into the ventricle during diastole (relaxation) and closes prior to systole (contraction) to prevent blood from re-entering the atrium (Figure 2.5). A normal heart rate is about 72 beats/min, and systole comprises about 40% of the cycle. The normal closed valve is able to withstand the high systolic pressure (100-150 mm Hg) of the left ventricle. Immediately after systole, the left ventricular pressure drops (<10 mm Hg), and the slightly greater left atrial pressure passively opens the mitral valve to increase left ventricular volume by approximately 60-70mL.

Figure 2.5: Mitral valve in its closed (left) and open (right) positions. The closed valve on the left shows the anterior leaflet (upper) and the posterior leaflet (lower). The valve opens to allow blood flow into the left ventricle.
The valve itself is divided into two major sections, the anterior and posterior leaflets (Figure 2.6). The anterior leaflet is positioned near the aorta, medial to the posterior leaflet. The posterior leaflet is attached to the posterior wall of the left ventricle and wraps around the anterior leaflet when the valve is closed. The chordae tendineae attach the underside of the valve leaflets to the papillary muscle of the ventricle and are separated into two categories: basal chordae, the large chordae that insert into the center of the leaflet; and the marginal chordae, the smaller chordae that insert into the free edge of the leaflet.

![Figure 2.6: Ventricular view of mitral valve excised from heart failure patient after transplant and cut open. The parts of the valve are labeled.](image)

The leaflet consists of three layers covered by vascular endothelial cells: the fibrosa, a thick collagenous layer on the ventricular side of the leaflet; the atrialis, a thin elastic layer on the atrial side of the leaflet; and the spongiosa, a middle layer rich in PGs and GAGs\(^4\) (Figure 2.7A). The atrialis and spongiosa
are less dense than the fibrosa and are sparsely populated by valvular interstitial cells, which display characteristics of both fibroblasts and smooth muscle cells\textsuperscript{25}. These cells are supported in a PG "gel" containing the randomly aligned collagen and elastin of the atrialis as well as some of the dense collagen bundles of the fibrosa\textsuperscript{24}.

The total thickness of the anterior leaflet, especially the fibrosa, is greater than that of the posterior leaflet, indicating that the anterior leaflet is designed to support greater tensile loads\textsuperscript{24}. The collagen fibers of the fibrosa prevent the prolapse of the leaflet into the atrium as the ventricle contracts\textsuperscript{2}, while the elastic fibers of the atrialis allow the valve to recoil from its closed position as the valve opens. The atrialis and spongiosa are thicker at the leaflet free edge than the leaflet center in order to handle the compressive stresses present at coaptation, or leaflet closure. In the anterior leaflet, the ECM structure of the leaflet center and free edge are very different, more so than in the posterior leaflet. The center region (Figure 2.7B) is very collagenous, which supports the tensile forces imparted upon the tissue, while the PGs and GAGs found in high concentrations in the free edge (Figure 2.7B) allow the tissue to bear the compressive forces present during leaflet coaptation. The different components of the leaflet layers cause the tissue to act as a composite, so that the bending resistance of the structure is lower than an equal thickness of pure collagen representative of the fibrosa\textsuperscript{24}. Unlike the leaflets, the chordae are rope-like structures of aligned collagen within a thin sheath of elastic fibers and endothelial cells\textsuperscript{25}. This design
is well-suited to the primary function of the chordae which is to bear tensile forces upon valve closure.

Figure 2.7: (A) Movat pentachrome stain of the mitral valve showing the layered construction of the leaflet. (B) Drawing of whole mitral valve, cut open and laid flat, with leaflet regions and chordae defined. A=atrialis, S=spongiosa, F=fibrosa.

The layered structure of the mitral valve supports the tissue bending as the valve opens and closes in addition to the tension applied to the leaflet center and the compression imparted upon the free edge. This tissue structure can change as the ECM is remodeled by the valve cells during a disease state. The mechanisms and result of tissue remodeling has yet to be completely characterized for the disease states highlighted in this project. Characterization of these ECM changes will help researchers find the causes of and improve treatments for mitral valve diseases.

2.2.2. Aortic Valve

At present, little research is available pertained to the effects of changes to the mechanical and chemical environments on the mitral valve. Structural and functional changes to the aortic valve in response to the same phenomenon, on
the other hand, have been well-characterized\textsuperscript{27-29}. The location of the aortic valve in the left heart and the layered tissue structure make this valve a good indicator of possible remodeling that the mitral valve may undergo.

The aortic valve is located at the exit of the left ventricle, just beneath the aorta (Figure 2.4). This valve prevents blood from leaving the ventricle until systole, when the chamber is emptied. The aortic valve contains three leaflets. The left semilunar (coronary) cusp corresponds to the location of the left coronary artery, inserted in the wall of the aorta above the cusp. The right semilunar (coronary) cusp corresponds to the location of the right coronary artery, and the posterior (noncoronary) semilunar cusp does not have an association with any of the smaller arteries that branch from the aorta.

The microstructure of the aortic valve is similar to the mitral valve in that there are three distinct layers of tissue. The ventricularis, which lies below the inflow surface, contains collagen with radially aligned elastin fibers. The central layer of the valve leaflet is the spongiosa, which contains both GAGs and loosely arranged collagen. The fibrosa lies below the outflow surface and is comprised of densely packed, circumferentially aligned collagen\textsuperscript{30}.

Aortic valve mechanical properties and ECM composition in both normal and pathological conditions have been characterized to a greater extent than those of the mitral valve. Porcine aortic valves are widely used as, or in the manufacture of, bioprosthetic valve replacements, making them a common subject of study. This additional research available on the aortic valve is also due to the prevalence of cusp calcification and other valve diseases.
2.2.3. **Valve Mechanics**

Valve tissue has been modeled using the quasi-linear viscoelasticity law, which relates changes in stress to step changes in strain to describe relaxation behavior of tissue\(^1\). As with traditional engineering materials such as steel, a stress-strain graph can be used to illustrate the role of the valve layers, specifically the collagen fiber structure, on the tissue mechanical behavior. Unlike steel, valve tissue exhibits different mechanical behavior depending on the magnitude of applied stress. In the region of lower strain, the slope of the stress-strain curve is lower than in the region of higher strain. A transition point exists in this graph after which the slope increases by several magnitudes\(^2\). The mechanical properties of the lower strain "toe region" are influenced by both the collagen and elastic fibers in the tissue, as the crimped structure of the collagen has yet to extend and absorb the full impact of the load and the elastic fibers are therefore bearing the load (Figure 2.8). Mechanical properties of the higher strain, or elastic, region are mostly due to the loading of the uncrimped collagen fibers\(^3\). The slope of the stress-strain curve in this region is the tissue’s Young’s modulus (E).

The valve tissue mechanical properties also depend upon the anatomical region of the valve and the orientation of the fibers. Collagen fibers in the mitral valve anterior leaflets are predominantly organized in the circumferential direction. Circumferential tissue samples therefore tend to have a greater Young’s modulus (anterior leaflet: 4.26±0.740 – 7.44±0.990 MPa; posterior leaflet: 1.55±0.168 – 2.09±0.191 MPa)\(^4\), as applied forces are taken up by the
collagen. Tissue samples aligned in the radial direction tend to be less stiff (anterior leaflet: 1.53±0.183 - 1.99±0.0671 MPa; posterior leaflet: 1.17±0.106 - 1.89±0.194 MPa)\textsuperscript{32}, as the intrafibrillar matrix handles a greater portion of the load and the fibers must rotate to bear load. The collagen fibers of the mitral valve posterior leaflet are more askew; therefore, the tissue is not quite as stiff in the circumferential direction as the anterior leaflet. The entire valve can be compared to a composite, where the fibers handle stresses imposed along their main axes while the matrix is depended upon to handle the forces in other directions\textsuperscript{32}.

![Sample stress-strain curve for a circumferential section of the posterior leaflet. The toe region is encircled, and elastic region is indicated by a straight dashed line. The slope of the elastic region is known as the Young's modulus (E) of the tissue.](image)

Figure 2.8: Sample stress-strain curve for a circumferential section of the posterior leaflet. The toe region is encircled, and elastic region is indicated by a straight dashed line. The slope of the elastic region is known as the Young’s modulus (E) of the tissue.
In general, chordal tissue has a shorter pre-transition region and a higher $E$ (basal chordae: $21.0\pm2.89 - 22.1\pm1.95$ MPa; marginal chordae: $39.8\pm6.20 - 40.1\pm4.67$ MPa)\textsuperscript{32} in the post-transition region than the leaflets\textsuperscript{32}. Chordae with smaller diameters are less extensible than those with greater diameters, due to greater collagen crimping within the larger chordae\textsuperscript{34,35}. The straightening of the collagen fibers contributes more to the stress-strain characteristics of the chordae than the elastic fibers, since the elastic fibers are merely a sheath surrounding a collagen core\textsuperscript{26}.

Since the mitral valve tissue structure strongly influences the tissue function, it is assumed that any tissue remodeling can be assessed by changes to the ECM and by resulting changes to the mechanical properties. Changes to mitral valve mechanical properties are therefore examined in this project as a means of assessing impact of tissue remodeling. Conversely, changes in the valve ECM can explain changes in mechanical properties and tissue behavior.

2.3. Heart Pathology

A wide range of diseases exist that can affect the performance of the heart and its valves. This project will focus on two pathologies, congestive heart failure (CHF) and serotonergic valve disease, that can directly and indirectly damage the left heart valves.

2.3.1. Congestive Heart Failure

CHF often results from other types of heart disease, especially coronary heart disease or myocardial infarction. CHF-associated enlargement of the left
ventricle can lead to mitral valve regurgitation, causing some blood to leak back into the left atrium after the mitral valve has closed. Additionally, the possibility exists that the left ventricular assist device (LVAD) used to treat CHF may cause complications with the left heart valves.

2.3.1.1. Disease Variations

CHF causes chronic volume overloading of the left ventricle and is most commonly caused by cardiomyopathy. Four types of cardiomyopathy exist: dilated (DCM), hypertrophic, ischemic (ICM), and restrictive.

DCM, the most common form of cardiomyopathy, can be caused by alcohol abuse, pregnancy, or myocarditis\textsuperscript{36, 37}. Most DCM cases are designated as idiopathic, meaning that there is no known cause. DCM is generally irreversible and is the underlying cause of approximately \( \frac{2}{3} \) of heart transplants performed since 1986\textsuperscript{38}. Heart muscle affected by DCM is weakened and cannot pump blood efficiently. Other body tissues try to compensate for DCM by retaining blood, and the body generates more blood as a result. The ventricular myocardium then dilates to allow collection of the extra fluid volume. The initial response is positive; the heart muscle stretches more, causing a stronger contraction. Unfortunately, the heart will also increase its pumping rate to force a greater volume of blood throughout the body. The muscle will eventually lose its ability to contract, and the body will retain fluid, causing edema\textsuperscript{36, 37}. Additionally, the plasma levels of angiotensin II, aldosterone, and arginine vasopressin are elevated; this altered blood chemistry is probably a compensatory mechanism to maintain the arterial blood pressure\textsuperscript{39}.
The second most common form of cardiomyopathy, hypertrophic, is usually inherited. This disease causes a thickening of the heart muscle more extreme than the moderate thickening seen in athletes. The muscles in both ventricles become larger, blocking blood flow through the aortic valve or preventing the heart from filling with blood during diastole. Hypertrophic cardiomyopathy may also cause damage to the mitral valve, or the valve may adhere to the septum, preventing blood flow. Standard treatments include medication and lifestyle changes, especially a major reduction in athletic activity.\textsuperscript{36, 37}

ICM is the weakening of heart muscle due to an ischemic event, such as the blockage of a coronary artery to prevent the entrance of oxygenated blood into the heart muscle. Treatment for ICM is similar to that for DCM, with emphasis on treatment for the underlying coronary heart disease. In cases of serious damage, transplant may be recommended.\textsuperscript{36, 37}

Restrictive cardiomyopathy is rare in the United States. It is usually a result of another disease, such as excessive iron buildup in the body. The heart rhythm and pumping action are normal, but the stiffened walls of the heart chamber prevent complete filling. Blood flow is reduced, and excess blood is backed up into the circulation. While the original cause of restrictive cardiomyopathy can be treated, the muscle damage cannot be reversed. Medication can control the symptoms, but transplant is an option if damage is extensive.\textsuperscript{36, 37}
In most cases of CHF, the ventricular muscle becomes progressively weakened and overloaded as the body’s original compensatory mechanisms fail to resolve the underlying cause of the disease. Remodeling of DCM hearts occurs, leading to upregulation of collagen types I and III in addition to associated turnover markers in the cardiac muscle. As DCM progresses, the loading conditions upon the ventricular muscle change the directions of strains along the wall, causing the chamber to function more inefficiently.

As the heart muscle becomes more overloaded, the mitral valve tissue itself undergoes fibrotic remodeling. An increase in DNA synthesis and collagen production has been noted in mitral valves, presumably an adaptation mechanism for excessive pressure. Mitral valves from CHF patients have a greater concentration of DNA than normal, indicating an increase in the cell density within the valve tissues. These valves also contain less water but more collagen and GAGs; it is not known if the PGs or GAG classes are different from those present in the healthy valves. Changes to DNA, collagen, and GAG content are most pronounced in DCM patients, while changes in hydration are more apparent in ICM patients. CHF patients also have been observed to have moderate mitral regurgitation, annular dilation, and thicker mitral valve leaflets. The fibrotic changes to the valve tissue indicate that the valve undergoes a dysfunctional remodeling process in response to the pathological changes to the left ventricular geometry during CHF, although the altered blood chemistry may also play a role.
2.3.1.2. Treatment via Mechanical Assist

The estimated prevalence of CHF in adults over age 20 in the United States was approximately 53 million in 2005. CHF was considered an underlying cause in 57,120 deaths in 2004\textsuperscript{45}. End-stage CHF is usually defined as New York Heart Association (NYHA) Class IV failure, characterized by a gross loss in cardiac myocyte function. Ambulatory patients with Class IV symptoms have a one-year mortality rate of 50\%\textsuperscript{46}. Short-term treatments for CHF include diet changes and medication, but these options can become exhausted quickly. At this point, the favored treatment option for DCM and ICM patients is a heart transplant; unfortunately, only 2500 donor hearts are available annually\textsuperscript{47}. This major discrepancy between needed and available donor hearts makes the mechanical assist device an attractive treatment option. Other heart ailments, including myocardial infarction, myocarditis, and postcardiotomy shock, can also require use of a mechanical assist device\textsuperscript{47}.

The left ventricular assist device (LVAD) removes blood from the left ventricle and pumps it into the aorta, bypassing the aortic valve. LVAD mechanics differ between manufacturers, but some common characteristics are outlined below (Figure 2.9). An inflow cannula is inserted into the left ventricle, usually at the apex. The body of the pump, which is usually installed in the upper left quadrant of the chest, fills passively. In automatic mode, the blood is forcefully ejected into the aorta once the pump is approximately 90\% full\textsuperscript{44,47}. All LVADs have a percutaneous driveline leading to an external power source, which is often worn at the patient's waist\textsuperscript{44}.
Figure 2.9: Novacor® Implantable LVAD. The device is connected between the left ventricular apex and the aorta to pump blood out of a weakened ventricle.

The first LVAD (Thermo Cardiosystems Inc, Woburn, MA) was implanted in 1966. The earliest LVADs did not differ greatly in function from those used today; however, the pneumatic console that powered these devices was so large that the patient was required to wheel it around with him. The LVAD therefore kept the patient alive, but did little to improve his physical strength or mental outlook since he could not fully return to his everyday activities. The first truly mobile LVAD was implanted in April 1996 (Baxter-Novacor, Oakland, CA). Final FDA approval to use the LVAD as a bridge-to-transplant was granted in
September 1998. Three commercially available LVADs currently in use are the Novacor (Baxter-Novacor, Oakland, CA), the Heartmate 1000 implantable pneumatic (IP) pump, and the Heartmate vented-electric (V-E) pump (Thermo Cardiosystems Inc, Woburn, MA). The Heartmate 1000 IP device was approved by the FDA in 1994, and the other two devices were approved in 1998.

The LVAD was designed for use as a bridge-to-transplant; in the United States, it is usually implanted into patients with NYHA Class IV CHF who have been approved for transplant, keeping the patient alive until a donor heart is available. The LVAD has been successful in offloading the cardiac muscle, increasing myocyte contraction and decreasing myocyte diameter, reducing CHF symptoms. Additionally, collagen deposition within the cardiac tissue has been found to decrease after LVAD use. Ventricular function, as measured by left ventricular ejection fraction and end-diastolic diameter, was shown to improve after 30-60 days of LVAD treatment but declined after 90-120 days. The LVAD increases cardiac output, decreases cardiac workload, and maximizes subendocardial perfusion, allowing the myocardial tissue to recover both its geometry and its functionality.

Since the use of the LVAD encourages structural and functional recovery of the left ventricle, it has been suggested that the device can be used as an end treatment, also called destination therapy. The Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) studies were conducted from 1998 to 2001 to determine the feasibility of the use of the LVAD as a destination treatment. These
studies have determined that the LVAD can alleviate CHF symptoms while stopping disease progression and improving survival rates\textsuperscript{55}. Patients receiving LVAD therapy had a significantly lower mortality rate than patients receiving oral medical management therapy\textsuperscript{46, 56, 57} and reported a better quality of life\textsuperscript{46, 57, 58}. Mortality among LVAD patients was often due to infection and device failure\textsuperscript{56-58}, however, it was decided that the risks of the device were outweighed by the benefits\textsuperscript{57}.

If the LVAD is to be implanted permanently, major problems such as infection, post-operative bleeding, and thromboemboli formation must be considered\textsuperscript{44, 49}. An alternate treatment option is to implant the LVAD until the heart shows signs of recovery, after which the patient is weaned from the device. Timing of LVAD implantation for this treatment is critical; if the device is implanted too early, the patient is subjected to unnecessary surgery, but delaying implantation can lead to complications\textsuperscript{49}, with lower survival rates for moribund patients.

Although LVAD implantation has led to recovery of the ventricular tissue in most patients\textsuperscript{44, 47, 50, 54, 59}, explantation of the device from a functionally recovered heart has often led to a recurrence of CHF symptoms\textsuperscript{38, 49, 59}. An optimal level of muscle function recovery has been noted in LVAD patients, unique to each person, after which continued treatment causes a reversal of LVAD benefits and a loss of ventricular function. All patients who have suffered a recurrence of CHF symptoms after weaning had LVAD treatment continued after this optimal recovery point in an attempt to further stabilize the patient\textsuperscript{38}.
Additionally, the time spent on the pump prior to this optimal recovery point was longer for the patients who were weaned unsuccessfully. Unfortunately, there was no difference between the two groups in duration, depth, type, or origin of heart disease prior to LVAD implantation, and investigators could not predict if later weaning would be successful prior to surgery\(^{38}\).

### 2.3.1.3. Effect of Mechanical Assist on Heart Valves

Since the LVAD pumps blood directly into the aorta, the aortic valve remains closed to blood flow after device implantation. If the patient has a mechanical valve, this valve is at risk for thrombosis, particularly on the aortic (as opposed to ventricular) side. This thrombus can propagate into the coronary arteries, causing myocardial infarction. Replacement of the mechanical valve with a tissue valve mitigates these risks. Additionally, severe insufficiency of the native aortic valve can cause a cycle of flow back through the pump and ventricle. Patients with this valve problem should have their native aortic valve replaced with a bioprosthetic tissue valve prior to use of the LVAD\(^{49}\).

Fusion of the commisures, the connections between two leaflets comprised of loose fibrous tissue, has been noted between the aortic valve cusps\(^{27,29,60}\) (Figure 2.10) and was most common with the Heartmate VE LVAD. The fusion, which can lead to valve insufficiency, has also been noted to a lesser extent in valves of CHF patients who have not had LVAD therapy. This effect may be reduced by periodically decreasing the pump rate or stopping pump function to force the use of the inoperative valve\(^{27}\). No correlation was found between duration of LVAD use and the presence of either aortic valve thrombosis.
or commissural fusion, and the overall effect of the LVAD on the ECM of the aortic valve remains a question\textsuperscript{29}.

![Image of aortic valve with LVAD support]

**Figure 2.10**: Aortic valve of patient with LVAD support. The arrow indicates fusion between two of the valve cusps\textsuperscript{27}.

As noted above, mitral regurgitation is a common side effect of CHF. Although the movement of the mitral valve in an LVAD-assisted heart resembles a flutter motion, regurgitation is not an issue following unloading of the left ventricle with LVAD implantation. The changes in loading of the ventricular muscle that lead to mitral regurgitation in CHF are alleviated with the use of the LVAD. Additionally, although the replacement of mechanical aortic valve prostheses with tissue valves has been recommended prior to LVAD implantation, continued blood flow through the native mitral valve should make a similar replacement of the native mitral valve unnecessary\textsuperscript{49}.
Many LVAD patients have suffered tricuspid valve regurgitation due to increased pulmonary artery pressure. As the patient remains on the LVAD, this initial high pressure can decrease. If the insufficiency does not recover, annuloplasty, a surgical technique used to reinforce the valve annulus after valve repair, can be performed[48]. An intermediate-length study (162 ± 29 days) has shown that, while the LVAD activity decreases left ventricular dimensions and mitral regurgitation, it increases right ventricle dimensions and tricuspid valve regurgitation[61,62]. These changes to the right heart likely stem from leftward septal shifting (Figure 2.4) with unloading of the left ventricle in addition to the volume loading of the right ventricle due to an increased venous return[62]. This change in function of the right heart can be difficult to quantify since the diminished left heart capability also increases afterload placed on the right heart[61].

In order for the LVAD to be an effective destination treatment, the heart and its valvular components must be able to function within a normal range after device removal. It is currently unknown whether the LVAD reverses the effects of CHF on the mitral valve, as it does with the left ventricular muscle, or if the LVAD has any adverse effects on the mitral valve that would cause poor functioning of the heart upon weaning. Any remodeling of the mitral valve due to LVAD placement should be identified before widespread use of the LVAD as a destination therapy in order to prevent any further health issues due to a regurgitant valve.
2.3.2. Valve Diseases – Serotonergic Valve Disease

Serotonergic valve disease is characterized by the presence of a plaque on the valve surface that leaves the underlying valve microstructure intact. This superficial plaque is comprised of a collection of myofibroblasts within a matrix consisting mainly of collagen and GAGs\textsuperscript{63,64}. Earlier reports of this disease indicated that the plaques contained no elastin fibers, but recent studies have shown that elastin is present in approximately 42% of the plaques, particularly within the chordae and even along the papillary muscles in advanced cases\textsuperscript{64}. Elastin in these pathological valves is less commonly observed in the leaflets and valve cusps, and it is located deep within the tissue, in longitudinal layers separate from the normal atrialis layer\textsuperscript{64}. Serotonergic valve disease has been associated with elevated serum serotonin levels, but the mechanism of the plaque formation is unknown.

2.3.2.1. Serotonin Pathway

Serotonin (5-hydroxytryptamine or 5HT) is a major neurotransmitter. Elevated blood serotonin levels are known to cause serotonergic valve disease, but the actual mechanism of the disease progression is still under investigation\textsuperscript{63-65}. Seven different families of receptors for serotonin, 5HT\textsubscript{1} through 5HT\textsubscript{7}, have been identified\textsuperscript{66}; the receptors of interest in serotonergic valve disease belong to the are 5HT\textsubscript{1} and 5HT\textsubscript{2} families. Immunostaining has confirmed the presence of the 5HT\textsubscript{1B} in diseased valves but not in normal valves\textsuperscript{67}. Reverse transcriptase-polymerase chain reaction (RT-PCR) has identified the 5HT\textsubscript{2A} and 5HT\textsubscript{2B} receptors in sheep aortic valve interstitial cells (VICs)\textsuperscript{63,65}. Further experiments
have suggested that the 5HT$_{2B}$ receptor is likely to be responsible for pathological changes to the valve tissue$^{68}$. It is believed that the presence of serotonin stimulates the VICs to produce TGF-β1 through G-protein signal transduction via Src kinase and extracellular regulated kinase (ERK) phosphorylation$^{63,69}$. The TGF-β expression leads to an increase in collagen and GAG production$^{63}$ and possibly the matrix changes noted in serotonergic valve disease. Since the mechanism of the serotonin on the heart valve is still unknown, a bioreactor would be very useful in studying the activity of this altered blood chemistry on the valve tissue.

2.3.2.2. Carcinoid Valve Disease

The mechanism behind the creation of the plaque characteristic of carcinoid valve disease is unknown but is associated with high serum levels of serotonin. Carcinoid valve disease can affect all four heart valves, but the majority of non-drug related cases appear to affect the pulmonary and tricuspid valves in the right heart (Figure 2.4). These firm, pearly white plaques often appear on the arterial sides of the pulmonary and aortic valves or the ventricular sides of the tricuspid and mitral valves$^{64}$ (Figure 2.11). The presence of these plaques on the tissue surface can lead to complications such as regurgitation and stenosis. The only current treatment for this condition is surgery. If left untreated, carcinoid valve disease can be fatal within one year$^{64}$. 

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Figure 2.11: Gross pathologic specimens from a 20 year study on carcinoid valve disease conducted by the Mayo Clinic\textsuperscript{64}. Plaques are indicated with arrows. All valves are from the same 58-year-old male patient. (A) Tricuspid leaflet with severe stenosis and regurgitation. (B) Whole pulmonary valve with severe regurgitation. (C) Whole mitral valve with severe regurgitation. (D) Two aortic valve cusps with moderate regurgitation.

2.3.2.3. Fen-Phen Valvulopathy

Fenfluramine-phentermine (fen-phen, Wyeth, Madison, NJ) is an anorectic drug cocktail voluntarily withdrawn from the market in September 1997 due to reports of carcinoid-like valve disease in patients. Symptoms of this fen-phen valvulopathy include plaques on the valve surface comprised of myofibroblasts in a myxoid matrix, with the underlying tissue structure left intact\textsuperscript{70} (Figure 2.12). These lesions are identical, grossly and histologically, to those characteristic of serotonergic valve disease.

As with carcinoid valve disease, fen-phen valvulopathy was thought to be caused by elevated levels of serotonin in the blood. The racemic (equal amounts
of left- and right-handed stereoisomers of a chiral molecule) form of the fenfluramine component, dexfenfluramine, is metabolized to dextrofenfluramine, which releases serotonin and activates the 5HT₂ receptors⁷¹. Recent research, however, has noted that chronic fenfluramine exposure causes elevations in plasma serotonin far less than that required to initiate valve disease⁷².

Figure 2.12: Valve sections showing difference between normal and fen-phen affected valves. Tissue stained with Verhoeff-van Gieson elastin. (A) Normal mitral valve with atrialis at top and fibrosa at bottom (magnification=40X). (B) Mitral valve with fen-phen plaque covering ventricular surface (10X). (C) Normal aortic valve with ventricularis below and fibrosa above (20X). (D) Aortic valve with fen-phen plaque on ventricular surface (40X). The underlying structure of both fen-phen affected valves are intact⁷⁰.

Unlike carcinoid valve disease, fen-phen valvulopathy tends to affect the valves of the left heart. This preferential effect has been hypothesized to be due to the role that the lungs play in clearing serotonin from the blood. When the lungs are functioning normally, as in patients with carcinoid valve disease, the serotonin is cleared from the blood in a single cycle. Previous studies have shown that the appetite suppressant chlorphentermine, chemically similar to
phentermine, compromises the lungs' ability to uptake and metabolize serotonin. When added to fenfluramine, the action of phentermine may allow higher concentrations of serotonin to reach the left heart\textsuperscript{71}.

\subsection{2.3.2.4. Ecstasy}

The club drug 3,4-methylenedioxymethamphetamine (MDMA), or “Ecstasy,” has been studied to determine similarities in activity to serotonin receptor agonists. It appears that MDMA can bind and activate the 5-HT\textsubscript{2B} receptor in a similar manner as the other serotonin receptor agonists. This receptor activation occurs at the same amount of chemical as is found in a standard recreational dose\textsuperscript{68}.

\subsection{2.3.2.5. Pergolide}

With the exception of fenfluramine, all drugs currently proven to stimulate the 5HT\textsubscript{2B} receptor to induce carcinoid-type changes in valve tissue are ergot derivatives\textsuperscript{68}. Pergolide is an ergot-derived dopamine receptor agonist used in the treatment of Parkinson’s Disease and restless legs syndrome\textsuperscript{73}. Previous research has determined that pergolide also interacts with this receptor. Histological analysis has shown that valve tissue exposed to pergolide is pathologically similar to carcinoid valve disease. Echocardiographs allowing the scientist to view regurgitation of the valves have shown that approximately 20\% of patients taking pergolide have suffered some sort of valvulopathy\textsuperscript{73}. Identification of valvular disease in the older generation that is more susceptible to Parkinson’s disease is difficult. Since degenerative and
calcification valvular diseases tend to affect the left heart valves of the elderly, the effect of pergolide is better assessed by focusing on the pathology, such as regurgitation, of the tricuspid valve. This analysis can be performed on living subjects using echocardiography. The reversibility of the effects of Pergolide have not been accurately assessed, as the patient and doctor tend to weigh the neurological benefits of the drug higher than the cardiac side effects and are reluctant to cease drug use.

2.3.3. **Summary of Relevant Heart and Valve Disease**

Previous studies have indicated that valvular ECM remolds in response to changes to the mechanical and chemical environments. Aortic valve response to alterations to the mechanical environment due to LVAD support has been well-characterized, but little research has been done on the mitral valve response. The formation of a plaque on the mitral valve stemming from increased serum serotonin levels has been documented, but the mechanism of this plaque disposition is undefined. This project will look specifically at the effects of LVAD treatment and serotonin agonist exposure on the mitral valve.

2.4. **Bioreactors Used in Valve Organ Culture and Tissue Engineering**

Bioreactors are useful for their ability to inexpensively reproduce the in vivo biochemical and mechanical valve environment. Bioreactors modeling the valvular environment have been used to create tissue engineered semilunar heart valves (Chapter 3) and to provide invaluable information about mitral
valve disease and recovery by characterizing structural remodeling due to environmental changes\textsuperscript{76}. Previous models used in heart valve research include flow loop\textsuperscript{77, 78}, cyclic pressure\textsuperscript{79, 80}, and cyclic flexure\textsuperscript{81-85} systems.

The mitral valve has not been frequently cultured using a bioreactor; however, aortic valve bioreactors have provided some valuable insight into the role of mechanical forces on valvular remodeling. In one experiment, aortic valve leaflets were exposed to static flow (no movement of media), steady flow, and pulsatile flow conditions in a tubular system similar to a flow loop. Leaflets were also exposed to shear flow using a parallel plate system. After 48 hours, valve leaflets were compared to tissue that had not undergone any incubation. The aortic valve that had not undergone any incubation showed low levels of protein, GAG, and DNA synthesis, while samples exposed to static flow showed higher levels of component production. Additionally, tissue exposed to static flow conditions had significantly greater synthesis of DNA and GAGs than all other groups of tissue. Tissue in the tubular and parallel plate systems tended to have protein assembly levels closer to, although not as low as, the control valves\textsuperscript{76}. An alternate system exposed aortic valve tissue to static pressure. These results were similar in that collagen synthesis was found to increase with pressure, but there was no appreciable change in either GAG or DNA synthesis\textsuperscript{74}.

With the end goals of repeatability and cost reduction, this project proposed to create a “splash” bioreactor, where the medium was moved across the mitral valve to mimic the fluid mechanics in the filling left ventricle. The forces across the valve were not as high as those in the left heart, but may have
more closely mimiced those seen in the right heart. The valve modeled with this proposed bioreactor saw more of a physiological force distribution, as opposed to the shear forces in the example above. The compact and inexpensive design allowed multiple bioreactors to be made at a low cost; therefore, multiple experiments could be conducted simultaneously.

2.5. Unanswered Questions in Current Research

The research currently available lacked characterization of the mitral valve, especially when compared to the information available on the aortic valve. CHF was proven to encourage mitral regurgitation, but the changes to the myocardium that influenced this pathology were unclear. A link between LVAD treatment and aortic valve commissural fusion was proposed, but few studies until this work existed to show any remodeling of the mitral valve tissue due to LVAD support.

Similarly, the exposure of aortic valves to the in vitro conditions of a bioreactor has been well documented. Organ culture of the mitral valve in similar circumstances, however, had not been performed to the same extent. The chordae of the valve created an additional element of tension to the valve; therefore the overall motion of the valve leaflets and chordae were an ideal subject for a bioreactor model.

The characteristics of the plaques that form in serotonergic diseases, such as fen-phen valvulopathy, have been studied; however, little information was available on the mechanism of the disease progression. Elevated blood serotonin levels were associated with this family of diseases, but, aside from the
presence of 5-HT receptors on the valve tissue, the remodeling process remained a mystery.

This research project was designed to define the remodeling of the mitral valve observed due to chemical and mechanical changes in its environment. Aortic valve remodeling has been well-characterized, but anatomical differences of the mitral valve warrant closer examination. The resulting knowledge of the causes and mechanisms of mitral valve disease obtained from this research should lead to more effective treatment options in cardiac disease patients.
Chapter 3. Age-Related Structural Changes in Cardiac Valves: Implications for Tissue-Engineered Repairs

3.1. Abstract

Elderly patients would receive substantial benefits from tissue engineered heart valves (TEHVs), but most TEHV research has not focused on applications for this growing patient population. There will be numerous technical challenges involved in developing TEHVs for the elderly, such as designing tissues to accommodate higher blood pressure and larger aortic roots that may be friable or calcified. Concomitant medications may also affect the biology of the TEHV. Due to the predominantly senescent behavior of cells from older persons, a non-autologous cell source may be required to develop the TEHV. Decellularized heart valve allograft from an elderly donor may not be durable enough to use as a scaffold, but several polymer and natural biodegradable scaffolds may provide promising alternatives. The selection of cell sources, scaffolds, and mechanical/biological conditioning will need to be precisely targeted to meet the diverse physiological, medical, and surgical requirements of elderly patients.

\[\text{\textsuperscript{1}Previously published in the American Journal of Geriatric Cardiology, Sept-Oct 2006}\]
3.2. Motivation for Tissue Engineered Heart Valve Development

Approximately 53,000 people over the age of 65 underwent a valve replacement or other type of valve surgery in 2003, an incidence higher than any other age group and almost double that of patients age 45-64.\textsuperscript{87} Valvular heart diseases common in the elderly include aortic stenosis (AS), mitral valve regurgitation (MR) and aortic valve regurgitation (AR).\textsuperscript{88} AS has multiple causes in younger patients; however, calcific degeneration is the most common cause in elderly patients. The standard treatment for AS patients able to tolerate surgery is an aortic valve replacement (AVR). MR in the elderly is often caused by calcification of the valve annulus, myxomatous degeneration of the tissue, or ischemic heart disease. While MR does not worsen over time in the majority of elderly patients, it can lead to an increased risk of valvular tissue deterioration. Minor cases of MR can be managed by digoxin and diuretics, whereas valve reconstruction or replacement surgeries are reserved for more severely ill patients. Mild to moderate AR can often be managed with a vasodilator, but AVR is often recommended for more severe cases.\textsuperscript{88}

When replacement is warranted, the standard implant choices are a mechanical or a bioprosthetic heart valve. The thrombogenic materials used in the mechanical heart valves require lifelong anticoagulation therapy. Bioprosthetic heart valve replacements require a significantly shorter course of anticoagulation therapy, but tend to calcify over time and must be replaced more
frequently. When selecting an appropriate valve replacement, therefore, the ability of the patient to tolerate anticoagulation therapy must be balanced with the ability to tolerate multiple surgical procedures. Despite the risk of a repeated operation, the use of bioprosthetic valves in elderly patients has been recommended. A study following elderly artificial heart valve recipients showed that the 5-year survival rates of patients receiving either type of valve were identical; however, valve-related morbidity events related to the mechanical valve occurred more frequently than those related to the bioprosthetic valve, mostly due to anticoagulant-related hemorrhage.\textsuperscript{89} A living, nonthrombogenic tissue engineered heart valve replacement (TEHV), which could be created from donated or autologous tissues and would only require a single surgical procedure, is a possible solution to these current dilemmas presented by artificial valves.

3.3. **Feasibility and Technical Challenges for Using TEHVs in Elderly Patients**

3.3.1. **Alterations to Mechanical Environment**

Aging-related changes in the overall function of the heart, such as reductions in left and right diastolic function,\textsuperscript{90} influence the behavior of the connected valves and arteries as well as the remodeling of the cardiac and valve tissue microstructure. Aortic compliance decreases with age, increasing the resistance to blood flow from the left ventricle.\textsuperscript{91} This decreased compliance,\textsuperscript{92} in
addition to the calcification of the aortic valve tissue with age, has been hypothesized to cause native valve failure. Due to the decreased aortic compliance, aortic systolic pressure also increases. An aortic TEHV for use in elderly patients should be strong enough to withstand these pressure changes.

Insertion of the valve replacement into an elderly patient's less compliant and more friable aortic root may also be an issue. The suture retention strength of the TEHV will also be a critical property, as the implant must be able to withstand the mechanical forces within its new environment without dislodging from the aortic root. Finally, the aortic root itself dilates with age, possibly requiring that larger TEHVs be designed for implantation into older patients.

3.3.2. Mechanical Behavior Mismatch

When creating a TEHV, especially one within a vascular conduit, biocompatibility will be improved by matching the material properties of the biomaterial and the surrounding native tissue. Since this environment changes with age, the TEHV specifications should likewise be altered to prevent a mismatch between the material properties of the TEHV and the in vivo structure to which it will be attached.

3.3.3. Alterations to Chemical Environment

The blood chemistry of the elderly patient should also be considered prior to the creation of a TEHV for a specific patient. For example, some of these patients may be undergoing concomitant treatment for Parkinson's disease with the ergot-derived drug pergolide, which may be linked to the formation of plaques.
on the mitral and aortic valves; other medications commonly taken by older patients could also affect the implanted TEHV.

3.4. Tissue Engineering Principles and Relevance to Aging Valves

The design of any tissue engineered structure generally combines cells, scaffold, and exogenous growth factors. A self-sustaining engineered tissue must contain cells that proliferate within the scaffold structure and secrete extracellular matrix (ECM) components. A scaffold is used in the beginning stages to provide support but will ideally degrade or become remodeled as these cells begin to secrete their own ECM. Finally, growth factors and mechanical stimulation are used to stimulate the cells to proliferate and differentiate appropriately.

3.4.1. Cells

TEHVs have been developed using cells from a variety of sources, including vascular smooth muscle and endothelial cells, stem and progenitor cells, and valvular cells. Heart valves are covered with a smooth layer of endothelial cells, but are populated internally by valvular interstitial cells (VICs), a characteristically heterogeneous set of cells. Much of the basis for the heterogeneity of VICs is unknown, although the regional variability in valve microstructure, anatomy, and material behavior may be a factor.
VICs are thought to have a phenotype similar to, yet distinct from, any other cell type.\textsuperscript{103} VICs are likely not classical fibroblasts, as they are coupled by communicating junctions and respond to vasoactive agents.\textsuperscript{105} Compared to either skin fibroblasts or smooth muscle cells, VICs release 10-fold more prostacyclin and exhibit a greater mitotic response when exposed to endothelial cell conditioned medium.\textsuperscript{103} In injury or disease, VICs' expression of smooth muscle alpha actin (SM\r{a}A) becomes elevated.\textsuperscript{104} Transgenic growth factor beta (TGF-\r{b}) can also stimulate VICs to differentiate into myofibroblast-like cells which express abundant SM\r{a}A, cytokines and ECM components.\textsuperscript{106}

The effects of advanced aging on valve cells are not well characterized. It has been observed that the density of cells (graded 0-4 semiquantitatively) drops from grade 3-4 in adolescents to grade 1 after age 60,\textsuperscript{93} and the remaining cells demonstrate irregularities in overall morphology as well as in their intracellular composition.\textsuperscript{107} The low cell density and surgical timing logistics would likely preclude the use of autologous VICs to develop TEHVs for elderly patients. Moreover, in our laboratory, VICs isolated from older patients grow more slowly and senesce earlier in culture than VICs from younger people, which would be a disadvantage for growing a TEHV from elderly valve cells. Alternative autologous elderly cell sources, such as the saphenous vein, have produced ECM of inadequate strength when used in a tissue engineered vascular graft,\textsuperscript{108} even after transfection of elderly human cells with telomerase was used to improve cell proliferation. Putative mesenchymal stem cells with very high proliferative capacity were isolated from the connective tissues of adult rats via
dilutional cloning, but this work has not yet been translated to older human adults. For these reasons, non-autologous cells may be preferable in developing TEHVs for elderly patients.

3.4.2. Scaffold

In the preparation of a TEHV, cells are seeded onto a scaffold, which serves a support function analogous to the valvular ECM. Various synthetic biodegradable polymers, natural decellularized scaffolds, and natural biodegradable polymers have been considered as support materials.

3.4.2.1. Synthetic Biodegradable Polymers

Many synthetic biodegradable polymers have been explored for use as TEHV scaffolds due to their ranges of initial mechanical strength and degradation times as well as their nontoxic degradation products. A TEHV constructed from polyglycolic acid (PGA) and seeded with ovine vascular endothelial cells and smooth muscle cells was successfully implanted into the pulmonary position of a sheep model, but this polymer has a high initial rigidity. A pulmonary valve conduit consisting of a composite of PGA and polyhydroxyoctanoate (PHO), with leaflets made of PHO seeded with ovine cells, was viable in a lamb for 6 months. The PHO did not degrade completely, possibly leading to a foreign body response. Alternatively, Sodian et al. used polyhydroxyalkanoates (PHAs) to create an entire valve. The PHA exhibited mechanical strength and elasticity appropriate for a TEHV scaffold. Poly(vinyl alcohol) (PVA) has also been evaluated for use in TEHVs. PVA is hydrophilic, preventing adhesion
of VICs; the incorporation of poly-L-lactic acid, a hydrophobic polymer, increased both hydrophobicity and cell adhesion, but also increased the degradation time of the macromer.\textsuperscript{99}

The composition and fabrication of the above polymeric scaffolds can be altered to obtain the proper material properties, which will be different for elderly patients than for younger patients. At this time, most of these valve structures have been tested either in bioreactors or in the pulmonary valve position of an animal model, leading to the concern that these implants will not be strong enough to withstand the forces imposed upon the aortic or mitral valves. Additionally, the required lifespan of the polymer is still an open question. A polymer scaffold would ideally degrade as cells secrete structural ECM components. This degradation time will likely be different in the elderly than in younger adults, but would also depend on the types of cells chosen to populate the scaffold.

3.4.2.2. Decellularized Animal Valve

A decellularized xenograft or human valve could be seeded with human cells or used alone to produce a TEHV. When implanted in the pulmonary position of a sheep model, decellularized porcine scaffolds (unseeded) showed little degradation or calcification.\textsuperscript{110} The method of decellularization used in this study is known as the SynerGraft™ procedure,\textsuperscript{111} and it has shown promising results in adults.\textsuperscript{112} Preseeding the decellularized valves with cells (i.e., human dermal fibroblasts) prior to implantation has improved the resulting TEHV
performance in a bioreactor, resulting in valves that contained more collagen at 4 weeks than decellularized structures that were unseeded at the start of the study.\textsuperscript{113}

Various enzymatic and detergent-based decellularization treatments have been compared, with mixed results. In one study, a combination of the non-ionic detergents Triton-X 100\textsuperscript{®} and sodium deoxycholate appeared to completely decellularize the matrix while maintaining the underlying structure.\textsuperscript{114} Other studies have shown that the decellularization process itself can weaken the scaffold through a loss of ECM components.\textsuperscript{115} Because collagen present in the scaffold could stimulate thrombus formation via a combination of platelet activation and coagulation factor XII,\textsuperscript{116} recent studies have combined the decellularised animal scaffold with biodegradable poly(hydroxybutyrate), a member of the PHA family, in order to strengthen the scaffold and reduce any inherent thrombogenicity.\textsuperscript{116}

The evidence of numerous age-related changes to the valvular ECM indicates that valve allografts from elderly donors would not be durable scaffolds for TEHVs. With age, the valves thicken\textsuperscript{117} and their relative proportion of collagen decreases.\textsuperscript{118} Collagen in the older aortic valve resists digestion with collagenase, potentially due to an increase in crosslinking\textsuperscript{118} which would be consistent with their reduced extensibility.\textsuperscript{119} In normal heart valves, individual collagen fibers were found to become thicker and less aligned with age. Additionally, lipid accumulation and calcification within normal valves increased with aging in a similar manner as valves afflicted with AS.\textsuperscript{93}
3.4.2.3. Natural Biodegradable Scaffold

An assortment of materials are currently under investigation for use as a natural biodegradable scaffold. Porcine small intestine submucosa has shown some promise in the replacement of mature pulmonary valve leaflets, forming a more valve-like structure over time, although some thrombus formation on the leaflet surface was observed after implantation in a porcine model.\textsuperscript{120} Collagen sponge\textsuperscript{101} and fibrin gel\textsuperscript{121} have also been shown to support valve cell growth. Fibrin has certain advantages in that it can be isolated from a patient’s blood (including from elderly patients) to remove the risk of an immune reaction, and its degradation is controllable with aprotinin. Unfortunately, fibrin gels have tended to shrink with time and have an initially low mechanical stiffness.\textsuperscript{121} Crosslinked hyaluronan gels have also been investigated as substrates for cell growth, with the possibility for use in a final TEHV product.\textsuperscript{122} While these natural scaffolds appear to be useful as a cell support structure, more work must be done to develop scaffolds with appropriate degradation, mechanical, and nonthrombogenic properties.

3.4.3. Factors Influencing Cell Growth

Chemical growth factors and mechanical stimulation have been frequently used to control proliferation and differentiation of cells, as well as the mechanical properties of the scaffold and resulting TEHV. These tools can likewise be used to tailor the final TEHV structure to the physical and chemical environment of the elderly patient. The growth factor TGF-\(\beta\) has been shown to mediate the
differentiation of VICs, promoting myofibroblast-like characteristics (SMαA expression, contractility), yet inhibiting proliferation and apoptosis. Fibroblast growth factor-2, which was upregulated six-fold by VICs in a scratch wounding experiment, is mitogenic and could also mediate the remodeling of nascent TEHVs. Mechanical stimulation of the cell/scaffold combination can affect the mechanical properties of the polymeric scaffold; for example, dynamic flexure can fragment PGA fibers or disrupt bonding of poly(hydroxybutyrate) to the polymer. Mechanical conditioning has also been shown to improve cell distribution throughout the scaffold, ECM development, and the resulting mechanical properties of the engineered tissue, and could therefore plausibly be used to control these characteristics so the TEHV will perform properly in the altered physiological environment of the elderly patient.

3.5. Conclusions

Interest in developing tissue engineered heart valves has grown exponentially over the last decade. Frequent reviews of this field have provided lively debate and, more importantly, close scrutiny of preclinical and clinical results. Although elderly patients have not yet been the focus of TEHV research, recent successes implanting TEHVs in adult patients should spur new efforts in developing living valve tissues for patients older than 65 years. The selection of cell sources, scaffolds, and mechanical/biological conditioning, however, will need to be precisely targeted to meet the physiological, medical, and surgical requirements of this diverse, large, and growing population.
Chapter 4. Effects of CHF and LVAD Use on the Mitral Valve

Specific Aim 1: Determine how LVAD support, and the resulting decrease in left ventricular load, affect the ECM composition of the mitral valve of CHF patients.

4.1. Background

Approximately 60,000 people are diagnosed with New York Heart Association Class IV congestive heart failure (CHF) each year; the yearly mortality rate is 50%.

In the United States, the most common underlying pathologies leading to CHF are dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ICM), both resulting in a loss of myocyte function. The body's compensatory mechanisms eventually cause the ventricular chamber to dilate, leading to heart failure. When a patient does not respond adequately to oral medical management therapies such as ACE-inhibitors and beta-blockers, a heart transplant is often advised. Unfortunately, only 2,300 donated hearts are available every year, indicating a great need for an alternative treatment.

The left ventricular assist device (LVAD) has been successful in offloading the cardiac muscle, increasing myocyte contraction and decreasing myocyte diameter, reducing intensity of CHF symptoms. Additionally, collagen deposition within the cardiac tissue has been found to decrease after LVAD use. Ventricular function, as measured by left ventricular ejection fraction
and end-diastolic diameter, was observed to improve after 30-60 days of LVAD treatment but declined after 90-120 days\textsuperscript{52, 53}.

Results from the Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (REMATCH) study have shown that the LVAD is more successful than oral medical management therapies at treating CHF; follow-up studies with new LVAD designs have shown a continued increase in long-term survival\textsuperscript{126, 127}. The success of the LVAD as a bridge-to-transplant device has suggested an alternative use of the device as destination therapy for end-stage CHF; certain devices have received FDA approval for this use. Unfortunately, it appears that sufficient recovery to permit LVAD explantation only takes place in approximately 4-9\% of patients\textsuperscript{53, 128}, and that a level of recovery exists after which further LVAD treatment is not beneficial to the patient\textsuperscript{38, 53}.

It was recently shown that the mitral valve tissue of patients with end-stage CHF contains less water, more cells, and more collagen than normal tissue\textsuperscript{42}. These fibrotic changes contribute to the mitral valve regurgitation often observed in the failing heart. Although severe mitral valve regurgitation worsens CHF prognosis\textsuperscript{129}, this condition functionally improves with LVAD treatment\textsuperscript{61, 62}. If the LVAD does not reverse the fibrotic remodeling of the valve tissue, however, a patient weaned from the device may experience continued valvular regurgitation. Additionally, previous studies have indicated cusp fusion and thrombus formation on the aortic valve with LVAD treatment\textsuperscript{27, 29}; it is unknown whether there is a similar effect on the mitral valve. Combined, the possibility of
continued mitral valve fibrosis and the known detrimental effect of the LVAD on the aortic valve motivate the investigation of mitral valve remodeling in LVAD patients.

The current study examined the effect of LVAD use on mitral valve microstructure. It was hypothesized that, while LVAD use promoted recovery of the cardiac tissue, restoration of the normal mitral valve extracellular matrix (ECM) would not be complete. A possible implication of this result would be continued mitral valve dysfunction, such as mitral regurgitation, after device explantation. Determination of valve pathologies due to mechanically-induced matrix remodeling could lead to recommended clinical workarounds, such as the LVAD cycling recommended in some patients to prevent aortic valve fusion.

4.2. **Methods**

The first specific aim compared mechanical, biochemical, and histological data from CHF patients who did or did not receive LVAD support prior to transplant. The purpose of this study was to determine if alterations in the mechanical environment stimulate cell-mediated remodeling of the ECM, resulting in changes to the mechanical properties and function of the tissue. Data gathered in support of Aim 1 was analyzed both alone and, for completeness, in conjunction with data obtained previously at the Cleveland Clinic.
4.2.1. **Tissue Selection**

Human test tissue was obtained from the Cleveland Clinic Foundation (CCF, Cleveland, OH), St. Luke’s Hospital (Houston, TX), and the Cooperative Human Tissue Network (CHTN); IRB approval was obtained as needed at each institution. Mitral valves were excised from the diseased hearts after transplant and stored in phosphate buffered saline (PBS) at 4°C until dissection. Once received, each valve was photographed and visible anomalies, such as calcification, were noted. Patient data, such as LVAD use, age, gender, and medical therapies, was gathered as available. Control tissue, from patients without history of heart disease, was obtained from CCF and Ben Taub Hospital (Houston, TX). After autopsy, the tissue was excised and treated in the same manner as diseased tissue.

4.2.2. **Mechanical Testing**

4.2.2.1. **Machine Setup**

During dissection, 5-10 mm wide radial and/or 5-10 mm wide circumferential tissue sections were removed from the anterior and posterior leaflets of the valve and set aside for mechanical testing. Additionally, anterior basal/marginal and posterior basal/marginal chordae were tested. Tissue could be tested up to 5 days after transplant or autopsy, since valve tissues kept at 4°C have been shown to retain their matrix-based mechanical properties up to 5 days\textsuperscript{130}. All mechanical testing was performed using an Instron (Norwood, MA) or EnduraTec ELF 3200 machine (EnduraTec, Minnetonka, MN).
generated by the mechanical tester were imported into Mathematica® 5.0 (Wolfram Research, Champaign, IL) programs for analysis.

![Mechanical Tester with Grips and Tissue Tested](image)

**Figure 4.1:** EnduraTec ELF 3200 mechanical testing machine. The grips, clamping a piece of mitral valve leaflet, are shown in the inset.

The machines performed uniaxial tensile testing on small pieces of tissue (Figure 4.1). The tips of the grips were rounded to prevent tissue tearing, and the grip face was given a sandblasted finish to tightly grip the tissue between the two faces without slipping. As an extra precaution against slipping, 600 grit wet/dry sandpaper was glued onto the grip faces. A saline chamber was available to keep the tissue at 37°C during long-term testing. The Cleveland Clinic used a similar bath for all mechanical tests, but a chamber was not available during the first mechanical tests at Rice; these early samples were therefore not included in data analysis.
4.2.2.2. Sample Preparation and Dimensions of Test Strips

Before testing, the thickness and width of the tissue were measured. The strip of tissue was then inserted between the two grips of the machine, and the grips were tightened as much as possible to prevent slipping. The distance between the two grips was adjusted until the tissue sample was only slightly slack, and the gap was measured to give the initial length of the tissue.

The gauge length was defined as the point at which collagen fibers began to uncrimp and support the load imparted upon the tissue (Appendix 1). The original method used to calculate gauge length was defined by Carew and Vesely. After comparing the mathematical gauge length obtained by the Carew/Vesely method to the tissue load-elongation curve, the original algorithm was modified to better fit the physical results by Meaghan McNeill, a 2008 NSF REU student from Baylor University (Appendix 1).

To reduce hysteresis, restoring physiological tissue behavior, all tissue was pre-conditioned prior to mechanical testing by cycling the sample at least three times from zero strain to a strain able to induce elastic behavior. If the tissue showed evidence of slip or failure, the sample was discarded.

4.2.2.3. Load-Elongation

The tissue was then subjected to load-elongation testing. All tissues were cycled at 1 Hz from a state of zero strain to a strain sufficient to induce elastic behavior. The first three waveforms of the load-elongation test, as with the stress-relaxation and failure tests, were designed to retain the conditioned state
of the tissue. Subsequent waveforms were used for data analysis. Samples that showed evidence of slipping or failure were discarded. Time, load, and displacement were recorded to a text file for analysis in Mathematica.

4.2.2.4. Stress-Relaxation

After load-elongation testing, the tissue was subjected to stress-relaxation testing. The maximum strain used for load-elongation testing was used for the stress-relaxation testing. The tissue was first pulled using 3 triangle waveforms at a frequency of 1 Hz to return tissue to its conditioned state. The tissue was then returned to the peak displacement and held for 100 seconds, then returned to the initial displacement. Time, load, and displacement were recorded to a text file for analysis in Mathematica.

4.2.2.5. Failure

After stress-relaxation testing, tissue was pulled until failure after exposure to three preconditioning waveforms. If failure was not obtained for a specimen before reaching the machine’s displacement limit, results for load-elongation and stress-relaxation testing only were analyzed. Time, load, and displacement were recorded to a text file for analysis in Mathematica.

4.2.2.6. Radius of Curvature

Radius of curvature is a relatively new phenomenon to be studied in valve tissue mechanics. As noted in Chapter 2, the valve tissue is anisotropic; the orientation of the collagen fibers in the circumferential direction causes greater
stiffness in the circumferential than the radial direction$^{132}$. When viewing a tissue
stress-strain curve of a circumferential section, the transition region is smaller. If
a circle were to be fit to this transition region, its radius would be smaller than if a
circle were fit to the transition region of the stress-strain curve of a radial
segment. The differences in the radii of curvature between these two tissue
orientations are due to the mechanisms of collagen uncrimping as the tissue
begins to bear load. Collagen fibers are preferentially aligned in the
circumferential direction in the valve leaflet and uncrimp together when tension is
applied to the tissue. On the other hand, the fibers are less aligned in the radial
direction and uncrimping occurs in a more heterogeneous manner as fibers
rotate before bearing load. This new tool to assess mechanical behavior is
therefore especially novel, as it can be used as a measure of collagen fiber
orientation within the leaflet. In this manner, a circle was fit to the transition
region of the strain-strain curve from load-elongation data obtained during testing
in Section 4.2.2.3. The radius of this circle was measured using Mathematica.

4.2.2.7. Data Analysis

All results were analyzed using a one-way analysis of variance (ANOVA)
between the two test groups of CHF patients with and without LVAD support in
addition to a group of normal subjects. Results were considered statistically
significant at p<0.05. A Holm-Sidak, or Dunn's test was then used as appropriate
to assess differences between subgroups showing a statistically significant
difference (p<0.05). Independent variables were the tissue regions tested and
the tissue displacement (strain) throughout the course of the experiment. The
dependent variable was the force response (stress) of the tissue sample to the
displacement.

4.2.3. **Histology and Immunohistochemistry**

4.2.3.1. **Sample Preparation**

A 5 mm radial section of the posterior and anterior leaflets of each valve, as available, was reserved for histology. Strips extended the entire radial length of the valve, from annulus to free edge. When possible, the strips were taken from the center of the leaflet. These strips sometimes contained chordae; however, chordal diameter is small compared to the width of the tissue sample, and the chordae was not present in many tissue sections from the microtome. As a result, chordal histological samples were often examined separately. Other tissue sections of interest, such as sites of commissural fusion, were taken when present. Histological sections were inserted into a tissue cassette and placed in HistoChoice™ Tissue Fixative (Amresco, Solon, OH) for at least 24 hours; use of HistoChoice™ prevented excessive cross-linking of the tissue.

Flex™ (Richard-Allen Scientific, Kalamazoo, MI), a mixture of isopropyl alcohol and methanol, was used in place of ethanol. After fixation, the tissue was dehydrated in preparation for embedding in paraffin. The sample was placed in 50% and 70% Flex™ 100 (Richard-Allen Scientific) solutions for 1 hour each. The sample was then placed in 80%, 90%, and 95% Flex solutions for 2 hours
each and in 100% Flex for 4 hours. Finally, the sample was placed in a 50:50 Flex:xylene solution and then 100% xylene for 4 hours each.

Prior to paraffin embedding, the sample was placed in liquid paraffin for 2 hours. The tissue was then placed in a mold with paraffin, which was allowed to set. After embedding, the sample was cut with a microtome into 5 μm sections and mounted on a slide. After the slides were dried, they were placed in an oven overnight and set aside for staining.

4.2.3.2. Hematoxylin and Eosin

Hematoxylin and eosin (H&E) staining (Appendix 2) was used to stain the cell nuclei blue (hematoxylin) and the cytoplasmic and ECM proteins pink (eosin) so that a change in cell concentration within a sample could easily be detected (Figure 4.2). Images of the H&E slides were created at 5X magnification. As available, at least three images each of the leaflet free edge, leaflet center, and chordae were created. Cell density in each valve layer was measured using ImageJ software (NIH, Bethesda, MD). Using the Movat sample (described below) as a guide to the locations of the atrialis, spongiosa, and fibrosa, regions of interest were defined on each image. The area of the region was measured, and the cells counted. Cell density was expressed in number/mm².

4.2.3.3. Movat Pentachrome

Movat staining (Appendix 2) was used to label the different ECM components in the sample. In the finished sample, elastin was stained black, smooth muscle cells and fibrin red, collagen yellow, glycosaminoglycals (GAGs)
and proteoglycans (PGs) blue-green, and cell nuclei a blue to black color (Figure 4.2). Rather than quantification of any one ECM component, this stain was used as a way to qualify any disruption to the layered valve structure.

![Figure 4.2: Posterior leaflet mitral valve samples from patient with ICM. H&E staining (left) indicates cellular density of tissue, while Movat staining (right) identifies ECM components in tissue.](image)

4.2.3.4. Immunohistochemistry (IHC)

Samples were stained for the presence of the PGs versican, decorin, biglycan, and collagen types I and III to ascertain the extent of ECM remodeling.
After capturing images of each sample, the images were blinded by another party to remove possible sources of bias. The samples were then semi-quantitatively graded for intensity on a scale of 0-4: 0 indicated no stain; 1 indicated a weak stain in <50% of sample; 2 indicated a weak stain in >50% of sample or strong stain in <10% of sample; 3 indicated a strong stain in <50% of sample, and 4 indicated a strong stain in >50% of sample. The samples were graded twice on two separate days. The difference between the two assigned grades was examined; a difference greater than 2 required a re-grade of the sample by microscope.

4.2.3.5. Data Analysis

Average cellular concentration was analyzed for significance among the three subject groups (normal, non-LVAD, and LVAD patients) using a one-way ANOVA. For immunohistochemical testing, the average grades for each subject group were also analyzed using a one-way ANOVA. Holm-Sidak, Tukey, or Dunn's post-hoc testing was used as appropriate, with p<0.05 defined as significant.

4.2.4. Biochemistry

Biochemical assays were used to quantify content of collagen, DNA, and GAGs in the tissue. Since biochemical data for normal tissue has already been

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1 Immunohistochemical data for PGs within the posterior leaflet of normal patients have been previously published133.
published by this lab\textsuperscript{12, 42}, the current work focused on comparing the differences between non-LVAD and LVAD patients.

4.2.4.1. Proteinase-K Digestion

Mitral valves excised from diseased and normal hearts were dissected into the following seven portions: anterior leaflet free edge (ALF); anterior leaflet center (ALC); anterior leaflet basal chordae (BC); anterior leaflet marginal chordae (MC); posterior leaflet (PL); posterior leaflet BC; and posterior leaflet MC. Any tissue left over after histological and mechanical testing samples were removed was set aside for biochemical analysis. The wet weight of each group was then recorded, and the tissue was dehydrated overnight. The dry weight of the tissue was recorded and percent hydration calculated.

Each sample was rehydrated with 1 mL 100 mM ammonium acetate (pH 7.0). More ammonium acetate was added as needed so that the liquid would cover the tissue. The tissue was minced, and a 100 \( \mu \)L aliquot of 10 mg/mL proteinase-K solution was added. Samples were allowed to digest for 16 hours at 60°C. If necessary, another 100 \( \mu \)L of proteinase-K solution was added to complete the digestion. The samples were heated for 30 minutes at a temperature slightly above 70°C to denature the enzyme. If desired, the samples were placed in a speed-vac to concentrate them to a volume less than 1 mL. This concentration step was not necessary, as it did not affect the amount of protein, collagen, DNA, or GAG in the sample; it just allowed simplified calculations using rounded volumes. After concentration, the sample was
leveled to 1.0 mL, 1.25 mL, or 1.5 mL using ammonium acetate. The sample was then set aside for use in the biochemical assays described below.

4.2.4.2. Collagen Determination

The hydroxyproline assay (Appendix 3), used to quantify collagen levels in tissue, works on the premise that heart valve collagen contains 13% hydroxyproline\textsuperscript{134}. The tissue was first digested with proteinase-K to break up the tissue structure and hydrolyzed with HCl to recover the peptide-bound hydroxyproline. The samples were mixed with a sodium N-chloro-p-toluene sulfonamide (Chloramine-T) and propanol solution to oxidize the hydroxyproline. After incubation with the Chloramine-T, the samples were incubated with an Erlich’s reagent (p-dimethylaminobenzaldehyde) and perchloric acid solution to form the chromophore. The assay samples were then analyzed using a spectrophotometer (Molecular Devices Corp, Sunnyvale, CA) to determine hydroxyproline content\textsuperscript{5}.

4.2.4.3. DNA Assay

The DNA assay was performed to give a measure of cellular density in the tissue\textsuperscript{135}. As for the hydroxyproline assay, an aliquot of tissue with a pre-estimated quantity of DNA\textsuperscript{42} was first digested with proteinase-K. Unlike the other biochemical assays, where the supernatant was tested, the DNA assay tested both the liquid and the residual solid matter, containing cells, from the proteinase digestion. The sample was sonicated to break up the individual cells.
and release the DNA, and the DNA was tagged with a fluorescent dye (Hoechst 33258) and read using a fluorimeter (Molecular Devices Corp, Sunnyvale, CA).

4.2.4.4. Carbohydrate Analysis

Fluorophore-assisted carbohydrate electrophoresis (FACE) is a process that uses a 1-D gel to differentiate GAG disaccharides by molecular weight and charge\(^{136}\). The tissue of interest was first digested with proteinase-K to break up the tissue structure and the PG core protein. Aliquots estimated from previously determined concentrations of total GAGs\(^{137}\) were then further digested with a chondroitinase to cleave the GAG chains into disaccharides of different sulfated amino sugars linked to \(N\)-acetyl-galactosamines or glucosamines. The terminal \(N\)-acetyl-galactosamine groups from the end of the GAG chain were also released. Chondroitinase ACII specifically cleaved HA and CS, and chondroitinase ABC specifically cleaved HA, CS, and DS\(^{138}\). After digestion, samples were fluorotagged using 2-aminoacridone (AMAC) (Molecular Probes, Eugene, OR) to replace the hydroxyl group on the disaccharide. After insertion into an acrylamide gel, the samples were electrophoresed and the gels viewed using a fluorescent light source\(^{136,138}\) (Figure 4.3).
Figure 4.3: FACE gel upon viewing with fluorescent light source. GAG disaccharides, as separated by charge and molecular weight, are indicated. C/D=Chondroitin/Dermatan Sulfate; GAG=glycosaminoglycan; GalNAc=N-acetyl-galactosamine.

4.2.4.5. Data Analysis

Biochemical data has been published for normal valves\textsuperscript{12}; data analysis in the present study was performed to assess differences in valve ECM between non-LVAD and LVAD patients. A Student’s t-test compared the mean values between the two patient groups. If an apparent difference was observed between short-term (<100 days) and long-term (>100 days) LVAD treatment, a one-way ANOVA was then performed between non-LVAD, short-term LVAD, and long-term LVAD patients with Tukey, Holm-Sidak, or Dunn’s post-hoc testing performed as appropriate. In both cases, p<0.05 was considered significant.
4.3. Results

This section first examines deviations from normal mitral valve function with CHF and LVAD treatment. After the mechanical testing results have been presented, the biochemical and immunohistological data describing changes in tissue structure will be examined. Since valve ECM structure and mechanical properties are related, these results will give a complete picture of the effects of the mechanical environment changes induced by CHF and subsequent LVAD treatment.

The data presented includes all patients examined during the course of this study (Table 4.1); however, not all groups are fully represented due to limitations on the availability of donated tissue. Additionally, the size of the LVAD limits its use in children and smaller women. It was decided, therefore, to define a cohort of these patients matched for gender, age, race, and pathology (Table 4.2). The results from this cohort will be presented as appropriate.

Due to the use of donated human tissue throughout this aim, obtaining an ideal sample size to achieve statistical significance was not always feasible. In the results below, if statistical analysis revealed a power value <0.80 (indicating a possible Type II error), the power is noted.

4.3.1. Mechanical Properties

Before testing, mitral valve tissue was divided into four groups: leaflet radial, leaflet circumferential, and the two types of chordae (BC and MC). The uniaxial testing of these two different leaflet orientations and of the two different
chordae was designed to give a clear picture of the changes in tissue behavior due to CHF and LVD treatment.

**Table 4.1: Patient Data.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>non-LVAD</th>
<th>LVAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>Mean Age</td>
<td>48.3±20.5</td>
<td>53.3±9.8</td>
<td>51.0±13.3</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Caucasian</td>
<td>13</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>Other/Unknown</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pathology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICM</td>
<td></td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>DCM</td>
<td></td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>RCM</td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>LVAD Support (days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100.2±72.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation.
Note: One patient was diagnosed with DCM and possible ICM.
CHF=congestive heart failure; DCM=dilated cardiomyopathy; ICM=ischemic cardiomyopathy; LVAD=left ventricular assist device; RCM=restrictive cardiomyopathy.

**Table 4.2: Patient Data – Caucasian Male Cohort**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>non-LVAD</th>
<th>LVAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Mean Age</td>
<td>56.0±21.3</td>
<td>53.0±10.3</td>
<td>57.3±6.2</td>
</tr>
<tr>
<td>LVAD Support (days)</td>
<td></td>
<td></td>
<td>111.8±78.5</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation.
CHF=congestive heart failure; LVAD=left ventricular assist device.

In the extensibility, failure strain, and radius of curvature leaflet data sets, data from a single LVAD subject will be presented. Calculations for samples
obtained at CCF were originally performed with the older gauge length algorithm. When the algorithm for determining gauge length was edited (Appendix 1), the gauge lengths resulting from execution of the two programs were very different for some samples. Analyses that relied heavily upon gauge length (i.e., extensibility, failure strain, and radius of curvature) therefore needed to be redone. Unfortunately, some of the original data files from CCF were missing. In the cases of stiffness, relaxation, and failure stress, the results of the calculations using the two different algorithms were similar enough to be able to confidently use CCF results. For the remaining analyses, CCF results were disregarded, and significance between control and non-LVAD subjects was assessed using the Student’s t-test.

4.3.1.1. Leaflet Radial

Radial leaflet stiffness (Figure 4.4) was significantly higher in CHF than in control patients. This stiffness remained elevated in patients treated with the LVAD (p<0.05, control vs. non-LVAD and control vs. LVAD). Conversely, extensibility was lower in CHF patients than control (p=0.03). Although only data from a single patient was available, this property also did not appear to recover with LVAD use. Although leaflet relaxation, a measure of viscous nature, did not vary significantly between test groups, it was positively correlated with age in non-LVAD patients (r²=0.73, p<0.01, data not shown).
Figure 4.4: Alterations to the mitral valve mechanical properties due to CHF and LVAD treatment. Stiffness (A) appeared to increase globally throughout the valve with CHF. Extensibility (B) decreased with CHF but the LVAD appeared to restore extensibility in the chordae. Relaxation (C) did not significantly change between any of the groups. While radial leaflet failure strain decreased (D) with CHF, circumferential leaflet failure tension (E) increased with CHF. Radius of curvature (F) generally decreased with CHF, with apparent recovery towards normal values with LVAD use. n=6-12 (control), n=7-18 (non-LVAD), n=1-6 (LVAD). Lack of error bar indicates n=1. Data represented as mean ± standard deviation. *p<0.05 (Dunn's post-hoc testing), †p<0.01 (Holm-Sidak post-hoc testing), ‡p<0.05 (Student's t-test), ^p<0.01 (Student's t-test), **p=0.03 between groups. CHF=congestive heart failure; LVAD=left ventricular assist device.
Although failure tension did not show any significant trends, failure strain was lower in CHF than control patients (p=0.02). Radius of curvature was also significantly lower with CHF (p=0.005), suggesting that the tissue underwent fibrotic remodeling radially. Effect of LVAD use on these mechanical properties could not be accurately discerned due to the small sample size.

Data from the matched patient cohort generally followed that of the entire population studied. Additionally, leaflet relaxation within the control patient group correlated negatively with age ($r^2=0.94$, $p=0.03$). It should also be noted that the leaflet thicknesses were not significantly different between the non-LVAD and LVAD patient groups (1.37±0.38 mm vs. 1.29±0.23 mm, non-LVAD vs. LVAD).

4.3.1.2. Leaflet Circumferential

Failure tension in the circumferential leaflet samples was close to reaching statistical significance ($p=0.08$ between groups, power=0.31). Since the number of LVAD patients was low ($n=3$), an additional comparison was made between the control and non-LVAD groups, and failure tension was found to be significantly greater in CHF than control patients ($p=0.04$, Student's t-test). LVAD use did not appear to return this tension to normal values. Stiffness, extensibility, relaxation, failure strain, and radius of curvature were not significantly different between the three subject groups (Figure 4.4). The non-LVAD radius of curvature tended to be lower than normal ($p=0.08$). No further trends were noted in the patient cohort, which had behavior consistent with the whole group.
4.3.1.3. Chordae

Chordal extensibility (Figure 4.4) was significantly different between the three test groups (BC: p<0.001, MC: p=0.03). Post-hoc testing showed that BC extensibility in LVAD patients was significantly greater than that in both control (p<0.01) and non-LVAD (p<0.01) patients. Stiffness was significantly greater in the MC of non-LVAD patients (p=0.03) and although the number of LVAD subjects was too small to confidently analyze (n=2), LVAD use did not appear to return stiffness to normal values.

Radius of curvature in the BC was significantly different between the three subject groups (p=0.02), and post-hoc testing showed that radius of curvature was greater in the LVAD patient group than the non-LVAD patient group (p<0.05). Radius of curvature was also lower in the MC of non-LVAD subjects than control subjects (p=0.004); since the number of LVAD patients was small (n=2), the effect of LVAD treatment could not be analyzed. Relaxation, failure strain, and failure tension were not significantly affected by either CHF or LVAD use.

The patient cohort held a number of interesting observations (data not shown). First, BC stiffness was positively correlated with LVAD support duration until 200 days, when a sharp drop occurred. MC relaxation in non-LVAD patients was significantly lower than that of controls (p=0.05); the number of LVAD subjects was too low to assess any impact of the device. Failure tension and age in the BC of LVAD patients tended to be positively related (r²=0.86, p=0.07).
Radius of curvature in MC tended to be negatively associated with age in the non-LVAD patients ($r^2=0.86$, $p=0.07$).

4.3.2. Structural Properties

After assessing mechanical behavior of mitral valve tissue with CHF and LVAD treatment, the microstructure and the overall matrix composition of the valves were examined. These changes to the valve structure illuminated possible causes of the observed changes to mechanical properties.

The different sections of the mitral valve have normal compositions relating to their function. The ALF and PL contain a more defined spongiosa, where the PGs and GAGs counteract the compression forces imposed upon the tissue during coaptation. Conversely, the ALC and chordae have a larger collagenous fibrosa layer to support the tension imparted upon the tissue. The following section will include a description of the changes to the layered structure of the valve and the relationship to the mechanical changes noted above.

4.3.2.1. Collagen

When measured biochemically, no change in overall mitral valve collagen content was noted between non-LVAD and LVAD patients in the general population; in the cohort, ALF collagen was significantly greater in LVAD patients than non-LVAD (52.0±18.5% vs. 40.5±6.3%, $p=0.05$). When compared to non-LVAD patients, however, short-term LVAD patients (55.0±27.2 days, $n=10$) showed significantly higher levels of collagen while long-term LVAD patients (190.6±35.5 days, $n=5$) showed significantly lower levels (Table 4.3) in the ALF
(p=0.03) and the PL (p=0.05). Mean collagen levels in the ALF of short-term LVAD patient groups were significantly higher than those of long-term LVAD patient groups (p<0.05). Post-hoc testing did not show any further significant changes in PL collagen levels between any paired combinations of the three subject groups. In the cohort, collagen in LVAD patients was significantly greater than that in non-LVAD patients in the ALF and PL. Additionally, collagen content in the posterior MC was negatively correlated with LVAD support duration ($r^2=0.64$, p=0.02).

**Table 4.3: Percentage collagen in mitral valve.**

<table>
<thead>
<tr>
<th></th>
<th>Non-LVAD</th>
<th>Short-Term LVAD</th>
<th>Long-Term LVAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior Leaflet Free Edge</td>
<td>46.0±12.2 (n=28)</td>
<td>57.1±13.9 (n=9)</td>
<td>39.4±10.6 ‡ (n=5)</td>
</tr>
<tr>
<td>Anterior Leaflet Center</td>
<td>47.2±9.0 (n=26)</td>
<td>49.6±7.2 (n=8)</td>
<td>44.2±17.2 (n=5)</td>
</tr>
<tr>
<td>Posterior Leaflet †</td>
<td>44.5±10.2 (n=28)</td>
<td>54.2±16.0 (n=10)</td>
<td>39.3±11.9 (n=5)</td>
</tr>
<tr>
<td>Anterior Chordae</td>
<td>50.4±10.4 (n=54)</td>
<td>50.9±7.2 (n=18)</td>
<td>50.9±8.1 (n=10)</td>
</tr>
<tr>
<td>Posterior Chordae</td>
<td>55.2±10.7 (n=55)</td>
<td>60.1±11.5 (n=16)</td>
<td>55.1±11.4 (n=8)</td>
</tr>
</tbody>
</table>

Data are given as mean ± standard deviation.
* p=0.03 among groups; † p=0.05 among groups; ‡ p<0.05 in comparison to short term LVAD samples.
LVAD = Left Ventricular Assist Device

When Movat slides from all CHF patients were compared to the control subjects, collagen and elastin staining in the CHF valves appeared stronger in the spongiosa, particularly at the free edge (Figure 4.5). These areas of collagen...
and elastin deposition within the normally PG-rich spongiosa were deemed "mixed spongiosa". Collagen found in the mixed spongiosa was mostly type I collagen and was combined with the PGs in a marbled pattern (Figure 4.6). Similarly, areas of PG and elastin deposition found within the normally collagenous fibrosa layer were called "mixed fibrosa". This mixed fibrosa showed greater PG staining in CHF patients. Collagen and PGs were also present within the normally elastin-rich atrialis layer of CHF patients; these changes were particularly noticeable near the free edge of the PL. When compared to control subjects, it was observed that expression of both collagen type I and type III was lower in the CHF AL atrialis while collagen I expression was higher in the CHF PL atrialis. Type I collagen was slightly greater in LVAD posterior chordae and type III collagen was greater in both anterior and posterior LVAD chordae; both types of collagen were found outside of the core, where collagen is observed in normal valve tissue.

Examination of Movat-stained mitral valve tissue showed no differences in overall amount or localization of total collagen between non-LVAD and LVAD patients. Intensity of type I and type III collagen staining also did not show a significant difference between the three subject groups.
Within short-term LVAD support, collagen content in the ALF of ICM patients was positively associated with LVAD support duration (Figure 4.7, $r^2=0.52, p=0.05$). In the PL, ICM patients older than 60 treated with the LVAD showed less staining for collagen I ($p<0.001$) and collagen III ($p=0.04$) when compared to patients younger than 60. No other trends were noted when patient data were analyzed by LVAD support duration or patient age.
Figure 4.6: Anterior leaflet free edge of LVAD patient. Spongiosa collagen noted in the Movat stain (A), when compared to a negative control (B), contained high levels of collagen I (C) and moderate levels of collagen III (D). Thick black arrows indicate collagen and thin white arrow indicates elastin deposition in the normally protoglycan-rich spongiosa of the free edge. a=atrial side; LVAD = Left Ventricular Assist Device; v=ventricular side.

Collagen Dependence on LVAD Support Duration

Figure 4.7: Change in collagen content in anterior leaflet of ischemic cardiomyopathy patients with LVAD support duration. Within the short-term and long-term LVAD support groups, collagen levels tended to be positively associated with LVAD support duration. Regression line of anterior leaflet free edge in patients undergoing short-term LVAD support had an r² value of 0.52 (p=0.05). LVAD = Left Ventricular Assist Device.
4.3.2.2. Hydration

Hydration was greater in the ALC (p<0.01) of LVAD patients than in non-LVAD patients (Figure 4.8). Hydration was also significantly elevated in the anterior MC (p=0.03). In the ALC, hydration in non-LVAD patients was lower than normal, and hydration was closer to normal in the LVAD patient group. In the chordae, however, the non-LVAD patient tissue had normal water content while LVAD patient tissue was more hydrated than normal. A similar trend was noticed in the cohort; additionally, the posterior BC displayed a difference in hydration (78.6±3.6% vs. 68.4±17.2%, LVAD vs. non-LVAD, p=0.05).

**Average Hydration**

![Average Hydration Graph](image)

Figure 4.8: Average hydration of mitral valve in non-LVAD and LVAD patients. Anterior leaflet center and anterior marginal chordae show significantly greater hydration with LVAD treatment. Mean hydration values for normal valve sections are represented by dashed lines $^{12}$. *p<0.01; †p=0.03. LVAD = Left Ventricular Assist Device.
Differences in ALC hydration were statistically significant between the three CHF subject groups (p=0.03), and hydration levels were higher in both long-term and short-term LVAD groups as compared to the non-LVAD (p<0.05) (Table 4.4). A similar result was noted in the grouped anterior (basal and marginal) chordae (p=0.003), with significantly greater hydration noted in both the short-term and long-term LVAD patient groups as compared to the non-LVAD patient group (p<0.05). Hydration level changes were not noted in either the ALF or PL, nor were they dependent upon patient age or support duration.

Table 4.4: Percentage water in mitral valve.

<table>
<thead>
<tr>
<th></th>
<th>Non-LVAD</th>
<th>Short-Term LVAD</th>
<th>Long-Term LVAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anterior Leaflet Free Edge</strong></td>
<td>85.4±3.0 (n=29)</td>
<td>84.9±3.8 (n=9)</td>
<td>87.3±1.7 (n=5)</td>
</tr>
<tr>
<td><strong>Anterior Leaflet Center</strong></td>
<td>77.9±4.5 (n=27)</td>
<td>81.2±2.7 ‡ (n=9)</td>
<td>82.3±4.3 ‡ (n=5)</td>
</tr>
<tr>
<td><strong>Posterior Leaflet</strong></td>
<td>86.0±3.6 (n=29)</td>
<td>85.3±3.4 (n=10)</td>
<td>86.2±2.8 (n=5)</td>
</tr>
<tr>
<td><strong>Anterior Chordae</strong> †</td>
<td>73.8±10.5 (n=56)</td>
<td>79.5±3.8 ‡ (n=18)</td>
<td>78.7±12.2 ‡ (n=10)</td>
</tr>
<tr>
<td><strong>Posterior Chordae</strong></td>
<td>73.7±9.4 (n=58)</td>
<td>78.1±3.4 (n=17)</td>
<td>78.0±1.8 (n=8)</td>
</tr>
</tbody>
</table>

Data are given as mean ± standard deviation.
* p=0.03 among groups; † p=0.003 among groups; ‡ p<0.05 in comparison to non-LVAD samples.
LVAD = Left Ventricular Assist Device
4.3.2.3. Cellularity

Changes in overall DNA concentration between groups, measured biochemically, were used to assess changes in overall tissue cellularity. DNA concentration of mitral valves from CHF patients did not change with LVAD treatment. In ICM patients, DNA content in the ALF was negatively associated with the duration of short-term LVAD support (Figure 4.9, \( r^2 = 0.80 \), \( p = 0.04 \)). In the ALC, DNA content was lower in DCM LVAD patients than in ICM LVAD patients (\( p = 0.02 \)). There was no change in DNA concentration of the entire population with age; however, non-LVAD patients from the cohort experienced a negative association in MC cell concentration with age (anterior MC: \( r^2 = 0.73 \), \( p = 0.03 \); posterior MC: \( r^2 = 0.76 \), \( p = 0.01 \)).

![Graph A](image)

**Figure 4.9:** Change in DNA concentration in anterior leaflet of ischemic cardiomyopathy patients with LVAD support duration. (A) Within the short-term and long-term LVAD support groups, DNA content tends to be negatively associated with LVAD support duration. (B) Regression line of ALF in patients undergoing short-term LVAD support has an \( r^2 \) value of 0.80 (\( p = 0.04 \)). ALC = Anterior Leaflet Center; ALF = Anterior Leaflet Free Edge; LVAD = Left Ventricular Assist Device.

Since the mitral valve is a heterogeneous structure, cells on H&E-stained samples were counted to determine if any changes by cell layer existed. In the
ALC, cell density was significantly lower in non-LVAD subjects compared to controls in the fibrosa (p=0.01 between groups, p<0.05 for control vs. non-LVAD) and atrialis (p<0.001 between groups, p<0.05 for control vs. non-LVAD and control vs. LVAD). While the cell density in the atrialis of the LVAD group appeared to be slightly greater than that of the non-LVAD group, the cell numbers in the fibrosa did not appear to recover with LVAD support. While not significant, a similar pattern was noted in the mixed-fibrosa and chordal regions (Figure 4.10). This trend for the fibrosa held in the patient cohort (p=0.03 between groups; p<0.05, control vs. LVAD). Cell density in the CHF valves tended to be lower than normal in the atrialis, but the control sample size was too small (n=2) to suggest significance. Similar patterns were also noted in the annular region of the PL (Figure 4.10). Non-LVAD subjects had a lower than normal cell density; while LVAD use appeared to reverse this trend in the fibrosa and mixed-fibrosa regions, the remaining sections did not recover.

![Figure 4.10](image)

Figure 4.10: Cell density of the anterior leaflet center (A) and the annular region of the posterior leaflet (B). The heart failure valves often had lower cell density than control. LVAD treatment did not always reverse these trends. Data represented as mean ± standard deviation. *p<0.05. LVAD=left ventricular assist device.
In the spongiosa of the ALF, non-LVAD cell density was negatively correlated with age ($r^2=0.90$, $p=0.01$). The reverse trend was true for the fibrosa of the PL annular region ($r^2=0.98$, $p=0.001$). The mixed-spongiosa region of the PL annular region demonstrated lower cell densities with LVAD support duration ($r^2=0.73$, $p=0.01$). These trends in the posterior leaflet were also noted in the cohort, where cell density of the anterior chordae also tended to be lower than normal in all CHF patients ($p=0.066$).

4.3.2.4. Glycosaminoglycans

Normal heart valve glycosaminoglycan values have been published previously$^{12}$; the current research compares these values with those obtained for non-LVAD and LVAD heart valves. As in the report, results were grouped based on the type of force resisted by the tissue; tension (Figure 4.11) or compression (Figure 4.12).

In tension-bearing tissues such as the ALC (Figure 4.11), the D4S and hyaluronan (HA) GAGs have been found to be the most abundant normally$^{12}$. Hyaluronan (HA) was slightly lower than normal values in all CHF patients regardless of treatment. In the ALC of CHF subjects, however, unsulfated chondroitin (C0S) was significantly greater in LVAD patients compared to non-LVAD ($p=0.01$). Conversely, D6S was slightly greater than normal values in all CHF patients. The values of all other individual GAGs, as well as the total GAG content, did not significantly deviate from normal values in CHF patients,
regardless of treatment. In the patient cohort, overall HA content was significantly greater in LVAD than non-LVAD patients (p=0.01).

GAG expression in the chordae tended to follow many of the trends observed in the ALC. In the BC, HA content was slightly lower than normal in all CHF patients, regardless of therapy, while HA values in the MC remained unchanged from normal (Figure 4.11). In both BC and MC, C0S remained constant in non-LVAD patients but was greater in LVAD patients. C6S content in both BC and MC was greater than normal in non-LVAD patients, and while not significant, C6S content in LVAD patients tended to be greater than non-LVAD. Conversely, BC/MC D6S content in non-LVAD valves was slightly greater than normal while reduced in LVAD patients; this difference was significant in the posterior MC (p=0.05). C2S was significantly greater in LVAD than non-LVAD posterior MC (p=0.05). C4S, D4S, and total GAGs in both the BC and MC were not significantly different either between the two CHF groups or from normal values. In BC, GAG chain lengths in chondroitinase-ABC digested samples were significantly lower in non-LVAD than LVAD patients (54.9±49.7 vs. 95.4±79.3 disaccharides, p=0.01). In the patient cohort, BC and MC HA content was significantly greater in non-LVAD than LVAD patients (BC: p=0.05; MC: p=0.03). C0S and D4S in the MC were also greater in non-LVAD patients (p=0.05, C0S and D4S), as was total GAG content (p=0.03).

In the ALF (Figure 4.12), total GAG content was significantly greater in non-LVAD valves than LVAD samples (p=0.01). All CHF samples contained slightly less HA than normal tissue. While D4S in the non-LVAD tissue was
slightly greater than normal, LVAD tissue contained normal amounts of this GAG. In the patient cohort, D4S in the non-LVAD tissue was significantly higher than LVAD (p=0.03). No other differences were noted either between normal and CHF tissue or between non-LVAD and LVAD tissue.

For both PL CHF treatment groups, HA and C6S contents were slightly elevated from normal values. Although C4S content in the non-LVAD samples was equivalent to that of normal tissue, LVAD C4S was significantly greater than in non-LVAD tissue; this trend also held for C2S. No other significant differences were noted in the remaining GAGs or in total GAG content.
Figure 4.11: GAG composition in the tension-bearing regions of the (A) anterior leaflet center and (B) chordae. Data presented compare GAGs from non-LVAD and LVAD patients. Dotted lines indicate normal GAG composition of the mitral valve. GAG=glycosaminoglycan; LVAD=left ventricular assist device.

\*p≤ 0.05.
Figure 4.12: GAG composition in the compression-bearing regions of the (A) anterior leaflet free edge and (B) posterior leaflet. Data presented compare GAGs from non-LVAD and LVAD patients. Dotted lines indicate normal GAG composition of the mitral valve\textsuperscript{12}. GAG=glycosaminoglycan; LVAD=left ventricular assist device. \( \dagger p \leq 0.05 \).
4.3.2.5. Proteoglycans

Proteoglycan content was assessed using a semi-quantitative method of grading IHC-stained tissue samples for overall intensity. Samples were separated into anterior and posterior leaflets. All three patient groups were tested and compared in this manner. It should be noted that, while the diaminobenzene (DAB) was made from powder for the immunohistochemical staining of most of the PG samples (see Methods), the lab eventually switched to use of a DAB kit for health and safety reasons. The three controls tissues obtained later into the study period, therefore, used a different DAB treatment, which may have skewed the control results slightly higher. It is therefore possible that the results are more significantly different than indicated below.

Decorin staining was greater in LVAD anterior leaflet tissue than control or non-LVAD tissue samples (p=0.05 among groups) (Figure 4.13). Versican staining in LVAD tissue was greater than in control tissue (p<0.01, power=0.072). Biglycan staining intensity was not significantly different between any of the groups. Similar trends were noted in the posterior leaflet, but there were no significant differences between groups (power ≤ 0.151 in PG testing of cohort PL). PG staining intensity in both classifications of CHF patients tended to be greater in both anterior and posterior chordae, infiltrating the collagen core. In general, versican staining was co-localized with elastin while decorin and biglycan were co-localized with collagen.
Figure 4.13: Proteoglycan content of the anterior leaflet. Versican and decorin staining intensities were both greater in the LVAD than normal samples. Negative control intensity staining=0.34±0.48 (all groups). * p<0.05 among all patient groups, † p<0.01. LVAD=left ventricular assist device.

No significant differences were observed in PG content with LVAD support duration or patient age. When comparing DCM and ICM patients, it is important to note that due to the small number of DCM LVAD patients in the study, statistical significance could not be reached. In ICM patients, there was a trend of lower biglycan staining in LVAD patients (2.4±0.8 vs. 1.8±0.4, non-LVAD vs. LVAD, p=0.065). In the anterior leaflet of ICM patients, versican staining intensity was greater in short-term LVAD patients (2.7±0.2 vs. 2.0±0.0, short-term vs. long-term, p=0.01). Decorin staining in the posterior leaflet was slightly greater in long-term LVAD patients, but significance could not be ascertained due to a small sample size (n=3).
4.4. Discussion

Previous research in this lab showed that the mitral valve underwent fibrotic remodeling in CHF patients\(^{42}\); this remodeling was likely due to changes in the geometry of the left ventricle and therefore in the forces imparted upon the valve during the cardiac cycle. The tissue itself became stiffer and less hydrated and contained more collagen and cells. It was unknown whether the unloading of the left ventricle due to LVAD treatment encouraged beneficial or continued pathological remodeling of the mitral valve tissue.

Tissue remodeling throughout the valve was varied in nature and did not always have the expected compensatory result on the tissue structure. Partial summaries of the structural remodeling are located below (leaflet center: Figure 4.14, chordae: Figure 4.15, leaflet free edge: Figure 4.16). LVAD use did not appear to reduce collagen content toward more normal values; however, tissue from long-term LVAD patients contained less collagen than short-term LVAD patients. The amount of collagen was greater in both the ALC, which was normally rich in collagen, and in the PG-rich ALF, which contained disorganized collagen and elastin. The greater than normal GAG content of the non-LVAD ALF suggested remodeling of the tissue, while the reduced GAGs in LVAD tissue showed a possible compensatory mechanism of the tissue. Conversely, GAG content was slightly greater in the non-LVAD posterior leaflet with no substantial recovery due to LVAD use. Unlike normal ALF and PL tissue, which contains more 6S GAGs than 4S GAGs\(^{12}\), the fact that CHF tissues contained similar quantities of both types of GAGs indicated that different PGs were involved in the
remodeling of the tissue. In particular, the shift from HA to D4S may have been related to ALF fibrosis in the non-LVAD patients. Greater versican and decorin expression in the LVAD anterior leaflet may have indicated the formation of new elastin and collagen, with elastin^{17} appearing in the short-term and collagen appearing with long-term support. The increase in collagen coupled with the decrease in DNA concentration with short-term LVAD support also indicated that the amount of collagen, and possibly other ECM components, produced per cell increased. A cohort of Caucasian males was defined to study trends within a matched patient subgroup. The results of this cohort shared many trends with the overall patient overall group, lending credence to the testing and analysis methods.

<table>
<thead>
<tr>
<th>Leaflet Center</th>
<th>Normal</th>
<th>CHF non-LVAD</th>
<th>CHF LVAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA (Hoechst)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells (H&amp;E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin (Unsulfated)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.14: Overview of selected biochemistry results for the anterior leaflet center. Arrows represent relative magnitude of component content between normal/non-LVAD patients and non-LVAD/LVAD patients. Red arrows indicate pathological remodeling, while green arrows indicate no change or beneficial remodeling.
Figure 4.15: Overview of selected biochemistry results for the chordae. Arrows represent relative magnitude of component content between normal/non-LVAD patients and non-LVAD/LVAD patients. Red arrows indicate pathological remodeling, while green arrows indicate no change or beneficial remodeling.

While the free edge remained fibrotic with LVAD treatment, the collagenous ALC and chordae began to recover normal hydration levels. It is interesting to note that HA, which is also associated with hydration, was lower in these tissues, suggesting that an upregulation of different GAG (i.e., sulfated) classes, such as the 6S GAGs found in the hydrated PG versican, was responsible for the higher tissue water content, especially in chordae. DNA concentration was lower in the ALC, suggesting that overall ECM upregulation, rather than greater cell proliferation, was the responsible mechanism. In LVAD ALC samples, the relative proportion of 4S and 6S GAGs was similar to that of the normal ALC\textsuperscript{12}; remodeling did not tend to alter the basic GAG composition of the tissue.
Figure 4.16: Overview of selected biochemistry results for the anterior and posterior leaflet free edges. Arrows represent relative magnitude of component content between normal/non-LVAD patients and non-LVAD/LVAD patients. Red arrows indicate pathological remodeling, while green arrows indicate no change or beneficial remodeling.

Mechanical testing results showed that the ECM remodeling described above worsened leaflet behavior while improving chordae behavior to a point similar to that observed in normal tissue. In the mitral valve leaflet, collagen fibers are normally aligned parallel to the annulus, in the circumferential direction. The orientation of the fibers shifts to align in the radial direction at the commissures and the strut (large basal) chordae. This alignment imparts a normal greater stiffness and lower extensibility in the circumferential direction than in the radial direction. While properties in the circumferential direction did not change drastically with either heart failure or LVAD use, radial stiffness was greater and extensibility lower in CHF subjects. This result suggested that any
new collagen incorporated into the CHF mitral valve structure was less organized than in normal valves and tended to be more oriented in the radial direction, a result corroborated by the decrease in the radius of curvature of the radial section load-elongation curve. The marbled collagen and elastin in the mixed spongiosa layer as well as the marbled elastin and PGs in the mixed fibrosa layer also indicated that these valve layers were bearing their respective compression and tension loads less efficiently than normal. Conversely, LVAD use appeared to return chordal mechanical behavior to normal. This result may have been due to the general uniaxial motion of the chordae and highly aligned collagen in the tissue.

It is interesting to note that the mitral valve segments that displayed the greatest increases in water content with LVAD treatment, the ALC and chordae tendinae, were the least hydrated tissues of the normal mitral valve. Conversely, the ALF and PL, which contained the least collagen in normal valve tissue, showed the greatest changes in collagen content with CHF and LVAD treatment. These results were likely due to changes in the types of forces imparted on the different valve sections due to the changing geometry of the left ventricle in CHF and in LVAD unloading. For example, decreased coaptation of the valve free edges in CHF due to a dilated left ventricular annulus apparently encouraged valve fibrosis. If the LVAD did not restore this coaptation, and the mitral valve instead fluttered during the cardiac cycle, normal valve structure and resulting functionality would not be restored.
Changes in hydration, collagen content, and cell concentration were often noted with longer LVAD support duration. In particular, mean collagen content was reduced below normal values after 100 days of LVAD treatment. The reduction in mean collagen concentration may also indicate that, with long-term LVAD support, cells upregulated production of other ECM components such as PGs, possibly leading to the lower proportion of collagen measured in the ALF and PL. The time scale of these changes to the valve microstructure were interesting when compared to the decline in ventricular function after 90-120 days of LVAD support previously noted \(^ {52,53}\); it was likely that this decline led to dysfunctional remodeling after long-term LVAD support.

The two measures of change in cellularity, DNA concentration and cell density, had very different outcomes. These apparently contradictory results can be clarified by understanding what exactly was measured. The Hoesch assay resulted in DNA concentration in the entire tissue sample. Unfortunately, the digestion of the tissue required for the assay removed the ability to examine any layer-specific cellularity changes. Tissue dehydration in preparation for digestion also negated the water-retention property of the spongiosa GAGs and PGs, leading to a lower initial tissue volume against which cellularity was normalized. In order to consider the effect of the layered valve structure, cell density in H&E-stained samples was measured by counting cell nuclei in each layer. The downfall of this cellularity measurement was its dependence on the point at which the tissue was mounted upon the slide. The two measures of cellularity combined gave a more complete assessment of changes in cell proliferation with
CHF and LVAD use. In the ALC, DNA concentration in CHF patients was greater than control\textsuperscript{42}, and did not appear to recover with LVAD use. Cell density in the fibrosa and atrialis, on the other hand, was lower in both CHF and LVAD patients when compared to control. These results suggested that cell number increased with CHF as well as increased ECM secretion, particularly elastin and collagen, per cell. This increased ECM per cell was also noted in the ALF, where DNA concentration decreased and collagen concentration increased with short-term LVAD support duration.

Remodeling of the valve tissue, while appearing to restore the biochemical tissue composition, did not improve leaflet mechanical behavior. Hydration and PG content both were greater in the LVAD leaflets. These results suggest that the valve tissue was undergoing remodeling towards a less fibrotic state, possibly to compensate for the lack of mobility in the radial direction. Additionally, overall collagen content appeared to have been negatively associated with prolonged LVAD use, but immunohistochemical staining of the normally PG-rich ALF indicated presence of disorganized type I. It was possible that the lower overall collagen was due to PG abundance rather than a decrease in collagen. Reduction of overall GAG content in the ALF further suggests that the tissue remodeling did not improve radial mechanical behavior of the tissue. Instead of the compartmentalized three-layer structure found in the normal valve, the non-LVAD and LVAD patient valves had a more amorphous composition. This disruption of the normal structure may explain the continued suboptimal
performance of the mitral valve tissue, particularly in the radial direction, with LVAD use.

One major limitation of this project was the small number of LVAD subjects available for analysis. A larger patient group would corroborate some of the trends reported while improving characterization of the LVAD effects on women and minorities. A more varied patient group could also allow the efficacy of different LVAD designs to be compared. An additional limitation was the inability to compare tissue effects of heart failure with LVAD treatment in the same patient. All tissue samples were obtained at transplant; therefore, the tissue properties at a final CHF state, but not multiple timepoints, could be studied. In the future, an interesting subject group would be patients weaned from the LVAD, as the use of tissue from transplant patients assumes a failed heart.

Overall, collagen was upregulated in the CHF tissue, resulting in a disorganized structure compared to the normal layered mitral valve and reduced radial motion of the leaflet. The LVAD induced further remodeling of the tissue, but in such a way that the normal tension-bearing ALC and chordae become more hydrated and the normal compression-bearing ALF and PL remained fibrotic, with the disorganized collagen dominating the tissue mechanical behavior. PG content was greater with LVAD treatment; this compensatory mechanism for radial movement could not overcome the disruption of the layered structure.
4.5. Conclusion

CHF induces fibrotic remodeling of the mitral valve; the tissue is stiffer, less hydrated, more collagenous, and more cellular. This remodeling is due to dilation of the left ventricle and resulting changes in the mechanical conditioning of the valve tissue. The LVAD unloads the left ventricle, allowing sufficient recovery of the tissue to bridge the patient to transplant. The LVAD has been successful as a bridge-to-transplant device, and an additional use has been proposed for the LVAD as a destination therapy. In order for the device to be successfully explanted after completion of treatment, normal function of the entire heart should be restored.

The current research has shown that the mitral valve did undergo continued remodeling as a result of the altered load imparted by the LVAD. This remodeling apparently benefitted the chordae. The tissue retained its usual hydration, and the normal mechanical properties were restored. Unfortunately, it does not appear that this remodeling restored the normal leaflet structure; the disrupted layered structure remained, especially in the normally PG-rich free edge. This altered leaflet structure translated to a continued suboptimal mechanical performance of the radial leaflet tissue. Before the LVAD can be widely used as a destination therapy, more work should be done to ensure that the mitral valve function can be restored prior to weaning the patient from the device to prevent further complications.
Chapter 5. Design and Validation of a Splashing Bioreactor

Specific Aim 2: Develop an inexpensive, efficient bioreactor to maintain mechanical stimulation to the mitral valve while modeling normal and altered chemical environments of the tissue.

5.1. Background

Although animal models have been used with great success in studying many forms of cardiovascular disease, the invasive nature of valve surgery creates an obstacle in studying the early stages of valve disease. Additionally, a large number of animals would be required to study multiple early timepoints. Alternatives to surgical methods, such as molecular imaging, have been explored\textsuperscript{140}. Cell studies are another less expensive alternative; current experimental research of valvular biology and remodeling generally employs cultures of valvular cells maintained in a monolayer\textsuperscript{106, 141, 142}. This widely accepted protocol, however, does not allow investigators to determine any changes to the layered valve structure. Additionally, a monolayer culture imposes a drastic change in environmental stimuli for the cells, and thus may not accurately elicit the \textit{in vivo} response. To compensate for these shortcomings, this aim focused on organ cultures, which maintain the valvular cells within the native physical structure of the tissue. Ideally, the organ cultures are grown in conditions engineered to mimic the native environment. Organ cultures have
successfully been established for a variety of tissues, including vascular intima\textsuperscript{143}, canine leptomeninges\textsuperscript{144}, mouse gallbladder epithelium\textsuperscript{145}, brain hippocampal slices\textsuperscript{146}, and human lacrimal gland tissue\textsuperscript{147}. In human and mammalian heart valve tissues, short-term (6 day) organ cultures have been used to investigate wound healing responses\textsuperscript{148-150}.

Heart valve tissues are particularly appropriate for organ culture research because the cells within adult valves are nourished primarily through diffusive transport of oxygen and nutrients (as opposed to through vascularization\textsuperscript{151}). Therefore, it was believed that designing a valvular organ culture system might be much simpler than for more complicated fully vascularized tissues. Previous work in our laboratory used a simple system for organ culture (based on work by Lester et al.\textsuperscript{148-150}) to demonstrate that the cells within the cultured valve tissues remained alive during culture periods up to 7 weeks\textsuperscript{152}.

The aforementioned work has also suggested, however, that static heart valve organ culture techniques did not maintain cell location. Fewer endothelial cells were noted on the cultured tissue. Although interstitial cells were still present and viable after seven weeks, fewer cells were located deep within the tissue, suggesting cell death or migration of these cells to the periphery to obtain nutrients\textsuperscript{152}. Results from a histological study performed by Anna Sophia McKenney, an undergraduate intern (manuscript in progress) indicated that with increased culture time, the layers of the valve became less defined. That work was performed on porcine tricuspid valves, which have a layered structure similar to that of the mitral valve. Expression of prolyl-4-hydroxylase (P4H), an indicator
of collagen formation, was consistently upregulated over a culture period of five weeks (Figure 5.1). Proteoglycan (PG) content in the spongiosa decreased dramatically as collagen content increased, creating a spongiosa layer more similar in structure to the fibrosa (Figure 5.2). Elastin, generally noted in the atrialis, also became more diffusely distributed within the tissue. A successful bioreactor design would limit this fibrotic remodeling.

Figure 5.1: Collagen synthesis mediator upregulation in porcine tricuspid valve organ cultures at 0, 1, 3, and 5 weeks (magnification=10X). The rightmost figures (magnification=40X) illustrate the accumulation of surface cells in damaged regions and crevices of the tissue blocks. (A) P4H, with negative control (B) beneath, (C) Movat pentachrome stained sections corresponding to the P4H images, suggesting that cells have migrated from the densely collagenous regions, but then begin to synthesize collagen (in yellow). P4H=prolyl-4-hydroxylase. Images courtesy of Jane Grande-Allen and Sophia McKenney.
Figure 5.2: Movat pentachrome stained sections of porcine organ cultured valve tissue blocks (magnification 10X). The ventricular surfaces are on the right and the atrial surfaces are on the left. (A) Collagen content (yellow) clearly dispersed from the normal structure and began to dominate the valves, usually becoming the only remaining matrix protein by the conclusion of culture. (B) PG and GAG content (blue-green) gradually disappeared, becoming just a diffuse haze in the mixture. (C) Elastin (black) became less localized and occasionally dominated the cross section, often together with collagen. Arrow indicates increased culture duration. GAG=glycosaminoglycan; PG=proteoglycan. Images courtesy of Jane Grande-Allan and Sophia McKenney.

5.2. Methods

5.2.1. Bioreactor Design Evolution

The design of the basic bioreactor was intended to be a subphysiological representation of a mitral valve environment; the emphasis was to be placed on creating a simple and easily reproducible model. The bioreactor design was motivated by the in vivo movement of the fluid past the valve providing both perfusion and gentle stretch to the tissue. The valve was to be positioned such
that it would "open" and "close" at regular intervals, although less frequently than observed clinically. The tissue would be attached slightly slack to prevent excess tension along the chordae during organ culture.

The original design of the bioreactor included a chamber made of transparent plexiglass (Figure 5.3A). This chamber would be sealed at the top with a 0.2 μm filter screw cap to allow air exchange while ensuring sterility. A valve, submerged in fluid, was to be mounted using sterile 4-0 polypropylene sutures (Ethicon, Somerville, NJ) onto customized ledges in the chamber. A silicone membrane attached to the bottom of the bioreactor would be jostled regularly to model blood flow over the mitral valve surface. The bottom of the membrane rested against a piston that moved up and down courtesy of a rocker plate. When the piston attached to the membrane was pushed up (Figure 5.3B), fluid would flow upward in the chamber, past a mitral valve leaflet segment and chordae. When the piston was pulled down (Figure 5.3C), the fluid would flow in the opposite direction. This action of the fluid would perfuse and exert gentle forces on the leaflet face and pull the chordae.
Figure 5.3: Original bioreactor design. The original chamber design (A) consisted of a hollow tube into which a mitral valve segment would be sutured, from annulus to chordae. The bottom of the tube would be covered with a silicone membrane, which rested atop a piston. The piston would cyclically move down (B) and up (C), forcing culture media inside to splash across the valve surface. Image courtesy of Jane Grande-Allen.

Construction and use of the proposed design, however, proved to be difficult and impractical. Suturing the valve segment into the long, thin tube was awkward. Additionally, the piston was moved by a rocker plate, and the motion was not easily reproducible between cycles. Additionally, most membrane materials tested failed within days. The final design combined user convenience with reproducible motion.

The original solid chamber design was first divided into multiple segments (Appendix 4 – ELSIE Version 1). The chamber was then filled with fluid (Figure 5.4) and allowed to sit for one week with no piston motion. When the chamber design proved to be leak-proof, a dynamic test was designed (Figure 5.5), where the goal was to expose the chamber to cyclic piston-driven fluid motion for a period of two weeks. The small diameter of the inner chamber required a thin,
flexible membrane that could deform to the extent necessary to force the culture media to move up into the chamber. Different membrane materials, including latex and nitrile, as well as a variety of membrane shapes were tested. Unfortunately, the nature of the long-term experiment required a more robust material, and no material or membrane shape was found that satisfied all these requirements.

![Figure 5.4: Static leak test. The segmented ELSIE design was allowed to sit for one week with fluid inside to determine water-tightness.](image)

At this time, it was decided to focus on the use of the chamber as an organ culture environment. The solid chamber top was replaced by a threaded top (Appendix 4 – Version 2) onto which a filtered cap from a tissue culture flask
could be placed. This cap design allowed for easy replacement of the culture media as well as air exchange. A nitrile membrane was attached to the base of a number of different ELSIE segments, with the intent of replacing membrane segments during media changes to circumvent the short lifespan of the membrane (Figure 5.6A, Appendix 4 – Version 3). This design was eventually discarded due to a concern of damaging the organ cultures with repeated dismantling of the chamber.

Figure 5.5: Leak test with dynamic motion. The ELSIE bioreactor was set up with the piston moving up (A) and down (B). After 3 days (C), the membrane began to show signs of wear (black arrow). Rupture occurred after 5 days (D, black arrow).
Throughout these design changes, the movement of a single plunger was powered by a simple 14V DC motor (Barber-Coleman, Rockford, IL) connected to a power source. In order to determine how well this motion translated to multiple chambers, the chambers were lined up in series (Figure 5.6B). The motion was weaker in chambers further from the motor, and membrane leakage was still a major issue. After the motion test, a lab rotator was found that could move the chambers 360°; the resulting fluid motion was similar to that applied by the piston and had the advantage of being replicable (Figure 5.6C). The membrane was therefore replaced by a solid piece in the final design, resolving the leakage issue.

Figure 5.6: Improving mechanical motion constancy and removing leak sources. (A) Using replaceable membrane segment to work around material fatigue issue. (B) Ascertaining reproducibility of motion between several chambers. (C) Replacing the piston with a lab rotator fixed both issues of material fatigue and motion reproducibility.
5.2.2. Final Design

The final bioreactor chamber design (Appendix 5) consisted of 3 polycarbonate segments (McMaster-Carr, Chicago, IL), each 2" in diameter (Figure 5.7). The segments were clamped together via four 4" long, ¼"-28 partially threaded screws and nuts spaced 90° around the circumference of the bioreactor. The two ends of each segment each had 3 1/16" neoprene washers (McMaster-Carr, Robbinsville, NJ) mounted using Loctite® glue. The washers were wrapped with Teflon tape upon chamber construction to prevent leaks.

The main body of segment “A” was 1.5” tall and had a ½” hole in the center to accommodate fluid flow. The top of “A” was 1” tall and threaded to fit the vented filter cap from a 25 cm² tissue culture treated flask (BD Biosciences, San Jose, CA) in order to allow gas exchange in the bioreactor. Segment “B” was 5/8” tall and had a ½” hole in the center. Polyester mesh squares (Sefar America Inc, Depew, NY) approximately 0.5x1 mm were glued between the neoprene washers to provide a mounting location for the mitral valve. Segment “C” was 1.25” tall, and a ½” hole was cut through the top 1” for fluid flow. The bottom ¼” of the segment was solid.
Figure 5.7: Splashing bioreactor chamber. The chamber was comprised of three hollow segments ("A", "B", and "C") into which the valve segment was mounted. The media (represented here by the green fluid) provided nutrients to the tissue. Valve attachment sites (inside chamber) are also indicated by ANN=annulus and CH=chordae.

Fluid motion was provided by a Labnet Mini LabRoller ™ (ISC Bioexpress, Kaysville, UT). This rotator fit two bioreactors on a pallet, secured with two 3/16" cable ties (Figure 5.8). The rotator moved a full 360° at an approximate speed of 0.33 revolutions/second. This rotation caused the media to splash across both valve surfaces, imparting a combination of normal and shear force to the valve. Turbulent flow in the physiological system generally indicates an abnormality,
such as valve regurgitation or stenosis\textsuperscript{153}. Media in the bioreactor tended to flow along the inner volume of the chamber; the result was a mostly laminar flow with turbulence at the chamber top and bottom, far from the tissue sample. The use of the rotator ensured that the motion was repeatable. A final volume of 4.5 mL was selected; while the media only partially bathed the tissue, this smaller volume (as opposed to completely filling the chamber with fluid) allowed greater forces to be imparted onto the valve surface. The presence of any bubbles in the media from the splashing motion improved oxygen pressure in the system.

![Figure 5.8: Bioreactor on rotator. The pallet moved a full 360 degrees, forcing media over the valve surfaces.](image)

Sterility testing of the bioreactor was performed by allowing media to remain in the chamber for one week. Initial tests were performed without valve tissue, and later tests included a mitral valve segment mounted in the bioreactor.
chamber. When the media was changed, a sample of the old media was placed into a 6-well plate and returned to the incubator. A sample of the fluid was also applied to an agar plate and allowed to incubate for 3 days. Additionally, media was spot-checked for contamination during the design validation studies (Section 5.2.3) by drawing media samples and incubating in a 6-well plate for 2-3 days.

5.2.3. Design Validation

Porcine mitral valves from animals approximately six months in age were used to validate the bioreactor design. Valves were extracted from the heart within 6-10 hours after sacrifice and rinsed in sterile PBS containing 5% antibiotic/antimycotic solution (Mediatech Inc, Herndon, VA) to remove bacteria. Two 5 mm wide radial sections (one surrounding each strut chordate, Figure 5.9A) were then removed from the anterior leaflets (n=19). The first section from each valve was cultured, under either static or dynamic conditions, for two weeks. Static cultures (negative control, n=10) were placed in a T-25 tissue culture flask, while dynamic cultures (n=9) were mounted in the bioreactor (Figure 5.9B,C). In both test cases, the tissue was bathed in organ culture media containing 5% antibiotic/antimycotic solution for the first 24 hours after dissection, after which it was replaced by media with 1% antibiotic/antimycotic solution. The second section from each valve was designated a baseline control sample. All tissues were fixed in Histochoice™ after two weeks (cultured samples) or immediately after dissection (baseline control samples). After fixation, all tissue
samples were dehydrated to xylene, paraffin embedded, sectioned into 5 μm slices, and mounted on slides (Section 4.2.3.1).

![Diagram of valve insertion](image)

**Figure 5.9: Valve insertion.** (A) A 5 mm radial valve section was removed from the AL. (B) The section was then mounted at the ANN and CH. The free edge was unattached. (C) Top view of valve in bioreactor. AL=anterior leaflet, ANN=annulus, CH=chordae, PL=posterior leaflet.

All samples were stained with H&E and Movat pentachrome (Appendix 2) to visualize the cells and extracellular matrix (ECM) components within the tissue layers. The PGs versican (VC), decorin (DCN), and biglycan (BGL), as well as collagen types 1 and 3 (COLL1/COLL3), were localized within the valve.
microstructure through immunohistochemical staining (Appendix 2). In addition to these ECM components, localization of the ECM precursors P4H, lysyl oxidase (LOX), and heat shock protein 47 (HSP47) was performed. P4H and HSP47 are associated with different stages of collagen synthesis, while LOX is involved in collagen and elastin cross-linking. Use of antibodies against matrix metalloproteinase-1 (MMP1), MMP2, MMP9, and MMP13 identified sites of enzymatic matrix degradation. An antibody against the tissue inhibitor of matrix metalloproteinase 1 (TIMP1) stain was also employed. An antibody sensitive to proliferating cell nuclear antigen (PCNA) determined the location of proliferating cells within the tissue to assess cell viability.

All sample slides were blinded and then graded for staining intensity on a scale of 0 (no stain) to 4 (heavy stain), as done in Section 4.2.3.4. In order to determine the effect of the mechanical stimulation throughout the tissue, intensity was assessed in all three valve leaflet layers: the elastin-rich atrialis, located at the atrial surface; the middle spongiosa layer, comprised of PGs and glycosaminoglycans (GAGs); and the collagenous fibrosa, located near the ventricular surface. Staining intensity was also measured in the chordae tendinae. Localization of the different ECM components and turnover mediators within the valve layers was performed via comparison of the immunohistochemically stained samples to the Movat samples.

The Movat samples were also used to determine sample thickness. Five sections along the radial sample were selected: ALC near valve annulus, middle ALC, strut chordae insertion (generally the midpoint of the valve, where the ALC
and ALF are separated), middle ALF, and ALF tip. Each section was measured at five different points to account for variability in thickness.

5.2.4. Statistical Analysis

Since each anterior valve leaflet has two strut chordae (Figure 5.9A), each valve yielded two sections for testing. For each valve obtained during the validation study, one section was treated as a baseline control, and the second section was cultured for two weeks in either the no mechanical stimulation or cyclic mechanical stimulation condition; therefore, each test sample had its own matched control. In each valve, staining intensity of each ECM component and turnover mediator of the cultured section was normalized to that of the baseline control section. A normalized staining intensity of 1.0±0.2 (100±20%) defined maintenance of the component over the treatment period. Normalized staining intensity of the dynamic organ culture was compared to that of the negative static control using a Student’s t-test, with statistical significance defined as p<0.05. Valve thickness was normalized to the baseline control in the same manner, and normalized static and dynamic sample thicknesses were compared using a Student’s t-test.

5.3. Results

After the bioreactor was designed and built, it was tested to verify that the mechanical stimulation would indeed maintain ECM structure and cell viability. Short-term sterility tests showed that the environment within the bioreactor
remained free from contamination during the test period (data not shown) both with and without tissue in the chamber.

In samples without mechanical stimulation, PCNA immunostaining indicated that fewer proliferating cells were located deep within the tissue, although proliferating cells were found near the tissue surface (Figure 5.10). H&E staining (not shown) displayed the same trend. After two weeks of static culture, the layered structure of the valve was also compromised. Although the Movat image showed maintenance of the atrialis, the fibrosa and spongiosa became less defined, with an increase in collagen content in the normally PG-rich free edge spongiosa. These results corroborated the earlier work performed in our lab.

The bioreactor appeared to counteract these static culture trends; after two weeks of culture, cell localization in the middle and periphery of the dynamically cultured samples were comparable to that in control tissue, as indicated by both H&E and PCNA staining. Positive PCNA immunostaining (Figure 5.11) also suggested that these cells remained viable during the culture period. Additionally, tissue samples cultured in the bioreactor displayed a similar ECM structure to that of the control samples; the collagenous fibrosa and PG-rich spongiosa layers were clearly delineated in the Movat images.
Figure 5.10: Comparison of a mitral valve free edge before (A, C) and after (B, D) two weeks of static organ culture (matched samples). When compared to the control (A), Movat staining showed an increase in collagen stain (in yellow, arrows) after two weeks of culture (B). PCNA stain in the control (C) identified proliferating cells (in brown) throughout the tissue. After two weeks (D), fewer cells were found deep within the tissue, although many cells were observed at the tissue periphery (arrow). V – Ventricular surface. Magnification = 5X.

The use of baseline controls for both the static and dynamic culture groups allowed the normalization of staining intensities of the ECM components and the turnover mediators for comparison of differences after two weeks of culture (Figure 5.12). A normalized value of 1 indicated that there was no change in the component after culture, while value <1 indicated reduced expression and value>1 indicated greater expression. The splashing bioreactor
was successful at maintaining most tested ECM components and turnover mediators; factors not maintained to within 20% (normalized staining intensity of 1.0±0.2) are presented in Table 5.1. The most compelling results are noted below.

Figure 5.11: Comparison of a mitral valve free edge before (A, C) and after (B, D) two weeks of dynamic organ culture (matched samples). When compared to the uncultured fresh control (A), Movat staining indicated maintenance of tissue structure after two weeks of culture (B). PCNA stain in the fresh control (C) and the cultured (D) tissues displayed proliferating cells (in brown) throughout the tissue. The thick arrows identify the spongiosa layer, while the thin arrows identify the fibrosa. V – Ventricular surface. Magnification = 5X.
In the atrialis (Figure 5.12), normalized COLL1 expression was maintained with dynamic culture and reduced in static culture (p=0.03). Normalized COLL3 expression tended to be greater with dynamic culture than static culture (p=0.06). PG expression remained constant compared to baseline with both static and dynamic culture, as did MMP1, MMP2, MMP9, and LOX content. TIMP1 expression was too varied between samples to draw a conclusion. MMP13 normalized expression was slightly greater in the dynamic samples, while HSP47 was reduced (Table 5.1).

Normalized spongiosa MMP9 (p=0.01) and TIMP1 (p=0.04) content (Figure 5.12) were both greater and closer to 1.0 with dynamic than static culture. Normalized MMP1 content, on the other hand, tended to be reduced in the dynamic culture samples compared to static culture samples (p=0.07). DCN and VC contents were comparable to the baseline control with both static and dynamic loading conditions, while BGL and COLL1/3 remained lower than the control (Table 5.1). MMP2, MMP9, and HSP47 expression was constant between the dynamic culture samples and baseline controls. Their resulting normalized stain intensity, which was approximately 1.0, was greater than and an improvement over that of the static samples. Normalized staining intensity was approximately 1.0 for MMP13, P4H, and LOX in both static and dynamic samples.

Normalized fibrosa MMP2 expression (Figure 5.12) was greatly elevated with dynamic culture compared to the baseline control (Table 5.1) as well as in comparison to the static culture (p<0.001, dynamic vs. static). In the fibrosa,
BGL and COLL1 expression remained constant between the baseline control, static culture, and dynamic culture samples. Normalized DCN expression in dynamic culture samples was slightly elevated compared to static culture, while VC content was lower (Table 5.1). Although not significantly different, normalized COLL3 expression (Table 5.1) in the fibrosa of dynamic culture samples was slightly lower than the elevated value found in static culture samples. P4H content was similar between the three sample groups, while expression of MMP1, MMP9, LOX, and HSP47 was increased toward baseline in dynamic samples. Dynamic MMP2 and MMP13 expression was elevated above baseline, while TIMP1 was reduced.

Normalized expression of LOX (Table 5.1) and BGL in the chordae (Figure 5.12) was greater in dynamic culture compared to both the baseline control and the static culture condition (p=0.02, LOX and p=0.02, BGL); however, COLL1 content was upregulated in the dynamic culture samples compared to static culture samples (p<0.01). COLL3 expression was similar to baseline in the dynamic culture samples, as was VC and DCN content. Content of HSP47, MMP1, MMP2, MMP9, MMP13, and TIMP1 in the dynamic culture and baseline control samples was approximately equal. Normalized P4H (Table 5.1) expression in the dynamic samples was greater than in static samples.

Although it was desired to show how the mechanical stimulation provided by the bioreactor improved cell viability and ECM structure compared to static culture conditions, it was also important to show that the bioreactor design would be a successful culture environment. Since a normalized value of 1 for stain
intensity indicated that there was no change in the expression of the specific component from baseline, the normalized expression for all components was tracked. The components that were not maintained with dynamic culture are listed in Table 1. Except for atrialis HSP47, which showed a slight improvement with dynamic over static culture, these components showed similar component expression with both static and dynamic culture. In general, however, the bioreactor was effective at maintaining ECM structure of the valve.

Figure 5.12: Staining intensity, normalized to paired baseline control, of selected ECM components and turnover mediators in the (A) atrialis, (B) spongiosa, (C) fibrosa, and (D) chordae. Data shown as mean ± standard deviation. For all data sets shown, the difference between the static and dynamic groups was significant (p<0.05).
Table 5.1: ECM components and turnover indicators not maintained by bioreactor

<table>
<thead>
<tr>
<th>Tissue Layer</th>
<th>Components (normalized stain intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrialis</td>
<td>HSP47 (0.77±0.26), MMP13 (1.6±0.57)</td>
</tr>
<tr>
<td>Spongiosa</td>
<td>BGL (0.79±0.28), COLL1 (0.71±0.28), COLL3 (0.53±0.27), MMP1 (0.54±0.57)</td>
</tr>
<tr>
<td>Fibrosa</td>
<td>VC (0.70±0.25), COLL3 (1.32±0.41), MMP2 (2.6±0.89)</td>
</tr>
<tr>
<td>Chordae</td>
<td>P4H (1.25±0.42), LOX (1.29±0.46)</td>
</tr>
</tbody>
</table>

Data represented as mean ± standard deviation.
BGL=biglycan, COLL=collagen, HSP47=heat shock protein 47, LOX=lysyl oxidase, MMP=matrix metalloproteinase, P4H=prolyl-4-hydroxylase, VC=versican.

Normalized valve thickness was greater near the annulus for static than dynamic organ culture samples (1.66±0.71mm vs. 0.93±0.33 mm, static vs. dynamic, p=0.01). At the valve tip, valve thicknesses for both culture groups were greater than for the baseline control (2.08±1.77 mm, normalized static and 1.65±0.94 mm, normalized dynamic); however, the difference between the two normalized values was not statistically significant. The normalized valve thicknesses at the middle ALC, chordal insertion, and middle ALF were approximately 1.0, and the static and dynamic normalized values were not significantly different from each other.

5.4. Discussion

Previous work in our lab has revealed that valve tissue cultured without mechanical stimulation did not maintain tissue structure. Proliferating cells deep within the tissue were depleted, and the layered structure definitive of the normal valve became less defined with increased culture time. The splashing bioreactor designed in this aim provided perfusion and gentle tension to the tissue. This
cyclic fluid motion was verified as sufficient to maintain valve structure for the two-week period of interest by assessing stain intensity of PGs, collagen, and ECM turnover mediators before and after culture. Fluid movement against tissue surfaces initiated by rotation of the bioreactor chamber provided the mechanical stimulation needed to maintain these aspects of tissue integrity. This novel bioreactor, though simple in design and construction, could become a powerful tool for studying initial valvulopathy mechanisms.

A comparison of Movat stains of the mitral valve before and after static culture indicated fibrotic remodeling similar to that observed in previous organ cultures of the tricuspid valve (Figure 5.2). Additionally, fewer proliferating cells were present in the deep layers of the tissue after two weeks of static culture compared to fresh tissue, although cells were noted at the tissue periphery. This result corroborates the previous work of Allison et al.\textsuperscript{152}, and may be attributed either to cell death or to a migration of cells for the purpose of reaching nutrients. After two weeks of mechanical stimulation provided by the splashing bioreactor, the layered structure of the valve remained intact. Proliferating cells were found throughout both the fresh and cultured tissue, indicating that the bioreactor was also successful in maintaining cell viability within the valve layers. These results show that the light stretching and tissue perfusion provided by the splashing bioreactor were sufficient to maintain the valve layered structure and cell viability that were lost with static organ culture methods.

Taken together, the role of MMP13 in collagen degradation and that of HSP47 in collagen production, in addition to the increase in normalized COLL1
and COLL3 expression, imply that the mechanical stimulation upregulated collagen production in the atrialis, and that the tissue was attempting to maintain collagen content via the appropriate turnover mediators. The greater normalized COLL3 expression lent further weight to the remodeling theory. It is interesting to note that although MMP13 is responsible for cleaving COLL1\textsuperscript{21,154}, there was no significant decrease in COLL1 staining intensity corresponding to an increase in MMP13 staining intensity in the fibrosa; as opposed to the behavior noted in the atrialis, this result suggests that collagen remodeling in the fibrosa of dynamic culture samples was limited after two weeks. Since MMP13 is associated with collagen turnover\textsuperscript{21}, this may have been an attempt to limit the excess collagen deposition noted in the earlier study. TIMP1 is an inhibitor of most MMPs\textsuperscript{21,154}; its decrease in the fibrosa was likely linked to the increase in MMP expression.

Expression of COLL1, BGL, LOX, and P4H indicated additional collagen remodeling within the chordae. In the chordae, BGL, LOX, and COLL1 normalized staining intensity in the dynamic samples was significantly greater than in the static samples. P4H normalized stain intensity was also slightly greater in dynamic than static samples. Since BGL, P4H, and LOX are associated with collagen formation, these results indicated that turnover of the matrix was occurring. Overall collagen content did not change; COLL1 normalized staining intensity was approximately 1.0, indicating that dynamic culture and baseline COLL1 expression were almost equal. This remodeling process therefore acted to maintain composition in this highly collagenous tissue.
Although the mechanical stimulation appeared to alter PG and collagen expression in the atrialis, fibrosa, and chordae, the contents of these ECM components remained unchanged in the spongiosa. Lack of improvement in BGL and collagen content in the spongiosa was likely due to the lack of coaptation in the leaflet free edge; while the bioreactor supplied mechanical stimulation from fluid flow, the tissue did not make contact with the opposing posterior leaflet as occurs in the heart. Elevated TIMP1 expression indicated the possibility that remodeling of the tissue might transpire with increased culture time.

Although the mitral valve tissue in the validation study demonstrated less COLL1 staining than baseline after two weeks of static culture, the tissue from previous studies in our lab showed more total collagen staining with increased culture time, as evidenced by the Movat safran stain (Figure 5.2). One possible explanation is that tricuspid valve tissue was used in the previous study. The two valves may have had different responses to the culture environment. The longer culture time in the earlier study may also have led to the greater collagen accumulation. As mentioned above, the use of the Movat stain also limited the results of the earlier study to a combination of all collagen types present in the valve. The validation study in the current research project measured both COLL1 and COLL3 in the tissue and suggested that an increase in collagen may have been due to COLL3 upregulation. COLL3 has been shown previously to be greater than normal in patients with myxomatous\textsuperscript{2} and rheumatic\textsuperscript{155} heart valves and has been presumed to indicate valve remodeling due to pathology; the
increase in COLL3 noted in the current study may have signaled a repair process of the tissue.

In general, valve thickness was maintained in both static and dynamic cultured samples when compared to the baseline control. At the annulus, the static samples did exhibit some thickening that was not noted in the dynamic organ cultures. Both treatments exhibited thickening at the tissue tip; however the variability between the measurements made this result less reliable. The main limitation in this method of measuring valve thickness was the variability of the valve thickness, both along the radial section and in the circumferential. A radial valve section taken at another point along the valve might show dramatically different results.

The novelty of this bioreactor design validation method, compared to those used to validate previous heart valve tissue engineering bioreactor designs, was in the longer time course of the study as well as the tissue analysis methods. Previous heart valve tissue engineering bioreactor studies usually focused on deposition of a collagen matrix or its precursors after 48 hours. Evidence of collagen formation in these earlier studies was a sign of improved strength of the engineered leaflet. Conversely, the current study used a longer time course to model an existing valvulopathy; this longer study duration has not been used as frequently in the heart valve literature. The heart valve tissue engineering bioreactor studies often used biochemical methods to assess ECM deposition. The research in Aim 2 is one of few bioreactor studies that examined changes to the layered valve structure. While the results from this
current study cannot be directly compared to those of the previous bioreactor studies, when the results are combined, a complete picture of valvular remodeling can be drawn.

Limitations of this project included the subjectivity of the semi-quantitative immunohistochemical analysis. An attempt was made to remove all bias through blinding of the samples. Repeatability was assessed by a second analysis of the tissues, and variability using this method has been previously documented as <15%\textsuperscript{133}. It was also possible that cell migration noted in the static culture studies was due to a wound response as opposed to nutrient access. This possible issue was mitigated by taking samples for staining deep within the tissue when mounting slides, away from the original cut. Time limitations prevented the use of multiple study durations in this aim. A longer study duration could follow the consequences of ECM turnover mediators that were not preserved, such as fibrosa MMP2. It is possible that the tissue would compensate for the abnormal expression, or that the ECM itself may remodel due to the effects of these mediators.

Organ culture can be a powerful tool to study physiological systems in \textit{vitro} that would otherwise be nearly impossible to observe. In the current study, maintenance of both ECM structure and cell concentration within the different layers of the tissue indicated successful organ culture. While valve tissue cultured without mechanical stimulation displayed both cellular loss deep within the tissue and an increase in collagen content, cell localization and ECM
structure of tissue cultured for two weeks in the bioreactor remained similar to that of fresh tissue.

5.5. Conclusion

Previous work in our lab has shown that valve tissue cultured without mechanical stimulation lost the normal layered structure; tissue became more collagenous with a reduction in PG content. Additionally, cell density deep within the tissue decreased. A splashing bioreactor was therefore designed with the intent of lightly stretching and perfusing the mitral valve tissue to maintain tissue structure. In general, mitral valve sections cultured in the bioreactor, when compared with tissue cultured in tissue culture flasks, maintained cell density within the layers as well as ECM structure. With this successful design, the splashing bioreactor was able to be used to study the early mechanisms of valvular heart disease in Specific Aim 3.
Chapter 6. Mechanisms of Serotonergic Valve Disease

Specific Aim 3: Determine receptor effects and early mitral valve ECM composition changes due to the introduction of two serotonin receptor agonists, serotonin and norfenfluramine.

6.1. Background

Carcinoid valve disease, caused by metastatic tumors within the gastrointestinal system and pancreas, is characterized by a superficial growth or "plaque" on the surface of the right heart valves, with the underlying leaflet structure remaining intact. The development of these plaques, which display cellular proliferation in addition to increased collagen, proteoglycan and elastin production, is associated with serotonin released by the carcinoid tumors. Fenfluramine-phentermine (fen-phen) valvulopathy, the valve disease named for the anorectic drug cocktail removed from the market in 1997, is defined by the presence of plaques on the left heart valves similar in structure to those observed in carcinoid valve syndrome. The Parkinson's disease drug pergolide was recently removed from the market in the United States due to a similar valve pathology, and the club drug "Ecstasy" has also been implicated in serotonergic valve disease.

The final disease state of serotonergic valve diseases such as carcinoid valve syndrome and fen-phen valvulopathy is clear; histological analysis of the
valve after surgical resection reveals the presence of a superficial plaque
impeding proper valve function. Definition of the early disease mechanism,
however, would be useful in the identification of similar drugs that may stimulate
or, conversely, prevent plaque formation. Clarification of the disease genesis
may also increase early treatment options, avoiding the need for valve surgery.

At present, very little is known about the early mechanism for serotonergic
valve disease. Studies on ovine aortic valve interstitial cells have indicated that
the activity of the 5HT$_{2A}$ receptor and consequent G-protein signal transduction
led to transforming growth factor (TGF)-β1 upregulation and possibly to the
valvulopathy$^{63}$. Further studies by this group have also suggested that an
additional mechanism, possibly via another member of the 5HT$_{2B}$ receptor family,
may have activated the extracellular signal-regulated kinase 1/2 (ERK 1/2)
pathway and encouraged cell proliferation$^{65}$. More recent work using human
embryonic kidney cells has highlighted 5HT$_{2B}$ as the likely receptor activated in
serotonergic valve disease$^{68}$. A schema published by Roth in 2007$^{69}$ has
suggested that 5HT$_{2B}$ activation leads to dissociation of the G-protein, whose
Gq subunit activates the ERK 1/2 pathway leading to cell proliferation, which
would then increase the overall amount of matrix produced by the cells. It is also
possible that the Gβy subunit causes Src phosphorylation to create Src-P, which
separately mediates TGF-β receptor activity to further cell proliferation. Recent
studies have further indicated a role of TGF-β in the upregulation of sulfated
GAGs$^{63,160,161}$. Further details regarding the early disease mechanism are under
investigation. It is also unknown why these drugs appear to have a preference
for the right or left heart valves, or how the plaques form on the valve surface. The literature does not agree on a link of serotonergic valve disease with length or amount of drug exposure, or whether cessation of drug use reverses the effects.\textsuperscript{162}

The goal of the third aim was to introduce an \textit{in vitro} method of studying serotonergic valve disease. To that end, mitral valve tissue in the splashing bioreactor from Aim 2 was exposed to the serotonin receptor agonists serotonin (5-hydroxytryptamine, 5HT) and norfenfluramine (NF, a metabolite of fenfluramine). These drugs were studied alone and in combination with a 5HT\textsubscript{2B} receptor antagonist to determine the role of the specific receptor in valve disease. It was believed that samples treated with the serotonergic drugs would have upregulated production of extracellular matrix within the tissue, especially glycosaminoglycan (GAG) content, while samples exposed to the receptor antagonist would show little remodeling. Additionally, it was hypothesized that the mitral valve used in the experiment would show greater NF-induced remodeling compared to 5HT, suggesting that an inherent difference between the right and left heart valves could be a reason for the differences in drug activity noted clinically.

\section*{6.2. Methods}

Porcine mitral valve segments were exposed to the serotonin receptor agonists 5HT, NF, and pergolide in order to simulate the early mechanisms of serotonergic valve disease. Combinations of the receptor agonists and a 5HT\textsubscript{2B}
receptor antagonist were used to confirm the role of this receptor in valve remodeling. The first phase of the project involved optimizing the drug concentration. The bioreactor created during Aim 2 was then used to provide consistent mechanical stimulation during a two-week culture period.

6.2.1. Drug Dosage Optimization

Since the available research pertained to cell culture studies, the drug concentration to be used in Aim 3 needed to be optimized for organ culture. A review of the literature indicated that 5HT$^{67, 163-166}$ and pergolide$^{68, 167}$ concentrations used in cell culture ranged from 0.01 µM to 10 µM, while NF$^{163, 164, 166}$ concentrations used in cell culture ranged from 0.1 µM to 100 µM.

To allow comparison of multiple valves treated with multiple concentrations of each drug, the optimization was performed under static culture conditions. The setup of the optimization experiment used 6-well plates instead of tissue culture flasks (as with the static culture condition of Aim 2); within each plate, sections from three different valves were exposed to two different drug concentrations. For each optimization experiment, two plates were used for each drug, allowing three valves to each be exposed to four different drug concentrations.

Mitrval valve tissue was excised from porcine hearts (Fisher Ham and Meat, Spring, TX) within 10 hours of sacrifice. The anterior leaflet was sectioned into four (5 mm) radial strips. After dissection, all strips were partially submerged in 1.5 mL cell culture media containing 5% antibiotic/antimycotic solution. After
24 hours incubation (Figure 6.1) at 37°C, the media was replaced by plain or serotonin receptor agonist culture media containing 1% antibiotic/antimycotic solution.

![Timeline of cell studies](image)

**Figure 6.1: Timeline of cell studies.** Round 1 (A) determined cell type and Rounds 2/3 (B) assessed cell proliferation.

The first optimization study (Round 1) compared four (0.01 μM, 0.1 μM, 1.0 μM, 10 μM) concentrations of 5HT hydrochloride (Sigma Aldrich, St. Louis, MO; n=3) and four (0.1 μM, 1.0 μM, 10 μM, 100 μM) concentrations of NF hydrochloride (Sigma Aldrich, n=3). Tissue samples were partially submerged in 1.5 mL of media, which was changed every 2-3 days. Since 5HT oxidizes in solution media, the solution was made fresh prior to each media change. After 11 days of culture, adherent cells were observed on the bottom of the plate;
culture and immunocytochemical analysis methods to identify these cells are described in Section 6.2.2 and Appendix 2.

The next round of drug optimization (Round 2) used the same concentrations of NF (n=3) as before and the same concentrations of pergolide methanesulfonate salt (Sigma Aldrich, n=3) as for 5HT. Unlike the previous round, the valve segments were completely submerged in 3.0 mL of media. Control samples, submerged within 1.5 mL (n=6) and 3.0 mL (n=6) of plain media for comparison against both partially and completely submerged tissues, were also cultured. Pergolide is insoluble in water but soluble in dimethyl sulfoxide (DMSO) (5.2 mg/mL) and ethanol (2.0 mg/mL); therefore, the solid was alternately dissolved in media solutions containing 0.08% DMSO and 0.42% ethanol\(^{167}\). The amount of DMSO/ethanol in the media was optimized to the minimum volume of liquid required to dissolve the desired mass of pergolide powder. Since pergolide settled immediately from the DMSO solution, the experiment was continued using pergolide in ethanol solution, and the pergolide control used tissues cultured within 1.5 mL (n=6) and 3.0 mL (n=6) media containing 0.42% ethanol. The third and final round of drug optimization (Round 3) cultured valve segments submerged in 3.0 mL 5HT (n=6), NF (n=3), and pergolide (n=3).

Optimization of the 5HT\(_{2B/2C}\) receptor antagonist (SB-206553 hydrochloride hydrate, Sigma Aldrich; later SB-206553 hydrochloride, Tocris Biosciences, Ellisville, MO) was performed in the same manner as for the drugs (n=3). Since 5HT\(_{2C}\) receptors are not expressed in heart valve cells\(^{168}\), SB-
206553 was considered a 5HT
 was considered a 5HT
2B receptor antagonist for the purposes of this study. A literature review showed that a concentration of 1 \( \mu M \) was frequently used in cell studies\textsuperscript{65, 68, 169}, and further optimization was performed for this study to account for possible differences in the chemical effect on cell and organ culture. Concentrations of the antagonist tested were 10 \( \mu M \), 1 \( \mu M \), and 0 \( \mu M \) (control); all valve sections were cultured in 10 \( \mu M \) NF media.

After 14 days of organ culture, all tissue samples were fixed in Histochoice\textsuperscript{TM}. After fixation, all tissue samples were dehydrated to xylene, paraffin embedded, sectioned into 5 \( \mu m \) slices, and mounted on slides as described in Section 4.2.3.1. Samples were stained with H&E and via immunohistochemistry (Appendix 2) for proliferating cell nuclear antigen (PCNA) to assess cell localization within the tissue layers. Tissue samples were also stained with Movat pentachrome to identify layers and review global changes in extracellular matrix (ECM) content. All samples were then viewed to visually determine the drug concentration causing the most change to the tissue structure.

6.2.2. Cell Studies

When adherent cells were observed on the tissue culture plates, it was desired to determine the cell type. On day 11 (Figure 6.1), organ cultures were removed from the 6-well plates. Before counting on day 19, the three wells containing cells from the three valve segments exposed to the same drug concentration were combined. Aliquots containing 5 \( X \) 10\(^5\) cells (from 1 \( \mu M \) NF,
0.1 μM NF, 0.1 μM 5HT, and 0.01 μM 5HT organ cultures) were seeded to four slides coated with fibronectin. After 2-3 days culture in plain media, cells were stained (Appendix 2) with acetylated low density lipoprotein (Dil-AC-LDL, one sample each NF and 5HT) to identify valvular endothelial cells (VECs) and an antibody against smooth muscle α-actin (SMαA, one sample each NF and 5HT) to identify valvular interstitial cells (VICs).

The remaining two cell studies were performed to assess differences in cell proliferation due to drug exposure. Cells from Rounds 2 and 3 were confluent on day 11 (Figure 6.1), and cells from all valve samples were counted and re-seeded to T-25 culture flasks at 8000 cells/mm² (the highest concentration possible for the flask with the smallest number of cells). Culture continued with the appropriate concentration of drugged media. On day 16 (Figure 6.1), cells were counted again to assess proliferation. To compare concentration, the proliferation cell counts were compared across groups using a one-way ANOVA with p<0.05 considered significant. Holm-Sidak post-hoc testing was used as appropriate to identify significant differences between individual concentrations and the control.

6.2.3. Dynamic Valve Culture

Based on observation of the Movat- and PCNA-stained samples, the results of the optimization experiments described in section 6.2.1 showed that a concentration of 10 μM for NF and 5HT elicited the most dramatic tissue remodeling (Figure 6.2). After fibrotic remodeling due to use of the static culture
method (explained in greater detail in Chapter 5) was taken into account, Movat stains of the 10 μM NF and 5HT organ cultures appeared to have deposits of proteoglycans (PGs)/GAGs on the valve surface and within the normally collagenous fibrosa. Localized clusters of proliferating cells were evident in samples immunostained with an antibody against PCNA. The receptor antagonist concentration of 10 μM also appeared to be the most effective at blocking the drug effects (data not shown). At this time, it was noted that the bovine growth serum used contains baseline levels of 5HT (>10μM), which might have affected the results of the drug-free media “control” case. The additional difficulties in keeping pergolide in solution, as well as a desire to explore more thoroughly the role of the 5HT_{2B} receptor, led to a replacement of the pergolide test condition with an alternative test condition, that of the 5HT_{2B} receptor antagonist only.

Organ culture in the splashing bioreactor was performed in the same manner as in Aim 2 (see section 5.2.3). The following four test cases were studied: NF only (n=7), NF + receptor antagonist (NF+BLK, n=8), 5HT only (n=6), and 5HT + receptor antagonist (5HT+BLK, n=6). In the NF+BLK and 5HT+BLK test cases, media containing only the antagonist (no drug) was added to the organ culture 30 minutes before adding the drug+BLK media at every media change^{169} to encourage receptor saturation with the antagonist before introduction of the receptor agonist.
Figure 6.2: Movat (A) and PCNA (B) stains comparing effects of varying concentrations of norfenfluramine on the mitral valve anterior leaflet center. The sample exposed to 10 μM norfenfluramine had greater PG/GAG and cell content in the tissue interior compared to control, which tended to be mostly collagenous with fewer cells. In all images, the atrialis is at the top right and the ventricularis is at the bottom left. Magnification = 5X.
Two control groups were prepared. The dynamic stimulation samples from Aim 2 (n=9) were used as the first control in this study. A small number of tissue samples (n=4) were cultured with the receptor antagonist only (BLK) in order to determine any effects of the 5HT inherent to the culture media.

After two weeks of culture, samples were fixed, stained for ECM components and turnover mediators, and graded as described in section 4.2.3. Immunohistochemical staining for MMP1 and MMP2 was replaced by staining for elastin in these samples, as the Movat stain indicated a possible upregulation of elastin in the NF-treated tissue (Appendix 2). In some instances, a foreign growth-like object was noted on the valve surface. In these cases, tissue samples were also stained for the presence of von Willebrand's Factor (vWF), SMoA, and fibrinogen (FB) to determine the possibility of an inflammatory response. A Masson's trichrome stain, which identifies nuclei, cytoplasm, erythrocytes, myelin and collagen, (Appendix 2) was also performed on these samples.

Prior to performing the organ culture studies, a power analysis was performed; the results of this analysis indicated that a subject size of 6 would be sufficient to attain statistical significance. Mean staining intensity was compared between each drug treatment and the associated agonist + antagonist group using the Student's t-test. A Bonferroni adjustment was made to account for the use of the three drug treatments in the study; therefore, significance was defined at p=0.017. Since the drug treatments were applied independently, however, differences with p<0.05 were considered strong trends and p<0.08 were
considered slight trends. The mean staining intensities of the three different drug groups and the three different agonist + antagonist groups were compared using a one-way ANOVA with Dunn’s or Holm-Sidak post-hoc testing performed as appropriate; the results of post-hoc analysis are shown below when significant. These comparisons were designed to assess the possibility that 5HT/NF exposure affected a secondary receptor to the 5HT$_{2B}$ receptor.

6.3. Results

6.3.1. Explant Culture Cell Proliferation

Cell number varied in a dose-dependent manner for 5HT, NF, and pergolide (Figure 6.3). Cell proliferation was lower than control for all 5HT drug concentrations except 1 μM. Cells exposed to 0.1 μM and 0.01 μM pergolide also had lower cell proliferation than control. For NF, maximum cell proliferation occurred at the 0.1 μM ($10^{-7}$M) concentration; proliferation appeared to decrease as NF concentration increased (Figure 6.4). In the cell proliferation studies (section 6.2.2), six valve sections were cultured in plain media and in media containing each tested concentration of the three drugs: serotonin, norfenfluramine, and pergolide. Prior to cell counting, cells cultured from three of the valve sections were combined. The first of these proliferation studies were pilot experiments, and it was decided to only compare one set of three valves exposed to each drug and to plain media, where the seeding densities and animal age were the most consistent between samples. A larger sample size would be needed to corroborate the trends shown here.
Cell type was identified via ICC stain with SMαA (VICs) and Dil-Ac-LDL (VECs). Cells stained strongly for SMαA (Figure 6.5), indicating that the cells were mostly VICs. Cell staining for Dil-AC-LDL was not distinguishable from the background (data not shown).

**Figure 6.3**: Effects of varying concentrations of serotonergic drugs on cell number. Data represented as mean. *: concentration not tested.
6.3.2. Organ Culture

The Movat stain of valves cultured with NF showed a strong upregulation of elastin in the leaflet free edge after two weeks of dynamic culture (Figure 6.6C). Staining with the PCNA antibody indicated a greater number of proliferating cells than the tissue cultured with plain media (Figure 6.6A) or with
the NF+BLK media. No other test group displayed a prominent difference in ECM composition or cell proliferation compared to the baseline control.

Figure 6.6: Comparison of anterior leaflet free edge of mitral valve leaflet exposed to (A) plain media, (B) media with the 5HT2B receptor antagonist, (C) norfenfluramine, (D) norfenfluramine + antagonist, (E) serotonin, and (F) serotonin + antagonist. Of all the test conditions, norfenfluramine exposure appeared to induce the most pronounced changes to the tissue structure, as noted by greater elastin upregulation (black stain) and cell proliferation (black arrows).
Of all tested ECM components and turnover mediators, PG expression (Figure 6.7) was most pronounced in the test groups. Biglycan (BGL) appeared to be elevated in the control and NF groups, while decorin (DCN) and versican (VC) appeared to be greatest in the two 5HT groups. BGL expression was greater in the control group compared to BLK for the fibrosa (p=0.001) and tended to be greater in the atrialis (p=0.04) and chordae (p=0.027). Expression in NF samples compared to the corresponding antagonist group was stronger in the atrialis (p=0.009), spongiosa (p=0.01), and chordae (p=0.002) with a strong trend noted in the fibrosa (p=0.03). A trend was also noted in BGL expression in the control (p=0.06) and 5HT (p=0.055) treatment groups in comparison with their respective antagonist groups. Within the drug only samples, 5HT BGL stain intensity in the fibrosa was lower than control (p<0.05). Chordae BGL stain intensity tended to be lower in 5HT samples than both control and NF (p=0.01 between groups).

5HT DCN expression in the atrialis tended to be greater than control in both the agonist (p=0.055 between groups) and the agonist + antagonist (p=0.055 between groups) treatment groups. In the spongiosa, DCN staining in the control samples tended to be greater than in the BLK group (p=0.02). VC expression in the atrialis (p=0.047 between groups, agonist treatments) and spongiosa (p=0.065 between groups, agonist and antagonist + antagonist treatments) tended to be greater in 5HT than NF. In the spongiosa, control VC staining also tended to be greater than control +BLK staining (p=0.04). Stain
intensity of DCN and VC in both fibrosa and chordae was not significantly different between groups.

Figure 6.7: Proteoglycan expression in tissue samples exposed to combinations of serotonin receptor agonists and antagonists. Staining intensity was assessed separately in the (A) atrialis, (B) spongiosa, (C) fibrosa, and (D) chordae. Values represented as mean stain intensity ± standard deviation. Bars sharing the same letter are significantly different. The letter indicates level of significance. a-d: p<0.017 (t-test), e-i: p<0.05 (t-test), j: p<0.05 (ANOVA post-hoc), k-l: p<0.05 (ANOVA), m-n: p=0.06 (t-test), o-r: p≤0.065 (ANOVA). SHT=serotonin; BGL=biglycan; BLK=serotonin receptor antagonist; DCN=decorin; NF=norfenfluramine; VC=versican.

Staining intensities of collagen and elastin (Figure 6.8) were comparable in magnitude to those of PGs. Elevations in component expression, if present, were most apparent in the NF treatment groups. In the spongiosa (p=0.04) and chordae (p=0.056), collagen type III (COLL3) stain intensity in control tended to be greater than in BLK. When looking spongiosa COLL3 staining in all drug
treatment groups, there was a significant difference (p=0.007 between groups), with a further difference noted between control and NF (p<0.05). In the fibrosa, BLK exposure tended to reduce collagen type I (COLL1) expression in the control (p=0.02) and NF (p=0.08) cases. Differences in atrialis elastin expression showed a trend of a decrease in NF+BLK compared to NF (p=0.04). Expression of COLL3 in the atrialis and fibrosa, COLL1 in the atrialis, spongiosa, and chordae and elastin in the spongiosa, fibrosa, and chordae was not different between groups.

Figure 6.8: Collagen and elastin expression in tissue samples exposed to combinations of serotonin receptor agonists and antagonists. Staining intensity was assessed separately in the (A) atrialis, (B) spongiosa, (C) fibrosa, and (D) chordae. Values represented as mean stain intensity ± standard deviation. Bars sharing the same letter are significantly different. The letter indicates level of significance. a-c: p<0.05 (t-test), d: p<0.05 (ANOVA post-hoc), e: p<0.05 (ANOVA), f-g: p≤0.08 (t-test). 5HT=serotonin; BLK=serotonin receptor antagonist; COLL=collagen; NF=norfenfluramine.
Staining intensity of the collagen and elastin synthesis mediators (Figure 6.9) was weaker than for the ECM components. Interestingly, the intensities in the two 5HT groups were often lower than control. Atrialis heat shock protein 47 (HSP47) staining intensity was greater in control than 5HT samples (p<0.05) and BLK samples (p<0.001). In the spongiosa, HSP47 expression was significantly different between all drug treatment groups (p=0.012), and post-hoc testing showed that stain intensity in 5HT was lower than control samples. Additionally, expression was greater in control than BLK (p=0.008). HSP47 staining in the fibrosa was greater in the control group than in BLK (p=0.009). Expression with drug treatment was also different between groups (p=0.003), with significant difference between the control and 5HT groups (p<0.05). Chordae HSP47 expression tended to be greater in control than BLK samples (p=0.07). Prolyl-4-hydroxylase (P4H) expression in the fibrosa was greater in control than BLK samples (p=0.016). In the atrialis, P4H expression in NF tended to be greater than in NF+BLK (p=0.047), and expression in 5HT tended to be greater than in 5HT+BLK in the chordae (p=0.025). LOX expression throughout the valve in addition to P4H expression in the spongiosa was not significantly different between groups.
Expression of the matrix metalloproteinases (MMPs) and the inhibitor (TIMP1) was weaker than the other ECM components and turnover mediators (Figure 6.10). Although not always significant, the combinations of serotonin receptor agonists and antagonist led to reduced expression when compared to control but greater expression compared to the BLK group. While MMP9 expression in the atrialis tended to be greater in control than BLK samples (p=0.06), it also tended to be lower in 5HT than 5HT+BLK (p=0.03). Stain intensity between the three drug treatment groups was significantly different.
(p=0.009), especially between control and 5HT (p<0.05). MMP9 expression in the fibrosa tended to be greater in the control than the BLK (p=0.055) but lower in the NF than NF+BLK (p=0.028) samples. ANOVA testing also showed an apparent difference between MMP9 expression in the three drug groups (p=0.029), but no further differences were noted with post-hoc testing. In the chordae, MMP9 staining intensity tended to be lower in NF than in NF+BLK (p=0.02). Atrialis MMP13 staining intensity was greater in control than in 5HT (p<0.05), NF (p<0.05), or BLK (p<0.001) samples. Likewise, MMP13 expression in the fibrosa and chordae was greater in control than in BLK (p<0.001, fibrosa and chordae), 5HT (p<0.05, fibrosa chordae), or NF (p<0.05, fibrosa and chordae). MMP13 expression was greater in control than BLK (p=0.007). TIMP1 expression in control tended to be greater than BLK in the atrialis (p=0.02) and spongiosa (p=0.03). TIMP1 stain intensity also tended to be greater in NF than NF+BLK (p=0.055). Stain intensity of spongiosa MMP9, fibrosa TIMP1, and chordae TIMP1 was not significantly different between treatment groups.
Figure 6.10: Expression of extracellular matrix turnover mediator in tissue samples exposed to combinations of serotonin receptor agonists and antagonists. Staining intensity was assessed separately in the (A) atrialis, (B) spongiosa, (C) fibrosa, and (D) chordae. Values represented as mean stain intensity ± standard deviation. Bars sharing the same letter are significantly different. The letter indicates level of significance. a-d: p<0.017 (t-test), e-i: p<0.05 (t-test), j-p: p<0.05 (ANOVA post-hoc), q: p<0.05 (ANOVA), r-t: p<0.06 (t-test). 5HT=serotonin; BLK=serotonin receptor antagonist; MMP=matrix metalloproteinase; NF=norfenfluramine; TIMP=tissue inhibitor of matrix metalloproteinase.

Foreign growth-like objects (plaques) were noted in two of the organ cultures treated with NF and one treated with NF and receptor antagonist (Figure 6.11). Throughout the culture period, pieces of the plaque would appear in the culture media, and a larger growth would be found affixed to the ventricular surface of the valve at the end of the culture period. A Movat stain of the plaque revealed elastin and PGs/GAGs (black and blue stain), while a Masson’s stain showed the presence of collagen and possible smooth muscle (green and red stain). The objects stained strongly for BGL (NF only), DCN, LOX, COLL1,
COLL3, FB, vWF, and HSP47 (NF only), indicating that this was more likely a tissue than a fungal growth (Figure 6.12). Whenever a plaque was noted, an additional organ culture run was performed; however, presence of the foreign object did not appear to influence staining intensity for any ECM component except elastin (elastin data analysis therefore excluded these samples). No such objects were noted in the control, BLK, 5HT, or 5HT+BLK treatment conditions.

![Image](image.jpg)

**Figure 6.11**: Plaque removed from bioreactor. Organ culture was exposed to norfenfluramine, and object was stuck onto valve surface. The appearance of the foreign object was similar to a thin layer of mitral valve tissue.
Figure 6.12: Characterization of plaque from bioreactor. Portions of the foreign object were obtained from a norfenfluramine-treated valve organ culture and stained with (A) Movat pentachrome (black=elastin, blue=proteoglycans/glycosaminoglycans, red=fibrin/smooth muscle), (B) negative control (anti-goat secondary, no primary antibody), (C) fibrinogen, (D) negative control (anti-rabbit secondary, no primary antibody), (E) lysyl oxidase, (F) von Willebrand Factor, (G) decorin, (H) type I collagen, and (I) type III collagen.

6.4. Discussion

Explant cultures and organ cultures were exposed to 5HT and NF for two weeks to determine the effect of these serotonin receptor agonists on cell proliferation and tissue structure. Both drugs appear to have had an effect on the mitral valve, but the two different drugs had very different impacts on the tissue. Tissues exposed to NF experienced an upregulation of BGL and collagen compared to control. On the other hand, tissues exposed to 5HT had greater DCN and VC expression and lower collagen and elastin content than in control tissue. The ECM components studied were regulated differently by the interaction between these agents and the 5HT₂B receptor, indicating the
possibility that another mechanism was involved in the valve microstructural remodeling.

In the cell studies, NF tended to increase proliferation. As drug concentration increased, however, cell proliferation decreased. Cells exposed to 5HT exposure showed a similar trend of lower cell number with greater drug concentration. These results tended to corroborate those of Hafizi et al.165, who proposed the possibility of receptor desensitization at higher drug concentrations. Since the cell studies in the current research were preliminary in nature, more samples should be included before reaching a final conclusion of the impact of drug concentration on cell proliferation. When determining the effects of these drugs on cells within the tissue structure, PCNA immunostaining showed that tissue cultured with NF contained a greater number of proliferating cells than either the control or the other test conditions. This greater number of proliferating cells increased the likelihood of ECM turnover within the valve.

Results of the Movat stain suggested that NF upregulated elastin production in the anterior leaflet free edge. It is interesting to note, however, that the atrialis and spongiosa of all treatment groups strongly expressed elastin with immunostaining methods. The antibody used is known to recognize tropoelastin and α-elastin, in addition to insoluble elastin; it is possible that a baseline quantity of tropoelastin exists in the normal spongiosa, and that NF exposure induced formation of the cross-linked elastin fiber. This explanation is supported by the presence of elastin within the plaques observed in serotonergic valve disease28.
BGL was also upregulated with NF exposure when compared to the BLK sample; the reduced expression in the NF+BLK sample showed that this was mediated by the 5HT$_{2B}$ receptor. This result, when compared to a recent study that suggested a role of TGF-β in BGL expression$^{170}$, lent strength to the effect of the ERK 1/2 pathway and Src phosphorylation on cell proliferation$^{63,69}$. In the spongiosa, DCN expression was slightly greater than control while VC expression was lower than the BLK case. In neither case did the NF+BLK case differ from the NF case, indicating that a mechanism separate from 5HT$_{2B}$ activation may have influenced DCN expression. The fact that DCN was upregulated with 5HT exposure also suggested involvement of a separate receptor (likely another family of 5HT receptors) or an alternate signaling method; these conclusions were also suggested by Xu et al.$^{65}$. As DCN is known to regulate collagen fibrillogenesis, and since 5HT exposure has been associated with increased collagen production in valve cells$^{169}$, it was also possible that the DCN upregulation observed in this study could have been due to an increase in valve collagen that would have been apparent with a longer culture time. Since the plaques involved with serotonergic valve disease have been observed to contain PGs, studying differences in the PG composition of plaques induced by exposure to different serotonergic drugs might also help to describe the early mechanism of the valve disease.

Spongiosa COLL3 expression was also greater in the NF samples compared to control and 5HT, and comparison of the NF and NF+BLK cases suggested the involvement of the 5HT$_{2B}$ receptor. Although LOX and P4H
expression did not differ significantly from control, the use of the receptor antagonist with NF decreased P4H content in the atrialis. HSP47 in the NF samples, however, tended to be greater than in the 5HT samples. Expression of MMP9 and MMP13 did not show much difference between NF and 5HT or NF and BLK, but was often lower in NF than control. Another interesting finding was the greater expression of MMP9 in the NF+BLK case than NF in the fibrosa and chordae; it appeared that NF exposure combined with inhibition of the 5HT2B receptor encouraged elastin turnover. A longer culture time might result in a change in elastin content in these valve layers.

5HT exposure had a considerably different effect on the valve tissue than NF exposure. When 5HT was added to the culture media, BGL content tended to be reduced compared to the control case, but approximately the same as the BLK case. This result was also noted with COL1, elastin, and P4H expression in the fibrosa as well as HSP47 expression throughout all valve layers. The decreased content of these components with increased 5HT content indicated that there was a possible desensitization of the 5HT2B receptor noted by Hafizi et al.165 and in the cell proliferation studies performed during the drug optimization studies at the beginning of this aim, leading to a reduced impact of the receptor agonist. 5HT exposure, with or without the combined use of the receptor antagonist, tended to increase content of atrialis DCN, atrialis VC, and spongiosa VC. LOX expression also appeared to decrease in both 5HT treatments as compared to both control treatments, although this result was not significant. This PG upregulation was consistent with the structure of the superficial plaques
indicative of carcinoid valve disease; the lack of difference in PG expression 
between the 5HT and 5HT+BLK groups suggested that an additional mechanism 
may have been responsible for this valve disease. The change in LOX 
expression was also intriguing; since LOX catalyzes cross-links in collagen and 
elastin fibers, its reduced expression may explain the reduced collagen and 
elastin content.

The BLK test case was introduced to give an indication of the cultured 
state of the mitral valve without activation of the 5HT\textsubscript{2B} receptor, and the 
comparison of these two control cases indicated that the use of the receptor 
antagonist in this experiment was effective in blocking activity of the receptor. 
Exposure of the tissue to the receptor antagonist, when compared to plain media, 
decreased BGL expression in all valve layers. There was little effect in DCN or 
VC production, except for a slight decrease in the spongiosa. COLL1 in the 
fibrosa decreased with BLK exposure and COLL3 decreased in the chordae. 
Although no change in LOX or P4H was noted with BLK use, except for fibrosa 
P4H, the decrease in HSP47 expression throughout the valve layers with plain 
media and BLK application indicated baseline activation of the 5HT\textsubscript{2B} receptor. 
The same results were found with MMP9 expression in the atrialis and fibrosa, 
MMP13 in all layers, and TIMP1 in the atrialis and spongiosa. The layer-specific 
response of the receptor antagonist showed both that the culture media was 
sufficiently perfusing even the deeper spongiosa layer and that the different valve 
layers can have very different responses to the drugs. It also showed that the 
introduction of the small quantity of 5HT in the tissue culture media was sufficient
to upregulate different ECM components and turnover mediator, particularly those associated with collagen production. These results corroborated those of Mekontso-Dessap et al.\textsuperscript{169}, who noted an increase in [\(^3\)H]proline incorporation (a measurement of collagen production) in human aortic and mitral VICs and VECs with 5HT exposure. Interestingly, their study also found no decrease in [\(^3\)H]proline incorporation with exposure to either 5HT\(_{2A}\) or 5HT\(_{2B/2C}\) receptor antagonists, indicating that activation of these receptors was not responsible for collagen upregulation.

The plaques found in the bioreactor chambers during organ culture with the NF and NF+BLK cases were also very interesting. At the start, the objects were assumed to be fungal growth or something similar; however, they stained positive for many of the same components as the NF and NF+BLK samples including COLL1, COLL3, DCN, and LOX. This result suggested that the foreign object was actually a type of excess tissue, similar to the plaques noted in patients with serotonergic valve disease. When the organ culture was removed from the bioreactor, the object appeared to be affixed to the ventricular side of the valve, as effort was required to remove it. The location of the growth on the ventricular side of the valve was consistent with clinical cases of fen-phen valvulopathy affecting the ventricular surface and chordae\textsuperscript{64, 70, 171}; the preferential location of the plaques may have been due to a structural difference in the ventricular and atrial surfaces of the valve. The proliferation of VICs noted in the preliminary cell studies as well as the presence of myofibroblasts in plaques removed from patients with serotonergic valve disease suggest that the
plaques removed from the bioreactor were generated due to excessive proliferation of VICs.

A limitation of this project was the discontinuation of the original antibody used against the MMP13 antigen, which required switching to an antibody supplied by a different manufacturer. In all valve layers, MMP13 expression was much greater in the control than in the other test cases. It was possible that the different antibody used affected the results; however, a comparison of samples of fresh valve tissue stained with the old (used in control) and new MMP13 antibodies showed that there was little to no difference in stain intensity.

This research has multiple exciting applications to the study of serotonergic valve disease. First, it showed that, although the histology of the plaques indicative of the disease has shown that the compositions are similar between the drugs, the early disease mechanisms due to NF and 5HT exposure were very different. This finding further disputed the original serotonin hypothesis\textsuperscript{72} and strongly suggested that the mitral valve responds differently to 5HT and NF. The difference in the mitral valve response between the two drugs could explain why fen-phen valvulopathy preferentially affects the left heart valves. A repeat of this study using the tricuspid valve would give further evidence for this proposed explanation.

This study also suggested that, while activation of the 5HT\textsubscript{2B} receptor was involved in the upregulation of the different ECM components and turnover mediators, it was not the only mechanism. Another 5HT receptor family or an alternate signaling pathway may have been involved. The cell culture and organ
culture results also further confirmed that a maximum drug concentration existed, above which effects to the tissue would be reduced due to desensitization of the 5HT\textsubscript{2B} receptor.

This research has shown that it was possible use the splashing bioreactor from Aim 2 to model the \textit{in vivo} chemical environment of the mitral valve leading to serotonergic valve disease. The results from this study not only displayed \textit{upregulation of the ECM components and turnover indicators from baseline} values, they showed a difference between NF and 5HT effects on the mitral valve. These results also suggested that activation of the 5HT\textsubscript{2B} receptor may not have been the only mechanism for these valve tissue changes.

\section*{6.5. Conclusion}

The goal of Aim 3 was to use the bioreactor designed in Aim 2 to model the effects of the serotonergic drugs norfenfluramine and serotonin on the mitral valve. The results of this study suggested that, not only did the two drugs instigate very different types of matrix remodeling, but that the activation of the 5HT\textsubscript{2B} receptor may not have been the only mechanism by which these drugs acted. Although this research did describe the genesis of serotonergic valve disease, the results led to other interesting questions that could lead to further work with this \textit{in vitro} system.
Chapter 7. Conclusion

The purpose of this project was to study the effects on the mitral valve extracellular matrix (ECM) of alterations to the normal mechanical and chemical stimuli provided by the heart and circulatory systems. The mitral valve is not merely a simple flap that randomly opens and closes to allow blood to pass. It is a complex living tissue that can remodel in a pathological manner preventing normal operation; this possibility has often been overlooked in medical treatments, with potentially dire consequences. Although much of the previous research on valve pathology and remodeling has focused on the aortic valve, the position of the mitral valve in the left heart between the two chambers, as well as the clinical emphasis on valve repair rather than replacement, make the mitral valve an intriguing research subject.

The first specific aim studied the effects of heart failure and the left ventricular assist device (LVAD) treatment on the mitral valve tissue. The results of this study are important to consider prior to widespread use of the LVAD as a destination therapy in addition to its original application as a bridge-to-transplant. The effects of the LVAD on the cardiac muscle and on the aortic valve have been well-characterized in the literature while the mitral valve has been less studied. Earlier research in our lab has shown that the mitral valve tends to undergo fibrotic remodeling in response to the change in ventricular shape and resulting change in mechanical forces imparted upon the tissue. If destination therapy is
to be successful, LVAD use should act to reverse this tissue state; therefore, the question studied was whether the LVAD induces further positive or negative remodeling of the mitral valve tissue. The results of this aim showed that the mitral valve does indeed remodel in response to the LVAD use. This remodeling improved some aspects of the valve function in the short-term while worsening others; however, it also resulted in a disruption to the normal layered tissue structure that would likely lead to malfunctioning of the valve in the long-term.

Results from the first specific aim showed that alterations to the normal mechanical environment of the mitral valve would lead to remodeling of the valve structure and altered function. An in vitro system to study the influence of chemical stimuli to the mitral valve would therefore require a design maintaining the in vivo mechanical environment. Previous work in our lab has shown that tissue cultured without mechanical stimulation underwent fibrotic remodeling. In this vein, the second specific aim entailed the design and verification of a splashing bioreactor for the mitral valve. The goal for this project was to create an inexpensive, easily replicable bioreactor that would lightly stretch and perfuse a valve segment, maintaining tissue structure over the test period. A period of two weeks was selected for this test because early tissue remodeling mechanisms were of greatest interest. The expression of multiple ECM components and turnover mediators was compared between fresh valve tissue, tissue cultured with no mechanical stimulation, and tissue cultured in the splashing bioreactor. When compared to the fresh tissue, the tissue cultured in the bioreactor showed fewer signs of remodeling over the two-week period. The
results of this aim produced a viable system for studying the effects of alterations to chemical stimuli in the third and final aim.

The activities of serotonin (5HT) and norfenfluramine were chosen to model in the third aim due to the upregulation of ECM components noted in histological analysis of patients with serotonergic valve disease. These two serotonin receptor agonists were used with and without a 5HT<sub>2B</sub> receptor antagonist in order to isolate the mechanism involved in the plaque formation associated with this disease. Results of this aim showed that, while the two drugs were similar in composition, their respective impact on mitral valve tissue was very different. While NF exposure led to an upregulation of biglycan that was apparently due to activation of the 5HT<sub>2B</sub> receptor, increased decorin expression observed with 5HT exposure was not impacted by inhibition of the 5HT<sub>2B</sub> receptor. These results were particularly exciting due to the preferential impact of serotonin on the right heart valves and of norfenfluramine on right heart valves. The use of the receptor antagonist indicated that although activation of the 5HT<sub>2B</sub> receptor was the likely mechanism for some aspects of plaque formation, an additional process was probably partly responsible as well.

This final chapter will summarize the work done for this research project. The novelty of these approaches and results will be examined as well as the role of the work in the current state of the literature. Although the questions posed in earlier chapters were answered, the results led to additional questions which will be presented here as possible future directions for this project. It is the hope of
the author that this work will not end here, but that it will serve as an inspiration for future scientists.

7.1. Effects of Mechanical and Chemical Stimuli on Valve Remodeling: Addressing Unanswered Questions in the Field

The current research literature pertaining to the success of the LVAD as a bridge-to-transplant focuses on cardiac tissue recovery\textsuperscript{44, 47, 50-54}. With heart failure, the left ventricle dilates, and the left heart valves remodel to compensate. The mitral valve in particular becomes more collagenous and less hydrated\textsuperscript{42}, which can lead to imperfect valve coaptation. Functionally, the LVAD offloads the ventricle, and the myocardial tissue recovers its original geometry and function. The aortic valve has unfortunately been shown to fuse at the commissures\textsuperscript{27, 29} with device use, and it has been recommended to replace the valve or alter the LVAD cycle to force bloodflow through the valve periodically. The current research was novel because of the emphasis on the mitral valve as opposed to the cardiac tissue or the aortic valve. Although commissural fusion is not a primary concern with the mitral valve, this study has shown that the disruption to the tissue that occurs with heart failure is not always reversed with LVAD use. Before a patient is weaned from the LVAD for destination therapy, the mitral valve should be functional to prevent further problems for the patient. The benefit of this approach was the study of the interaction between the structure and function of the mitral valve; the faulty function of the remodeled valve was likely due, ironically, to the valve’s compensatory remodeling.
As mechanical loading is apparently necessary in the successful *in vitro* model, multiple bioreactor designs have been described in the literature\textsuperscript{74-76}, many intended for use in the tissue engineering of a heart valve. The novelty of the current project lies in the simplicity of the design; the system is small, easily replicable with the intention of performing multiple organ cultures simultaneously. Many of the bioreactors in the literature focus on the culture of the semilunar valves, or the aortic and pulmonary valves. The splashing bioreactor is one of the few that are designed specifically for organ culture of the atroventricular, or the mitral and tricuspid, valves. Finally, the analysis of the individual layers in the verification steps not only showed that the perfusion was effective in the deep as well as shallow tissue layers, it indicated that any drug therapies modeled would have an effect throughout the entire valve.

Although valve tissue from patients with serotonergic valve disease has been extensively characterized\textsuperscript{28}, little is currently known about the initial mechanisms behind these pathologies. The current research focuses on cell proliferation studies, exposing valve cells to serotonin receptor agonists and antagonists\textsuperscript{164, 166} and assessing the uptake of markers indicating upregulation of ECM components. The use of an organ culture in the current research introduces a new level of complexity to this body of research. While the earlier works of Setola and Rothman focus on the response of the cells, the organ culture approach also studied cell proliferation and ECM regulation within the different valve layers. This work also looked at multiple ECM components and turnover mediators to give a more complete picture of the initiation of
serotonergic valve disease. Although the current work examined some outstanding questions regarding the mechanisms of the different effects of serotonin and norfenfluramine on the mitral valve, a whole new set of questions has risen, and these will be addressed in a later section.

7.2. **Advancing the Understanding of Valve Remodeling**

The results of Aim 1 extended our lab’s previous work quantifying structural and functional remodeling of the mitral valve as a result of congestive heart failure. Additionally, it compared the structure and function of the valve in patients treated with the LVAD in order to ascertain whether the valve would revert to its normal state. While the remodeling of the valve did lead to improved chordal function, the structure and function of the leaflet was still suboptimal. This work augments that of Connelly and Rose, who have noted pathological remodeling of the aortic valve\textsuperscript{27, 29}, and indicates that the function of the valves should be among the factors considered in determining use of the LVAD as a destination therapy. Furthermore, functional remodeling of the mitral valve may actually be an indicator of a successful candidate for destination therapy.

Aim 2 designed a splashing bioreactor to maintain mitral valve structure over a period of two weeks, and the design success was verified by a comparison of tissue cultured in the bioreactor with fresh tissue and tissue cultured without mechanical stimulation. This splashing bioreactor design provided the mechanical stimulation necessary to maintain tissue structure over two weeks. This research introduced a viable alternative *in vitro* method to the
flow loops\textsuperscript{77, 78}, cyclic pressure\textsuperscript{79, 80}, and cyclic flexure\textsuperscript{81-85} systems currently available for organ culture and tissue engineering applications. Validation of these earlier bioreactor designs often involved biochemical analysis of whole valve tissues or qualitative immunohistochemical analysis of the overall valve structure to verify success of the bioreactor. The current body of work used a semi-quantitative analysis method to compare immunohistochemical staining intensities within the individual valve layers, which is important due to their different structure and function. A final point of interest is that, while the bioreactor tissue engineering studies ideally encouraged collagen deposition to strengthen the valve construct, success of the splashing bioreactor was assessed by a lack of fibrotic remodeling previously noted in static cultures.

Aim 3 results showed that norfenfluramine and serotonin could initiate remodeling of mitral valve tissue in as little as two weeks. The drug optimization studies added evidence to Hafizi's theory\textsuperscript{185} that the effects of these two drugs were dose-dependent, and that a concentration existed above which the affected receptor is saturated and no further remodeling would be initiated. In organ-cultured samples, NF exposure led to an upregulation of biglycan that was inhibited by use of the 5HT\textsubscript{2B} receptor antagonist. On the other hand, 5HT exposure led to an upregulation of decorin that was unaffected by the receptor antagonist treatment. The varied effects of the two drugs on the mitral valve tissue, although they have a similar structure, laid groundwork for further studies regarding preferential remodeling of the right heart valves by serotonin and of the left heart valves by norfenfluramine. This result also provided further evidence
against the “serotonin hypothesis” previously used to explain the difference in right and left heart valve remodeling\textsuperscript{72}. The incomplete blocking of this remodeling with exposure of the tissue to the 5HT\textsubscript{2b} receptor antagonist has both confirmed the role of this receptor in serotonergic valve disease and introduced the idea of an additional involved mechanism, including activation of a separate receptor\textsuperscript{65}. This combined body of work has corroborated the work of others in the valve biology field while further defining the effects on the mitral valve structure and function of alterations to the mechanical and chemical environment.

7.3. \textbf{Future Directions}

The Discussion and Conclusions section of the earlier chapters in this body of research have noted where holes still exist in the knowledge base or where the current research has led to further questions. Additional ideas for future directions to this work have been collected during discussions and presentations of this data at public forums. Possible future directions of this work will be listed below according to the aim that inspired the idea.

Aim 1 was unfortunately limited by the availability of donor tissue. If the patient pool could be expanded, an interesting study would be the effect of the different LVAD types (i.e., continuous versus pulsatile flow). In discussions with other colleagues, the idea was presented to look at the pressure differences over the MV and AV to determine if there is any coupling involved in the valve remodeling. Another colleague suggested the use of proliferating cell nuclear antigen to augment the results of the biochemical (Hoescht) and histological
(H&E) analysis of cellular concentration in CHF and LVAD patients. The factors influencing the success of destination therapy should also be further examined; the extent of recovery of the left heart valves could identify patients who would benefit from this practice.

A slight change to the design of the splashing bioreactor could make it another system available for use in the tissue engineering of a heart valve with the introduction of a treated scaffold material and donor cells¹ (Chapter 3). The entire system could also be scaled up to accommodate an entire valve to better model the coaptation of the leaflet free edges.

The splashing bioreactor can be used in the future to replicate the conditions for the foreign object formation observed with norfenfluramine exposure. The system might also be used to isolate alternate mechanisms of serotonergic heart disease. Further assessment of the activation of the ERK1/2 pathways in these samples might provide more evidence on the early disease mechanism⁶⁹. A simple test to study preference of drug effects on the different valves would be the replacement of the mitral valve with a tricuspid valve section in the Aim 3 tests; the modular design of the bioreactor would easily accommodate the longer leaflet.

This study of the serotonergic drug effects on the right and left heart valves should be expanded to include the aortic and pulmonary valves. A cell proliferation or organ culture study comparing norfenfluramine and serotonin exposure on all four heart valves might highlight any inherent differences in the valves that would account for the preferential drug effects. Since the plaques in
fen-phen valvulopathy tend to occur on the ventricular surface, another cell proliferation study could be designed modeling exposure of the exposure of norfenfluramine and serotonin on endothelial cells from the atrial and ventricular surfaces. The lack of knowledge on the effects of fen-phen duration or drug cessation suggests another couple of studies to further define the effects of the drug. First, a cell proliferation or organ culture study could be designed to assess remodeling of the mitral valve after varying durations of drug exposure. Next, drug administration should be ceased at intervals while continuing the study to determine if the valve remodeling reverses or if it continues. Since pergolide use in this study was thwarted by the low solubility of the chemical, development of a reliable method of dissolving pergolide in culture media would give an additional type of serotonin receptor agonist to study. These additional studies would give a more complete picture of the effects of serotonergic drug exposure to the mitral valve, ideally limiting further episodes of serotonergic valve disease among the general public.

7.4. Conclusion

Taken together, the three specific aims give a complementary picture of mitral valve remodeling due to alterations to the normal mechanical and chemical environments. The first aim affirmed that LVAD treatment did not completely reverse the fibrotic remodeling of the mitral valve observed with heart failure. This result is less of a concern with bridge-to-transplant use than it is with destination therapy, where the patient should be left with a functional valve to
prevent ongoing cardiac health issues after weaning from the device. This impact of the change in left ventricular shape further emphasized the importance of maintaining the mechanical environment in an in vitro organ culture system, such as the one designed for the second aim. The splashing bioreactor was successful at maintaining the valve structure for the two weeks desired to examine the early mechanism of serotonergic valve disease. After two weeks, the serotonin and norfenfluramine caused remodeling of the mitral valve tissue, but the effects of the two drugs on the mitral valve were dramatically different, consistent with clinical observations that carcinoid valve disease (caused by excess serum serotonin) targets the right heart valves while fen-phen valvulopathy targets the left heart valves.

The normal chemical and mechanical environments of the mitral valve play an important role in maintaining tissue structure and therefore function. When the valve surroundings are altered, the tissue remolds to compensate. This ability of the tissue to remodel is the basis behind a number of valve pathologies and should be considered when exposing a patient to drug or medical device therapies that could modify the valve structure and function.
Appendix A1. Mechanical Testing Data Analysis

A1.1. Determination of Gauge Length – Original Assessment

In tensile mechanical testing data analysis, the length at which the sample began to resist load was used as a reference point to allow comparison of mechanical properties between samples. This reference point, the gauge length \( \ell_g \), has been difficult to define in the soft tissue of the mitral valve in an objective and easily repeatable manner. Carew and Vesely have defined \( \ell_g \) as the transition from compression to tension (Figure A1.1) as the sample was stretched from a partially wrinkled starting point \( \ell_0 \). Since testing was initiated with the tissue in compression (slack), \( \ell_g \) was always greater than \( \ell_0 \).

The resulting load-elongation curve was first fitted to the cubic line of best fit

\[
F(\ell) = a\ell^3 + b\ell^2 + c\ell + d
\]

where \( F \) was load, \( \ell \) was specimen length, and \( a, b, c, \) and \( d \) were the parameters of fit. The correlation coefficient \( r^2 \) was then calculated to determine the quality of fit.

\( \ell_g \) was estimated to be the stationary (inflection) point on the slope of the loading curve, or the value of \( \ell \) that satisfied

\[
\ddot{F}(\ell) = 0
\]

The estimation of gauge length was therefore
\[ \ell = -\frac{b}{3a} \]

In the case that the initial test region did not give the best fit (maximum \( r^2 \)) or the best estimate of \( \ell_g \), the test region was systematically reduced by dropping points in the direction of decreasing length until \( \ell_0 \) was greater than \( \ell_g \). At this point, data points were returned in the direction of increasing length. Once the maximum local \( r^2 \) was identified, the corresponding gauge length was returned\(^{131}\). These calculations were performed using Mathematica (Wolfram Research, Champaign, IL).

Figure A1.1: Determination of gauge length (A), defined by the point at which the second derivative of the cubic line of best fit was equal to 0 (vertical line). Location of gauge length with respect to entire data set is indicted in (B)\(^{131}\).
A1.2. Determination of Gauge Length – Final Algorithm

Unfortunately, the results obtained from these programs did not always make physical sense; the value obtained through hand calculations for properties such as stiffness did not match the values obtained by the Mathematica program. The Carew/Vesely algorithm was therefore edited by summer student Meaghan McNeill. The new program first determined the last datapoint in the set where the load was less than an earlier measured load ("negpoint") in order to negate the effects of any noise in the toe region. The load therefore increased monotonically after "negpoint." A test region of the data set was selected relative to negpoint, beginning at a point in the file 7% before negpoint and ending 70% after negpoint. The $\ell_g$ was defined as the minimum, as opposed to the inflection point, of the cubic function fit to this test region. Repeated testing verified that the algorithm gave a more reasonable approximation of $\ell_g$. As with the Carew/Vesely method, the test region was then adjusted to optimize $r^2$. 
Appendix A2. Histology and Immunohistochemistry

The details of the familiar histology stains H&E, Movat pentachrome, and Masson’s trichrome are presented in greater detail here for the reader. The immunohistochemical methods performed and a primary antibody chart are also included. This appendix ends with a brief treatment of the immunocytochemistry methods used in the cell characterization during the explant cell culture protocol in Aim 3.

A2.1. Hematoxylin and Eosin (H&E)

H&E was used to identify cell nuclei (purple-hematoxylin) and cytoplasmic or extracellular matrix (ECM) proteins (eosin-pink). The samples were first rehydrated and deparaffinized by washing in xylene three times for three minutes each, then in Flex three times for one minute each. The slides were then placed in a 95% Flex solution for one minute and rinsed briefly in first tap water, then ultrapure water. The slides were placed in filtered hematoxylin for five minutes and rinsed in tap water to remove the excess stain. The slides were placed in clarifier for 30 seconds and rinsed in running tap water for 30 seconds, then placed in bluing reagent for one minute and rinsed in running tap water for one minute. They were rinsed in 95% Flex and placed in eosin for one minute. Finally, the samples were dehydrated by washing in the 100% Flex three times.
for one minute each and in xylene three times for one minute each. After removal from xylene, the slides were coverslipped.

**A2.2. Movat Pentachrome**

Movat pentachrome stain was used to identify the ECM components within the layers of the valve tissue. The elastin (atrialis and chordae sheath) was stained black, and collagen (fibrosa and chordae core) was stained yellow. Proteoglycans and glycosaminoglycans (PGs/GAGs) in the spongiosa were a blue-green color. Any fibrin or smooth muscle present in the tissue was stained red, and cells were black.

Samples to be stained were deparaffinized in the same manner as above. The first stain used was alcian blue (1% alcian blue powder, 3% acetic acid in distilled water) to identify PGs/GAGs; the samples were stained for five minutes and washed in running tap water for five minutes. The slides were rinsed briefly in distilled water and incubated in alkaline alcohol (10% ammonium hydroxide in 95% ethanol) at 80°C for two minutes to solidify the alcian blue. After incubation, the slides were washed in running tap water for 20 minutes to completely remove the alkaline alcohol.

After briefly rinsing the samples in distilled water, they were placed in Verhoeff's hematoxylin solution (50% hematoxylin solution, 25% iodine solution, 25% aqueous ferric chloride solution) for 30-60 minutes (depending upon freshness of solution) to stain the elastin and cells black. The hematoxylin solution contained 5% hematoxylin in absolute ethanol, the iodine solution
contained 2% iodine and 4% potassium iodide in distilled water, and the aqueous ferric chloride solution contained 10% aqueous ferric chloride in distilled water. After rinsing in distilled water, the samples were differentiated with a 2% aqueous ferric chloride solution until individual elastic fibers were visible and then rinsed in distilled water to prevent over-differentiation.

The slides were then incubated in 5% aqueous sodium thiosulfate for one minute, washed in tap water for five minutes, and rinsed in distilled water. To stain fibrin and smooth muscle, the samples were stained with crocein scarlet – acid fuschin for 1-4 minutes, depending on the age of the solution. The stain was made of two stock solutions: 80% stock solution containing 0.1% Crocein Scarlet in 0.5% acetic acid and 20% stock solution containing 0.1% Acid Fuschin in 0.5% acetic acid. After staining, the slides were dipped twice in distilled water, agitating 1-2 seconds. The slides were then dipped twice in 0.5% acetic acid, agitating 1-2 seconds.

The samples were incubated in 5% phosphotungstenic acid for five minutes and dipped in 0.5% acetic acid twice, agitating 1-2 seconds. The samples were then dehydrated with three changes of absolute alcohol, 5-6 dips for each change. The slides were then placed in Safran (6% Safran du Gratinais in absolute ethanol) for five minutes or in a premade Safran solution (Poly Scientific, Bay Shore, NY) for 10 minutes to stain collagen. After staining, the samples were dehydrated again with absolute alcohol, with 5-6 dips for each change. Finally, the samples were cleared with three changes of 100% xylene and coverslipped.
A2.3. Masson's Trichrome

The Masson's trichrome stain was used to obtain more information on the ECM structure of the foreign objects noted in Aim 3. The protocol would stain collagen blue-green and mucus a lighter green. Erythrocytes and myelin would stain scarlet, while cytoplasm would stain pink, red, or brown. Cell nuclei would stain black.

Clearing and hydration of the slide were performed in the same manner as for Movat (Section A2.2). After rinsing in distilled water for one minute, the tissue was exposed for three minutes to Wigart's Fe hematoxylin, which was a combination of equal parts hematoxylin (5 g hematoxylin in 500 mL EtOH) and FeCl (5.8 g FeCl₃·6H₂O in 495 mL H₂O and 5 mL HCl) solutions. Excess stain was then washing away in running tap water for one minute.

Next, the samples were stained with 1% biebrich scarlet (5 g biebrich scarlet in 495 mL H₂O and 5 mL glacial acetic acid) for four minutes. After a brief rinse in running tap water to remove excess stain, tissue samples were immersed in PMA-PTA solution (5 g phosphomolybdic acid and 5 g phosphotungstic acid in 200 mL H₂O). After one minute, samples were removed from solution and viewed under a microscope to ensure removal of red dye from collagen fibers. Differentiation in PMA-PTA was continued until collagen was free of red stain. The slides were then exposed to 2.5% fast green FCF (5 g fast green FCF in 195 mL H₂O and 5 mL glacial acetic acid) for four minutes.
After staining, slides were immersed in two changes of acidified water (5 mL glacial acetic acid for every 1 L H₂O) for 30 s each. Slides were then dehydrated, cleared, and coverslipped as in Section A2.2.

A2.4. Immunochemistry (IHC)

IHC allowed the localization of specific ECM components and synthesis indicators within the valve tissue. The following protocol has been generalized to include all antibodies used in immunohistochemical staining. The antibody concentration, pretreatment method, and secondary antibody used for each primary antibody are listed in Table A2.1.

All samples were first deparaffinized with three changes of xylene and placed in two different washes of 100% Flex for one minute each. If the antigen decloaker or chymotrypsin pretreatments were not to be used, endogenous peroxidase was quenched by placing the slides in 0.7% H₂O₂ in 100% MeOH for 20-30 minutes and rinsed in 100% Flex for five minutes. All samples were then hydrated to 50% Flex over a span of 10 minutes.

One of three pretreatments was available for use, depending on the antigen of interest (Table 2A.1). Most antibodies required that the tissue be incubated in an antigen decloaker solution (Biocare Medical, Concord, CA) for 30 minutes in an 80°C water bath. The slides were then washed in tap water for five minutes and in PBS for one minute. Samples were exposed to a 3% hydrogen peroxide solution for five minutes and rinsed in two washes of phosphate-buffer saline (PBS) for two minutes apiece. PG samples were incubated in
Chondroitinase ABC (Seikagaku, Tokyo, Japan) to expose the binding site for the antibody. First, slides were washed in tris-buffered saline (TBS) for 10 minutes. The enzyme solution (1% Chondroitinase ABC, 2% BSA, 20% Tris solution in water) was then applied to the samples, which were then incubated at 37°C for one hour. TBS provided the appropriate pH for the enzyme activity, and its use was continued through many of the subsequent steps for PG staining. After deparaffination and rinsing in both 100% Flex and water, tissue samples to be treated with the elastin antibody were warmed and exposed to a chymotrypsin solution (0.4 g chymotrypsin and 0.4 g CaCl₂ in 400 mL water) for 10 minutes. This treatment was followed by a rinse in cold tap water.

In the following steps, use of the general term "buffer" indicates that TBS was used for PG samples while PBS was used for all other samples. After the applicable antigen retrieval step, wells were drawn around the individual tissue samples using a hydrophobic marker. In order to block nonspecific binding of the secondary antibody, a 10% goat serum buffer solution (rabbit serum was used for fibrinogen) was applied to the samples and allowed to incubate for one hour, then removed from the test samples. Negative staining controls were created by leaving the serum solution on a sample in lieu of application of a primary antibody. The primary antibody (in buffer and 0.1% bovine serum albumin) was then added to the wells of the test samples. The antibody then incubated overnight at 4°C (Aim 1) or at 37°C for one hour (Aims 2 and 3).

After incubation, the slides were rinsed and washed in two washes of buffer, for five minutes each. The appropriate biotinylated secondary antibody
(Jackson ImmunoResearch, West Grove, PA) was then added to all samples and allowed to incubate for one hour at room temperature. After incubation with the secondary antibody, the slides were rinsed and washed twice in PBS, for five minutes each. Samples were then incubated for 30 minutes at room temperature with an avidin-biotin complex, made from a Vectastain kit (Vector Laboratories, Burlingame, CA). The slides were rinsed and washed twice in PBS, for five minutes each.

The final step, tagging the antibody complex with diaminobenzene (DAB) solution, allowed visualization of the antibody binding sites. At the beginning of Aim 1, the DAB solution was made from a powder. Samples were washed in TBS for five minutes, then in tris buffer (0.05 M, pH 7.6) for 10 minutes at 37°C. All samples were then incubated with the DAB solution (100 mg DAB, 100 μL 30% H₂O₂, 0.08 g NiCl₂, 200 mL tris buffer) for 10 minutes at 37°C. After incubation, the samples were returned to the tris buffer and then rinsed in water. A DAB kit (Vector Laboratories) that required less physical contact with and disposal of the carcinogen was eventually purchased. Since many of the PG samples had already been stained, the powdered DAB solution was used to color all of these slides to reduce sources of batch variation. All collagen samples from Aim 1 were stained with the DAB kit. Use of the DAB kit was extended to Aims 2 and 3. All samples were finally counterstained with hematoxylin before coverslipping.
A2.5. Fluorescent Cell Markers and Immunocytochemistry

Cell staining methods were used briefly during Aim 3 to characterize the cells obtained from the explant cell culture as either valvular endothelial cells (VECs) or interstitial cells (VICs). Cells from culture were first cultured for 2-3 days on slides treated with fibronectin. Cells were washed with PBS and cells were exposed to DMEM containing 10 µg/mL acetylated low density lipoprotein (Dil-AC-LDL) to identify VECs. The cells were incubated at 37°C for four hours then washed with PBS to remove free Dil-AC-LDL. Cells were then fixed with 5% formalin in PBS for 10 minutes, washed in PBS, and mounted using Vectashield with DAPI (4',6 diamidino-2-phenylindole, Vector Labs), a nuclear marker that fluoresced blue. When viewed using a rhodamine filter, cells staining positive for Dil-AC-LDL appeared red.

VICs were identified using immunocytochemistry with an antibody against smooth muscle α-actin (SMαA). Cells were fixed with two PBS rinses followed by two 10 minute acetone treatments. As with IHC, cells were incubated at room temperature for one hour each in 10% goat serum in PBS and in a 1:100 dilution of the SMαA antibody in PBS containing 1% BSA. To create a negative control, cells were incubated for one hour in a 1:100 dilution of IgG in PBS containing 1% BSA. After incubation in the primary antibody, cells were washed in three washes of PBS for five minutes each. In order to create a permanent slide, cells were incubated at room temperature in a 1:1000 dilution of biotinylated goat-anti-mouse secondary antibody (as opposed to a fluorescein isothiocyanate, or FITC,
conjugated antibody) in PBS with 1% BSA for one hour. After rinsing the slide with three changes of PBS, the IHC protocol described in Section A2.4 was followed, beginning with application of the avidin-biotin complex. The stain could be viewed with a light microscope, and the slides could be stored indefinitely.
Table A2.1: Antibodies used in immunohistochemical staining

<table>
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<th>Primary Antibody</th>
<th>Dilution</th>
<th>Vendor Location (Cat. Number)</th>
<th>Pretreat Method</th>
<th>Secondary Antibody</th>
<th>Aim</th>
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<td>1:2000</td>
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<td>cABC</td>
<td>Goat anti-Rabbit</td>
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</tr>
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<td>Goat anti-Rabbit</td>
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<tr>
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</tr>
<tr>
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<td>cABC</td>
<td>Goat anti-Rabbit</td>
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<td>cABC</td>
<td>Goat anti-Rabbit</td>
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<td>Chymo</td>
<td>Goat anti-Mouse</td>
<td>2,3</td>
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<td>Rabbit anti-Goat (Abcam)</td>
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<td>AgR</td>
<td>Goat anti-Mouse</td>
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</tr>
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<td>LOX</td>
<td>1:250</td>
<td>Imugenex San Diego, CA (IMX-5121)</td>
<td>AgR</td>
<td>Goat anti-Rabbit</td>
<td>2,3</td>
</tr>
<tr>
<td>MMP1</td>
<td>1:200</td>
<td>Assay Designs Ann Arbor, MI (905-472)</td>
<td>AgR</td>
<td>Goat anti-Rabbit</td>
<td>2</td>
</tr>
<tr>
<td>MMP2</td>
<td>1:100</td>
<td>Assay Designs Ann Arbor, MI (905-482)</td>
<td>AgR</td>
<td>Goat anti-Rabbit</td>
<td>2</td>
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<tr>
<td>MMP9</td>
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<td>AgR</td>
<td>Goat anti-Rabbit</td>
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<tr>
<td>MMP13</td>
<td>1:200</td>
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<td>None</td>
<td>Goat anti-Mouse</td>
<td>2</td>
</tr>
<tr>
<td>MMP13</td>
<td>1:50</td>
<td>R&amp;D Systems Minneapolis, MN (MAB511)</td>
<td>AgR</td>
<td>Goat anti-Mouse</td>
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Table A2.1 (cont): Antibodies used in immunohistochemical staining

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Vendor Location (Cat. Number)</th>
<th>Pretreat Method</th>
<th>Secondary Antibody</th>
<th>Aim</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4H</td>
<td>1:200</td>
<td>Chemicon Billerica, MA (MAB2701)</td>
<td>AgR</td>
<td>Goat anti-Mouse</td>
<td>2,3</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1:100</td>
<td>Assay Designs Ann Arbor, MI (905-484)</td>
<td>AgR</td>
<td>Goat anti-Rabbit</td>
<td>2,3</td>
</tr>
<tr>
<td>VC</td>
<td>1:5000 (Aim1) 1:500 (Aim2,3)</td>
<td>Seikagaku Tokyo, Japan (270428)</td>
<td>cABC</td>
<td>Goat anti-Mouse</td>
<td>1,2,3</td>
</tr>
<tr>
<td>vWF</td>
<td>1:100</td>
<td>Sigma St. Louis, MO F3520</td>
<td>None</td>
<td>Goat anti-Rabbit</td>
<td>3</td>
</tr>
</tbody>
</table>

AgR=antigen decloaker treatment; BGL=biglycan; cABC=chondroitinase ABC enzyme treatment; Chymo=chymotrypsin treatment; COLL=collagen; DCN=decorin; FB=fibrinogen; HSP47=heat shock protein 47; LOX=lysyl oxidase; MMP=matrix metalloproteinase; P4H=prolyl-4-hydroxylase; TIMP=tissue inhibitor of MMP; VC=versican; vWF=von Willebrand Factor.
Appendix A3. Biochemical Methods

A3.1. Collagen Quantification

A3.1.1. Hydroxyproline Assay Protocol

The general purpose of the hydroxyproline assay was to quantify collagen content in the valve tissue by measuring the hydroxyproline prevalent in primary structure of the collagen protein. After digestion with proteinase-K, the samples were centrifuged to form a pellet of undigested tissue (elastin residues, cells, and calcium) at the bottom of the tube. An aliquot was taken based on the presumed amount of collagen in the tissue sample in order to have the experiment results fall within the range of a standard curve. This aliquot was placed into each of two 2 mL closed glass vials with a large enough amount of 6N HCl to hydrolyze the collagen (approximately 250 μL HCl for a 20-25 μL sample aliquot). The samples were hydrolyzed for 4 hours at 115°C. The vial cap was removed, and the vials were returned to the 115°C heating block to allow the HCl to boil away. The residue was then dissolved in enough ultrapure water to bring the concentration of hydroxyproline in the sample below 300 μg/mL.

The sample in each glass vial was assayed in two separate test tubes, so that each original tissue sample would be assayed using a total of four test tubes. The following assay steps were also performed on an aliquot of each of seven known concentrations of hydroxyproline (0, 10, 25, 50, 100, 150, and 300 μg/mL) used to form the standard curve. 500 μL dH₂O was measured into each test
tube, and a 20 µL of sample or standard was then added. 250 µL of chloramine-t reagent (44.06 mg chloramine-t, 0.625 mL dH₂O, 0.625 mL n-propanol, 5 mL hydroxyproline buffer) was added to each tube. The tubes were then vortexed and allowed to sit for 20 minutes at room temperature. 250 µL of aldehyde/perchloric acid was added to all tubes, which were then vortexed and incubated at 60°C for 15 minutes (0.5 g Erlich's reagent, 2 mL n-propanol, 0.867 mL 70% perchloric acid). The tubes were vortexed again, and two 250 µL aliquots from each tube were placed in a 96-well plate. The absorbance of each well was read at 558 nm. The absorbances of the 7 standards were graphed against the known quantity of hydroxyproline, and the coefficients of the linear fit were used to calculate the amount of hydroxyproline in each sample. The amount of hydroxyproline in the original sample was then back-calculated using the amount found in the sample and the known level of dilution. The amount of collagen was calculated using the amount of hydroxyproline found.

A3.1.2. Estimation of Collagen in Tissue

The measurement of hydroxyproline in collagen begins by hydrolyzing the sample in HCl then evaporating away the HCl. Before dissolving the hydrolyzate in dH₂O, it is important to have an idea of the amount of collagen in the original tissue sample. In order to obtain meaningful results, the concentration of hydroxyproline in the sample, after assay preparation, must fall within the limits of the standard curve (<300 µg/mL).
Before the tissue is hydrolyzed, the dry weight is measured. This value is multiplied by the known percentage of collagen for the particular tissue type to estimate the total amount of collagen in the tissue. Chordae and leaflets are assumed to contain approximately 80% and 60% collagen, respectively. Heart valve collagen is assumed to be approximately 13% hydroxyproline\(^2\). This proportion is based on the known mass percentage of the amino acid hydroxyproline among all amino acids in collagen, in addition to collagen composition of the normal mitral valve (74% type I, 24% type III, and 2% type V collagen)\(^2\). The total amount of collagen is therefore multiplied by 0.13 to give the total amount of hydroxyproline in the tissue.

The final step is to determine the amount of hydroxyproline in the aliquot taken for the hydroxyproline assay. The amount of collagen in the aliquot taken is determined, then the amount of water required to bring the concentration below 300 \(\mu\)g/mL is calculated.

**Example:**

ALF sample dry weight: 0.0293 g

Final volume of proteinase-digested tissue: 1.5 mL

Total collagen = 0.0293 g \(*\) 60% = 0.0176 g

Total hydroxyproline = 0.01758 g \(*\) 13% = 0.00229 g

Hydroxyproline in 50 \(\mu\)L aliquot: 0.00229 g \(*\) 50 \(\mu\)L / 1500 \(\mu\)L = 7.62 \(\times\) 10\(^{-5}\) g = 76.2 \(\mu\)g

Minimum final volume = 76.2 \(\mu\)g / 300 \(\mu\)g/mL = 0.254 mL = 254 \(\mu\)L
At least 254 μL dH₂O must be added to bring the concentration of hydroxyproline in the sample below 300 μg/mL for a meaningful result when compared against the standard curve.

A3.2. DNA (Hoescht) Assay Protocol

Samples were tested for DNA content as a measure of tissue cellularity. An aliquot of sample was determined based on presumed amount of DNA in the sample. This process was trial-and-error, as the aliquot was chosen so that the sample fit within the limits of the standard curve. This aliquot and an appropriate volume phosphate (Pi) buffer (50 mM Na₂HPO₄, 2 mM EDTA, 0.02% NaN₃, pH 7.4) were placed in a 1.5 mL centrifuge tube. Two different dilutions per sample were tested for repeatability. Samples were then sonicated for 3 minutes to rupture the cell membrane and release DNA into the solution.

Standards were made from calf thymus double-stranded DNA. The 100 μg/mL stock solution was diluted 10:1 in ultrapure water to make 1 mL of 10 μg/mL DNA solution. The DNA solution was then mixed with Pi buffer to create 100 ng/mL, 200 ng/mL, 500 ng/mL, 750 ng/mL, 1000 ng/mL, and 2000 ng/mL standards.

The dye stock solution was made using 0.2 mg Bisbenzimide (Hoechst 33258) in 1 mL ultrapure water. This stock was then diluted 1:100 in NaCl/Pi buffer (4M NaCl, 50 mM Na₂HPO₄, 2 mM EDTA, 0.02% NaN₃, pH 7.4). This dye was added to the samples and standards, and the tubes were vortexed. The
tubes were then protected from light and allowed to sit at room temperature for 15-30 minutes.

The standards and samples were aliquoted into black 96-well plates in duplicate 270 μL volumes. The plates were then read by a fluorocolorimeter with excitation set to 356 nm and emission set to 458 nm. The data obtained was imported into Excel (Microsoft Corp., Redmond, WA) for analysis.

A3.3. Glycosaminoglycan (GAG) Quantification

A3.3.1. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)

The purpose of the FACE assay was to first cleave the GAG disaccharides from the proteoglycan (PG) core protein, then to measure the measure content of individual GAGs via electrophoresis. After the samples had been digested with the proteinase-K solution, they were centrifuged to form a pellet of undigested tissue at the bottom of the tube. The samples were then digested using either chondroitinase ACII alone (to cleave HA and CS) or a combination of chondroitinase ACII and chondroitinase ABC (to cleave HA, CS, and DS). One aliquot of sample, estimated from previously determined concentrations of total GAGs, was used for each type of digest. The sample was mixed with dH₂O, 1 M ammonium acetate (pH 7.0), and 3 μL of 10 mU/μL chondroitinase ACII. Samples undergoing chondroitinase ACII/ABC digestion had an additional 3 μL of 10 mU/μL chondroitinase ABC added. The tubes were then allowed to incubate in a 37°C water bath for 3 hours, frozen, and placed in a speed-vac until dry. Emphasis was placed on maintaining pH<7.0 throughout the
process for complete digestion of the hyaluronan and unsulfated chondroitin by the chondroitinases. The unsaturated disaccharides that were the product of the enzymes were also unstable at a basic pH. Phenol red, which was present in the sample to verify that the pH of the samples was no greater than 7.0\textsuperscript{136}, was reduced to a colorless compound with cyanoborohydride to prevent detection in the FACE gel.

The next step was to label the GAG disaccharides with the fluorescent tag AMAC. A 0.0125 M AMAC solution in 3:17 v/v acetic acid/dimethylsulfoxide (DMSO) was prepared, and 40 \mu L of this solution was added to each tube. The samples were allowed to incubate at room temperature for 10-15 minutes. 40 \mu L of 1.25M sodium cyanoborohydride (NaCNBH\textsubscript{4}) was then added, and the samples were incubated 15-16 hours at 37°C. The samples were then cooled to room temperature, and 20 \mu L glycerol were added.

Prior to inserting samples into the FACE gel, the samples were diluted 1:4. A 2 \mu L aliquot of sample was mixed with 2 \mu L maltotriose standard and 4 \mu L AMAC buffer (34% by volume DMSO, 6% glacial acetic acid, 40% 1.2M NaCNBH\textsubscript{4}, 20% glycerol). The maltotriose standard was available in concentrations of 200, 100, 50 and 25 (concentrations in 4X of pmol/5 \mu L), and a different maltotriose standard was added to each sample in order to allow create a fluorescent standard curve.

Gels appropriate for running FACE analysis were not readily available, and were therefore made in the lab (Section A3.3.3.). To perform FACE, the gel box was placed in the center of a basin (Figure A3.1). Ice was added to the
basin to surround the gel box, and cold water was added to a level approximately one-third that of the ice. 1 L 4°C TBE buffer (100 mM Tris, 90 mM Boric Acid, 1 mM ethylenediaminetetraacetic acid, pH 8.3) was added to the gel box, and a stir bar was added to the gel box to maintain temperature equilibrium. The comb was removed from the gels. The gel glass and wells were then rinsed out with the TBE buffer. One gel was placed in each side of the FACE apparatus with the lower side facing inward. The gels were secured in the apparatus, and the apparatus was placed in the FACE box. 100 mL buffer was added to the top of the apparatus to fully cover the gel wells.

Figure A3.1: FACE experimental setup. The gel was secured within the FACE apparatus, buffer was added to the FACE box, and an electrical current was run through the gel to separate the GAG classes by molecular weight and charge.

A power source was programmed to provide a current of 500 V, 100 W and 400 mA to the apparatus. The gels were pre-run to verify that the actual
current was approximately 40-60 mA for 2 gels. A higher current indicated a shorter run time for the gel. A multipipetter was then used to add 5 μL of each sample to each well. The power source was turned on, and the gels were allowed to run for 30 min – 1.5 hours, depending on the pre-test current.

The gels were immediately imaged after removal from the FACE apparatus. First, a gel was rinsed with tap water and dried. The gel was then exposed to a UV transilluminator. Images were captured using a Kodak Gel Logic System (Eastman-Kodak, Rochester, NY) with a charge-coupled device (CCD) camera and Kodak 1D 3.6 software. Three exposures were taken: one low, one medium, and one high. Generally, the low exposure was selected such that none of the bands were saturated. The medium exposure was made at a time double that of the low exposure, and the high exposure was made at a time double that of the medium exposure, so that less abundant bands could be visualized. Band integrated optical density (IOD) was then measured using Gel-Pro software version 3.1 (Media Cybernetics).

**A3.3.2. Estimation of Uronic Acid**

Preparation for the FACE assay begins by digesting an aliquot of proteinase-K digested tissue with a GAG enzyme (Chondroitinase ACII and/or Chondroitinase ABC) to cleave the GAG chains into the disaccharides. As with collagen, GAG content in tissue should be estimated prior to taking an aliquot from the proteinase-K digested tissue in order to ensure that the results will fall within the confines of a standard curve. First, the wet weight of the sample is
determined. The wet weight for a leaflet is multiplied by 1200, or the wet weight for chordae is multiplied by 700, to give the amount of uronic acid, in μg, in 1 mL of sample.

The total amount of uronic acid is divided into 1000 μL to get the volume (in μL) of 1 μg uronic acid. This volume is multiplied by 5 to get the volume of 5 μg uronic acid. If the final volume is less than 30 μL, it is rounded to the next whole number, and reduced by 5. If the final volume is less than 210 μL, it is rounded to the next whole number, and reduced by 10. If the final volume is less than 1 mL, it is converted to 200 μL; otherwise, the volume is converted to 250 μL. This algorithm has been noted to work well as the 5 μg uronic acid is an ideal amount of substrate for the 3 μL of GAG enzyme used in the FACE preparation, based on the unit activity of the enzyme and a stock enzyme concentration of 10 mU/μL).

Example:

ALF sample wet weight: 0.1925 g
Concentration uronic acid = 0.1925 g * 1200 = 231 μg/mL
Volume of 1 μg uronic acid = 1000 / 231 μg/mL = 4.33 μL
Volume of 5 μg uronic acid = 5 * 4.33 μL = 21.64 μL
Round volume up to next whole number: 22 μL
Final volume = 22 μL − 5 μL = 17 μL

An aliquot of 17 μL should be used to obtain results that are meaningful within the standard curve produced in FACE.
A3.3.3. Preparation of FACE Gels

Gels appropriate for running FACE analysis were not readily available, and were therefore made in the lab. Two glass panes, one square and one with two pegs at the top (Figure A3.2A), were cleaned with alcohol. A thin layer of petroleum jelly was applied to both sides of two plastic spacers, and a spacer was placed on each side of the pegged pane (Figure A3.2B). The square pane was carefully placed on top of the pegged pane so that the spacers were between the two panes of glass. One side of the cassette was clamped and the other sealed with 3M™ Plastic Tape. The taped side was then clamped, and the other side was sealed with tape and re-clamped. The bottom of the cassette was wrapped with Parafilm® (SPI Supplies, West Chester, PA) to prevent unpolymerized gel from leaking. The cassette was then unclamped and placed in a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) apparatus (Figure A3.2C).

A resolving gel was made of 5.0 mL 40% Acrylamide/bis-acrylamide (Acryl/bis, 37.5:1), 500 μL 50% glycerol, 1120 μL 400 mM Tris Acetate (pH 7.0), 3.38 mL dH₂O, 70 μL 10% ammonium persulfate and 15.0 μL Temed. The resolving gel was poured into the glass cassette to a height of 1.5 cm below the top of the pegged plate. After polymerization of the resolving gel, a stacking gel was made of 1.0 mL 40% Acryl/bis (37.5:1), 250 μL 50% glycerol, 560 μL 400 mM Tris Acetate (pH 7.0), 440 μL 50% w/v polyethylene glycol (PEG), 2.75 mL dH₂O, 28 μL 10% ammonium persulfate and 7.5 μL Temed. The resolving gel was poured to the top of the pegged plate, and an 8-well comb was placed
between the two plates. After polymerization, the gel cassette was wrapped in plastic wrap and stored at 4°C until use (Figure A3.2D).

Figure A3.2: Creation of FACE gel. (A) Pegged pane of glass. (B) Glass with two spacers. (C) Taped gel in Bio-Rad apparatus. (D) Completed FACE gel.
Appendix A4. ELSIE Chamber Design Evolution

A4.1. Version 1: Initial Test of Piston/Crank Mechanism

Figure A4.1: Initial bioreactor design with motor and power supply. Arrows represent rotation of the motor causing lateral movement of the piston. The individual chamber components are labeled on the next page.
Figure A4.2: Close-up view of the chamber. A membrane was mounted to the top of section “D*” and affixed to the top of the piston. Fluid (represented in green) filled the chamber and the membrane, and the lateral movement of the piston forced the fluid to move up and down within the chamber. Design schematics of the lettered segments are depicted on the following pages. Note that the modular design of the chamber allowed flexibility of testing parameters (i.e., distance between tissue mounting locations) and as such, not all chamber pieces are represented in the photographs.
A - A
Top View
(symmetrical)
(make 2)

A - A
Side View

B - B
Top View
(symmetrical)
(make 1)
To view (symmetrical)
(make 1)

To view (symmetrical)
(make 2)
Top View
(symmetrical)
(make 1)

Bottom View
(symmetrical)
(make 1)

Side View
(rotated 90 deg)
A4.2. Version 2: Introduction of Filtered Cap

Figure A4.3: Bioreactor design with filtered cap closure. A membrane was mounted to the top of section “A” and affixed to the top of the piston using magnets (one each glued to the membrane and piston) to allow easy removal of the chamber from the piston/crank assembly. The designs of the lettered segments are depicted with the “Version 1” sketches; segment “C” was replaced with segment “C*” pictured below. As with the “Version 1” photo, not all chamber pieces are represented in the image.

C* – C*
Bottom View
(symmetrical)

C* – C*
Side View
(symmetrical)
Thread top (see bottle)

Figure A4.4: Front (top) and side (bottom) views of final bioreactor design using piston and silicone membrane assembly. After testing of this design, the crank/piston assembly was abandoned, and the number of segments used in the chamber was reduced to discourage contamination during assembly and media changes. The new segment schematics are presented on the following pages.
A - A  
Bottom View  
(symmetrical)

B - B  
Top View  
(symmetrical)

A - A  
Side View  
(symmetrical)

Thread top (see bottle)  
Cap to fit on top of stack

B - B  
Side View
C – C
Top View
(symmetrical)

C – C
Side View

D-D
Bottom View
(symmetrical)

D-D
Side View
(rotated 90 deg)
E-E
Bottom View
(symmetric)

E-E
Side View
(rotated 90 deg)
Figure A5.1: Finished bioreactor chamber design. The chamber was comprised of three hollow segments ("A", "B", and "C") into which the valve segment was mounted. The media (represented here by the green fluid) provided nutrients to the tissue. Valve attachment sites (inside chamber) are indicated by ANN=annulus and CH=chordae. Design schematics for the individual segments are presented on the following pages.
A - A
Bottom View
(symmetrical)

B - B
Top View
(symmetrical)
(outer holes ¼" or 1.250 BCD)

A - A
Side View
(symmetrical)
Thread top (see bottle)
Cap to fit on top of stack

B - B
Side View
C - C
Top View
(symmetrical)

C - C
Side View
(1/2" dia hole through top 1" of piece only
- ¼" holes go through entire piece)
References


