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Extracellular Matrix Organization, Structure, and Function

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1. Introduction

Heart valves are thin, complex, layered connective tissues that direct blood flow in one direction through the heart. There are four valves in the heart, located at the entrance to and exit from the ventricular chambers. The normal function of the heart valves is essential to cardiovascular and cardiopulmonary physiology. The opening and closing of valve leaflets at precise times during the cardiac cycles contributes to the generation of sufficiently high pressure to eject blood from the ventricles, and also prevents blood from flowing backwards into the heart instead of forward towards the systemic circulation and the lungs.

The ability of heart valves to open and close repeatedly, as well as the maintenance of the phenotypes of valvular cells, is made possible by their tissue microstructure, specifically the composition and orientation of extracellular matrix (ECM). The ECM within heart valves is primarily comprised of collagen, elastic fibers, and proteoglycans and glycosaminoglycans, although other ECM components are present as well. Taken together, the ECM performs several roles in heart valves. First, the ECM plays a biomechanical role: it is responsible for the unique mechanical behavior of the valve tissue and thus the overall valve function. Second, the valvular cells are bound to and surrounded by the ECM that is located within the immediate vicinity of the cell; this ECM is specifically known as the pericellular matrix (PCM). The PCM influences cell function by serving as a source of ligands for cell surface receptors, which transfers mechanical strains (experienced by the leaflet tissues) to the cells and initiates intracellular signaling pathways. Third, the various types of ECM have different innate mechanical behaviors, for example with collagen being stiffer than elastic fibers,

and a growing body of research has demonstrated that the phenotype and function of cells, including valve cells, are influenced by the stiffness of the substrate to which they are adhered [1]. These two latter functions of the ECM are considered to be mechanobiological as opposed to merely biomechanical since they affect cell behavior. Fourth, ECM has binding sites for growth factors and other soluble molecules found in the extracellular space, and thus the ECM serves as a reservoir for numerous bioactive factors that can affect cell behavior if they are released (such as when the ECM is degraded) or if a cell migrates close to this ECM reservoir.

Overall, the heart valve field is beginning to appreciate that there are numerous interactions between the ECM, valve cells, and valve mechanics. Given the complicated relationships that are being demonstrated, it is not surprising that alterations to the normal arrangement or composition of ECM, which frequently occur in valve disease, significantly and detrimentally impact valve function in a rather vicious cycle. For this reason, there has been an increasing effort to characterize the ECM within normal heart valves not only to elucidate valve biomechanics and mechanobiology, but also to obtain a solid basis for comparison with diseased valves.

This chapter will provide an overview of the ECM within heart valves, focusing on the aortic valve. After detailing the layered structure of the valve leaflets, each type of ECM component will be described and discussed in relation to its role in valve function and, in some instances, valve dysfunction.

2. The aortic valve leaflets are layered structures

Aortic valve leaflets consist of three main layers: the fibrosa, spongiosa, and ventricularis. Each layer has a distinct composition that aids in the normal mechanical and biochemical behavior of the valve. In diseased states, however, the composition of the layered structures can be altered compared to healthy tissues.

The fibrosa layer, close to the outflow surface, is mainly composed of collagen fibers with a small amount of elastic fibers, which are the major stress-bearing components and provide strength to maintain coaptation during diastole [2]. The circumferential alignment and orientation of collagen fibers contribute to the biological stress-strain relationship for aortic valve leaflets (Figure 1). This bilinear stress-strain curve represents the high extension with a low load and high elastic modulus with a high load applied [3]. Moreover, the particular architecture of collagen fibers contributes to the anisotropic mechanical behavior of the fibrosa layer. It has been found that the fibrosa is 4-6 times stiffer in the circumferential direction than in the radial direction [4].

The middle spongiosa layer of the leaflet predominantly consists of glycosaminoglycans and proteoglycans, particularly hyaluronan, which form a foam-like structure and bind a large amount of water. The spongiosa layer absorbs energy during compression, and facilitates the arrangement of collagen fibrils in the fibrosa and elastin in the ventricularis during the cardiac cycles [5].

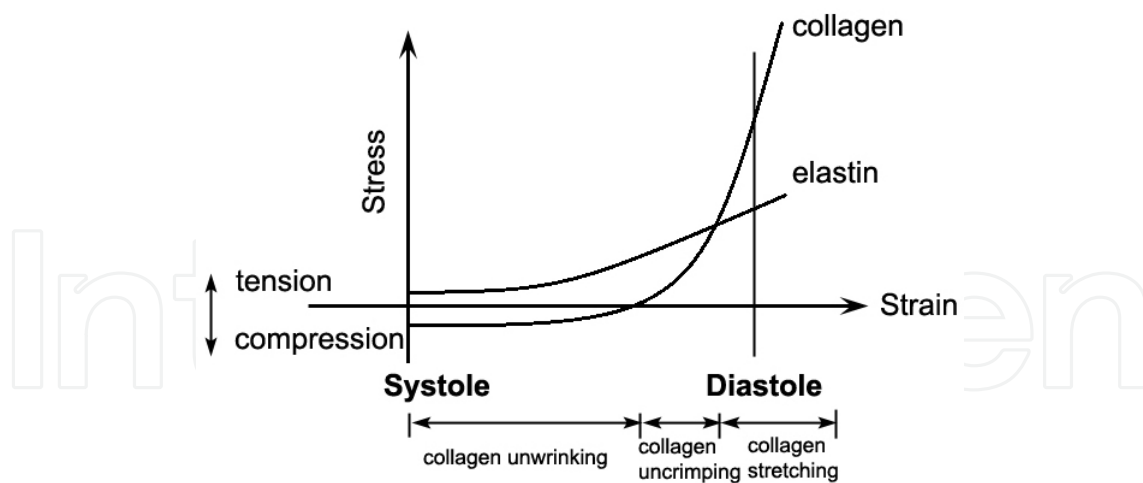


Figure 1. Schematic drawing of stress-strain relationships for collagen and elastin fibers during valve motion, reproduced with permission [3]

The ventricularis layer, close to the inflow surface, is rich in elastin with a moderate amount of collagen, which extends in diastole and recoils during systole [6]. The recoil of elastin restores the crimp of collagen fibrils and decreases the surface area of the stretched tissue from the closing phase [5]. The thickness of the three layers varies from the base to the free edge of the cusp [7].

It is worth noting that elastic fibers were found to span the whole leaflet, and connect or anchor three discrete layers together [6,8]. In addition, elastin provides intrafibrillar connections between collagen bundles in the fibrosa layer, whereas it forms a three-dimensional interconnected network in the spongiosa layer [8]. During unloading, the intrafiber elastin, which has high extensibility, helps the collagen fibers return to their wavy and crimped state [6]. These interconnected structures of elastic fibers anchor the discrete layers together, and prevent delamination, which therefore improves the continuity of material behavior of the whole leaflet. Table 1 summarizes the key ECM components in the layers and their major functions.

Location	Main Component(s)	Major Function(s)
Fibrosa	Collagen	Stress bearing
Spongiosa	Glycosaminoglycans and proteoglycans	Conferring flexibility, dampening vibrations from closing, and resisting delamination
Ventricularis	Elastin	Restoration of the wavy and crimped state of collagen fibers

Table 1. The key ECM components in each layer of the leaflet and their major functions

The structures of the leaflets described above provide the following critical functions [6,9–11]: 1) anisotropic mechanical behavior withstanding circumferential stress and extending radially; 2) bilinear biological stress-strain behavior allowing the leaflet to extend before bearing load in the closed phase; 3) elastic recoil to fully open the valve and restore the layer

structures for the next cycle. The particular shape of the leaflets and their unique macro- and micro-structures cause the anisotropic mechanical behavior along the circumferential and radial directions of the leaflets [9–11].

During the closed phase (diastole), the leaflets experience the maximum load. Collagen bundles in the fibrosa layer are the major stress-bearing component withstanding approximately 80 mm Hg pressure while the valve is closed and bulging back towards the ventricle [3]. Collagen fibrils are assembled into parallel collagen fiber bundles oriented along the circumferential direction in the leaflet, which are able to withstand such high tensile forces. However, collagen fibers cannot be compressed, making the alignment of collagen (waviness and crimping) important for decreasing the area of the stretched fibrosa layer. Although the collagen fibrils have limited extensibility (approximately 1-2% yield strain), the waviness and crimping allows the fibrosa to withstand roughly 40% strain under loading. Straightening of wavy fibers provides approximately 17% strain, whereas the crimping allows additional approximately 23% strain [6]. In addition, the strains of the cusps in the closed phase are anisotropic, i.e., the strains differ in the radial and circumferential directions [11].

During valve opening, cusps become relaxed through recoil of the elongated, taut elastin. This restores the wavy and crimped state of collagen fibers while decreasing the surface area of the cusps. The GAG-rich spongiosa layer facilitates the rearrangements of the collagen and elastic fibers during the cardiac cycle, dampens vibration from closing, and resists delamination between layers [6,8].

It is evident that normal aortic valve function is maintained, in part, by not only the composition but also the arrangement and orientation of ECM components, particularly collagen, elastin, and GAGs, in the leaflets. Furthermore, it is important to note that alteration of the composition [12] and mechanics [13] of ECM in the aortic valve leaflets was found in diseased conditions. In calcific aortic valve disease (CAVD), collagen bundles and elastin fibers in the fibrosa layer were disrupted and disorganized [14]; meanwhile, there was increased proteoglycan deposition [12]. Matrix metalloproteinases (MMPs) [14,15] and the potent elastase cathepsin S [16], which are produced by macrophages, contribute to this ECM remodeling. Moreover, ECM proteins related to bone, i.e., osteocalcin and osteonectin, were present in the calcified fibrosa layer [17]. These proteins promote mineralization, and their presence suggests the osteoblastic differentiation of valve interstitial cells (VICs).

In addition, excessive myofibroblast differentiation from VICs, leading to ECM accumulation and fibrosis, was influenced by remodeling of ECM in the fibrosa and facilitated by elastin degradation [18]. Furthermore, myofibroblast differentiation from VICs and calcification *in vitro* have been shown to be dependent on ECM composition [19].

Taken together, the macroscopic layered structure and the microscopic structure in each layer of the leaflets impart pronounced anisotropic mechanical behavior that allows the valve to open and close during a great number of cardiac cycles throughout life. These structures are tailored to fulfill the normal functions and maintain the homeostasis of the leaflets in a healthy condition. However, abnormal alteration of composition and mechanics of ECM in these structures may lead to calcific heart valve disease.

3. Collagen comprises a significant portion of the aortic valve leaflet fibrosa

Collagen is an essential component of the aortic valve's layered structure and is vital for maintaining the tissue's mechanical integrity. Mainly responsible for tensile strength, collagen is a strong load-bearing protein created and regulated by VICs. Although present throughout the entire valve, collagen is largely located in the fibrosa where it reduces high tensile stresses. In addition to its central role in valve mechanics, collagen acts as a regulator of VIC phenotype and calcification. Insight into the structure of collagen reveals its unique mechanical properties that support aortic valve function.

Fibrillar collagens are high strength fibers that comprise nearly all of the valve's collagen content. Fibrillar collagens are groups of 3 coiled polypeptide chains that assemble together in tightly packed parallel arrangements. These coils are approximately 300 nm long and join together in a staggered banding pattern with a periodicity of 67 nm [20]. The aortic valve is mainly composed of fibrillar collagen types I, III, and V. Each of these collagens is constructed from different types of alpha chains that govern the overall function of the collagen molecule. Together, these three collagen types work to provide the aortic valve with unique mechanical properties suited for maintaining unidirectional blood flow.

Synthesis of fibrillar collagen is an essential mechanism for maintaining the valve's mechanical integrity. This complex process originates within VICs and is completed in the valve ECM. Production of collagen begins with the intracellular creation of polypeptide alpha chains. There exist ten distinct polypeptide chains that consist of approximately 300 consecutive Gly-X-Y amino acid sequences flanked by small terminal domains. The secondary structure of collagen is created by folding alpha chains into a right-handed alpha helix with the peptide bonds localized at the backbone of the helix and the amino acid side chains facing outward. With slightly less than three residues per turn and a pitch of approximately 8.6 nm, glycine residues are positioned in such a way that the side chains of these residues allow for the formation of the helix. The single hydrogen side chains of these glycine residues allows for the formation of a triple helix structure [21].

The tertiary structure of collagen involves the formation of a left-handed triple helix constructed in the C to N direction. These triple helices exist as both homotrimers and heterotrimers of alpha chains. Collagen type III is a homotrimer of $\alpha 1(\text{III})$ while collagen type I is a heterotrimer of $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$. Additionally, collagen type V is a heterotrimer of $\alpha 1(\text{V})$ and $\alpha 2(\text{V})$. Known as procollagen, the tertiary structure molecule is approximately 1.5 nm wide and longer than 300 nm. For creation of the final supramolecular structure, the procollagen molecule is transported into the ECM for crosslinking and fibril formation. After modification in the extracellular space, procollagen is converted into tropocollagen, which undergoes fibrillogenesis where the triple helices are packed together into bundles. Crosslinking of the fibrils ensures the stability of the complex [21].

The arrangement of collagen fiber bundles is crucial to the proper functioning of the aortic valve. Collagen fibers are organized into multilayer structures linked by thin membranes

containing variably aligned collagen. Ranging from 10 to 50 μm in size, these membranes are believed to be much more extensible than the collagen fiber bundles they connect. These multilayer structures can easily slide past one another during valve movement, providing the combination of flexibility and tensile strength necessary for the required mechanics during valve opening and closing [22].

Collagen constitutes approximately 90% of the protein content of the valve insoluble matrix [23]. The vast majority of the valve's content is composed of collagens type I, III, and V. Together, these fibrillar collagens account for 60% of the valve's dry weight [24]. There is approximately 74% collagen type I, 24% collagen type III, and 2% collagen type V distributed throughout the valve [25–27]. Whereas collagen type I mainly exists in the fibrosa, collagen type III is expressed ubiquitously throughout all three layers [25].

Collagen fibers mainly function to reduce stress on the leaflets during systole and diastole. While elastin controls initial valve opening and closing, collagen fibers reduce peak stresses in the leaflet matrix by an estimated 60%. These fibers have an important role in stabilizing leaflet motion [28]. Throughout leaflet movement, collagen fibers adjust position to resist tensile forces. As transvalvular pressure increases, the ventricularis expands in the circumferential direction, causing collagen fibers to become highly aligned. This is believed to increase the cuspal stiffness of the valve during diastole and prevent overextension of the valve [29].

The heterogeneous distribution of collagen throughout the aortic valve provides high strength in areas of greater stress while also allowing the valve to achieve a large degree of flexibility. Within the fibrosa, the primary tensile load-bearing layer, collagen fibers are highly aligned in the circumferential direction, resulting in tissue anisotropy. The arrangement of these fibers corresponds to the direction of highest tensile stress. In contrast, the ventricularis endures smaller tensile forces involved with initial opening and closing of the valve [30]. In addition to circumferentially oriented collagen, the largest and strongest collagen fiber bundles are localized in the areas of greatest tensile stress along the lower part of the commissure and coapting regions [22]. This unique arrangement and positioning of collagen reduces high tensile loads on the valve while allowing flexibility to open and close.

Comparisons between the fibrosa and ventricularis indicate that the fibrosa has a greater elastic modulus in the circumferential direction but a similar elastic modulus in the radial direction. These mechanical differences are largely the result of the number of aligned collagen fibers in each direction. With fewer collagen fibers, the ventricularis is approximately half as stiff as the fibrosa in the circumferential direction. In the radial direction, however, each layer contains approximately the same amount of collagen fibers and has similar elastic moduli [31]. Taken together, the multilayer valve structure causes aortic valves to be less stiff and more extensible radially than circumferentially [32].

Collagen achieves high strength and extensibility with the aid of additional mechanisms that contribute to the valve's mechanical properties. These include collagen cross-links, collagen crimp, and layer corrugations. Collagen cross-links function to increase the strength of aligned collagen. In the circumferential direction, the number of collagen cross-links per col-

lagen molecule directly corresponds to the elastic modulus. However, this relationship does not apply to the radial direction, possibly due to the presence of elastin [31]. When there is no mechanical stress on the leaflet, the fibrosa exists as a number of folds in the radial direction known as corrugations. Large extensibility is achieved through these collagen corrugations in combination with collagen crimp. When stress is applied to the leaflet, initial extension is accomplished by straightening of the collagen crimp. Further stress causes the corrugations to unfold in the radial direction [30]. Together, collagen crimp and corrugations allow the fibrosa to extend further in the radial direction when compared to the circumferential direction.

Throughout the lifetime of the aortic valve, collagen synthesis and degradation are responsible for maintaining adequate valve strength and extensibility. Constant turnover of collagen allows the valve to adapt to regional changes in tensile strength. *In vitro* studies show that VICs respond to cyclic mechanical loading as a way to balance collagen synthesis and degradation. Cyclic stretch of valve leaflets stimulates VIC collagen type III production. In particular, the amount and duration of the stretching can have an effect on the amount of collagen produced [27]. Additionally, VICs in culture express collagen type I and collagen type III mRNA for new matrix synthesis [33]. New collagen production is localized to specific regions of the valve depending on the collagen type that is produced. Collagen type I synthesis occurs in the fibrosa around, but not within, areas of mature collagen. Collagen type III synthesis, however, mainly occurs outside of the fibrosa [34]. Collagen degradation is also an important function of VICs and acts as an essential control to collagen production. Studies have shown that VICs seeded into collagen scaffolds express MMPs that degrade the scaffold in a heterogeneous manner [33]. Thus, VICs continuously regulate the mechanical properties of the surrounding ECM through collagen synthesis and degradation.

Aside from its mechanical functions, collagen has been shown to regulate VIC phenotype and calcification potential. *In vitro* studies were unable to induce calcification in VICs cultured on collagen proteins in standard media. It is believed that collagen actively inhibits VIC calcification [19]. Other studies have shown that scaffold collagen content also affects VIC proliferation. Specifically, one study reported that VICs adhered and spread on collagen surfaces but were not able to proliferate [35]. Another study showed that VIC proliferation decreased on scaffolds containing higher collagen content [36]. An *in vitro* study indicated that matrix stiffness regulates VIC differentiation to myofibroblastic or osteogenic phenotypes in calcific conditions [37].

4. Elastic fibers comprise a significant portion of the ventricularis layer of the aortic valve leaflets

Elastic fibers are macromolecular assemblies of several different molecules. The majority of the elastic fiber consists of elastin, an insoluble protein generated by lysyl oxidase crosslinking of soluble tropoelastin monomers (approximately 70 kDa). The elastin tends to be located in the inner core of the elastic fiber and is surrounded by a fine mesh of microfibrils.

These microfibrils are predominantly fibrillin-1, but to a lesser extent Fibrillin-2. Microfibril associated glycoproteins (MAGPs), fibulins, and other proteins are also present in the microfibrillar sheath [38]. At the light microscope level, one can observe the fine elastic fibers by histological staining with Voerhoff's stain or related methods, but when tissue sections are viewed with transmission electron microscopy, there is a clear distinction between the electron-dense elastin core and the microfibrillar sheath [39].

The unique mechanical behavior of the elastic fiber is conferred primarily by the mechanical function of elastin and fibrillin. Crosslinked elastin is remarkable for its ability to undergo high amounts of deformation when subjected to small amounts of load, as well as to recoil back to its original dimensions, when the load is removed, with very little loss of energy. Fibrillin-1, the most widely studied of the microfibrillar components, is also highly extensible. Fibrillin and the other microfibrillar components also coordinate, in a complicated manner still under investigation [38], to aid in the cross-linking of tropoelastin and assemble the final elastic fiber. Interestingly, fibrillin is not always associated with elastic fibers. Fibrillin can often be found by itself, in which it may independently function as a mechanical, load-bearing but highly extensible scaffold [40]. Numerous domains in fibrillin exist for binding integrins, heparan sulfate proteoglycans, and growth factors, which point to substantial roles for alone fibrillin and mature elastic fibers in mediating cell signaling and adhesion [41].

In semilunar heart valves, elastin is found primarily within the ventricularis layer on the inflow side of the leaflet, but is also abundant in the middle spongiosa layer. A thin, frequently imperceptible layer of elastic fibers, the arterialis, is found atop the collagen-rich fibrosa layer. These elastic fibers merge with the intima of the adjacent arterial wall, but the overall function of the arterialis has not been well characterized [42].

In the ventricularis, elastic fibers are present in dense and continuous sheets across the whole of the leaflet. These fiber sheets are the most significant contributor to the mechanical properties of the ventricularis [6,30], which can be demonstrated when all ECM components but elastin are removed when using NaOH digestion. After this treatment, the digested ventricularis matches the mechanical behavior of the undigested ventricularis radially, indicating a strong presence of elastin in the radial direction [30]. The elastic fibers within the ventricularis undergo considerable, continual stretch from the initial stage of closure, when blood flow vortices are starting to push the leaflets towards the valve orifice, to the final coapted position of the leaflets. The extension of these elastic fibers accommodates the unfolding of the fibrosa layer, which is normally corrugated in the unloaded position. During this unfolding process, the elastic fibers are bearing the loading of the entire leaflet [43]. Even at high strains, when the collagen in the fibrosa is considered to dominate mechanical properties, the elastin in the ventricularis still plays a significant role. This effect was shown when separated ventricularis was preloaded to mimic its intact configuration; the separated ventricularis was shown to bear load before the separated fibrosa [44]. It has been speculated that this response acts as a safety mechanism to prevent radial overextension of the aortic valve leaflet. Then, when the pressure across the valve is reduced, the elastic fiber sheet in the ventricularis recoils and retracts the leaflets back toward the annular attachment to the

arterial wall, a process that involves the re-folding of the corrugations in of the fibrosa. This action restores the original shape and orientation of collagen quickly and consistently to prepare for the next cycle of valve closing. Although the elastic sheet in the ventricularis has fibers that are also oriented circumferentially as well as radially, elastin does not appear to play an important role in the mechanical behavior of the leaflet in the circumferential direction. Valve leaflets exposed to cyclic circumferential stretch and cultured under flow for 48 hours maintained a constant concentration of elastic, suggesting that elastogenesis was not activated during the duration of stretch [45]. However, it is speculated that connections between the elastic fiber and collagen networks facilitate the radial extensibility of the ventricularis layer and the overall leaflet [43]. There are also some elastic fibers in the fibrosa, which surround and connect the collagen fibers, thus preserving collagen crimp and the characteristic corrugated nature of the fibrosa [6,46,47].

The elastic fiber structure in the spongiosa has been characterized much less than in the ventricularis, partly due to the difficulty in isolating its structure from the rest of the leaflet [30]. This elastic structure, however, has been observed during microdissection separating the leaflet [30,48], with scanning electron microscopy (SEM) [6,46], micro-computed tomography (micro-CT) [6], immunohistochemistry (IHC) [25], and autofluorescence imaging [49,50], which all have shown a fine elastic fiber network emanating from the ventricularis and connecting to the fibrosa. We have recently reported that the thickness of this elastic fiber network in the spongiosa is significantly thicker in the hinge and coaptation region than in the belly region of the aortic valve leaflet [8]. We also found two distinct patterns of spongiosa elastic fibers within the leaflet: (i) a rectilinear pattern in the hinge and coaptation region; and (ii) a radially oriented stripe pattern in the belly. Overall, it is believed that the elastic fibers in the spongiosa contribute to valve function in three ways. First, they connect the elastic fibers in the ventricularis to collagen in the fibrosa, which allows coupling of the mechanics of the two layers and matrix components, while using elastic recoil to exert preload on the fibrosa. Second, they distribute stress between collagen and elastic fibers, particularly at low strains. Third, they passively allow relative movement and shear between the outer layers [5,6,48].

Given the presence of a thick, rectilinearly-arranged structure of elastic fibers in the spongiosa of the hinge and coaptation regions, it is speculated that this elastin structure plays a role in leaflet flexure [5,30]. Flexure of the leaflet towards the outflow direction compresses the fibrosa and applies tension to the ventricularis. Rather than undergoing compression, however, the fibrosa may attempt to buckle separately from the leaflet, thereby exaggerating its corrugated configuration. The leaflet would subsequently bend at the troughs of this corrugation, where the second moment of inertia would be locally reduced, albeit temporarily. Buckling would only occur with shearing between the fibrosa and ventricularis, which is allowed by both the compliant elastic fibers in the spongiosa connecting the two outer layers as well as by GAGs in the spongiosa lubricating the outer layer movement [5,30,51,52]. Recoil from the elastic fibers in the spongiosa would then return the fibrosa to its original configuration so it could undergo the next cycle of loading [5,30]. At the hinge, where bending occurs in the opposite direction, it is speculated that the elastic fiber-rich ventricularis com-

presses readily without buckling, most likely due to the tensile preload already exerted on the ventricularis, but that leaflet deflection may be limited by the stiff fibrosa, which would not allow the leaflet to bend [4,53]. Limited flexure at the hinge would allow the leaflet to absorb pressure from reverse blood flow in diastole, but prevents distention of the leaflet. Thus, our finding of a thicker spongiosa and elastic fiber structure in flexural regions provides evidence of a significant role for elastin in flexure [8]. In addition, the thick network of elastic fibers that we have observed in the spongiosa of the coaptation region may play a role in dampening vibrations that result from valve closing [5].

5. The middle layer, the spongiosa, is comprised mainly of glycosaminoglycans and proteoglycans

Glycosaminoglycans and proteoglycans (GAGs and PGs, respectively) comprise a significant part of the aortic valve leaflets. PGs and GAGs are mainly found in the spongiosa layer of the valve, located between the ventricularis and fibrosa, where they play a vital role in maintaining normal valve function. Previous work has shown that GAGs and PGs serve to not only provide mechanical support to the tissue but also aid in the normal biological functions of the valve [54]. Therefore, it is crucial to fully understand the function of GAGs and PGs in both the normal and possible diseased states of tissues.

GAGs are composed of long and unbranched chains of repeating disaccharides, which consist of a hexosamine and either, depending on the GAG type, uronic acid or galactose. There exist the following families of GAGs with each group being defined by its composition: hyaluronan (HA), heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS) (Table 2) [55–58].

GAGs are primarily formed in the lumen of the Golgi apparatus. The formation process occurs, except in the case of HA, with glycosyltransferases alternatively adding a uronic acid or galactose with a hexosamine to a protein core. The attachment to the protein core varies based on the GAG type. Heparin, HS, CS, and DS are attached to a serine residue, connected to the protein core, via xylose. KS can attach to the protein core either by an asparagine residue at the N-terminus or linked to serine or threonine at the O-terminus. HA does not attach to a protein core. It is synthesized by the addition of sugars to the non-reducing termini of the forming polysaccharide by HA synthase, without a protein backbone. In all cases, modifications can be made to the resulting polysaccharides. Two noteworthy changes include sulfation of the chains and epimerization of the uronic acid. These changes do not occur, however, with HA. Sulfation and epimerization modifications can give a more distinct characteristic to the GAG chains. The epimerization of the uronic acid of CS leads to the production of DS. Epimerization also occurs on heparin and HS. Sulfation can occur in CS, DS, heparin, HS, and KS. N-sulfation takes place in heparin and HS; whereas, O-sulfation can take place in heparin, HS, CS, and DS. In addition to epimerization and sulfation, phosphorylation of the xylose linkage—occurring among CS, DS, heparin, and HS to their respective protein cores—can take place [54,58,59]. Through gel electrophoresis, it has been found that

HA comprises approximately half of the total GAG content in aortic valves [60]. It is important to note that all GAGs, with the exception of HA, exist *in vivo* as components of PGs.

Glycosaminoglycan	Uronic acid	Galactose	Hexosamine
Hyaluronan	Glucuronic	-	N-acetylglucosamine
Heparin	Glucuronic Iduronic	-	N-acetylglucosamine
Heparan sulfate	Glucuronic Iduronic	-	N-acetylglucosamine
Chondroitin sulfate	Glucuronic	-	N-acetylgalactosamine
Dermatan sulfate	Glucuronic Iduronic	-	N-acetylgalactosamine
Keratan sulfate	-	+	N-acetylglucosamine

Table 2. List of glycosaminoglycans and their composition [59]

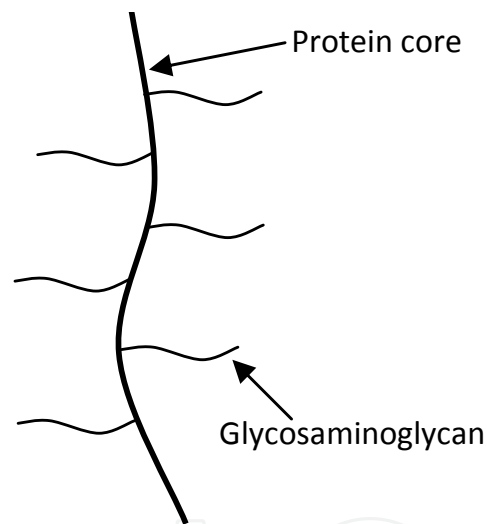


Figure 2. Proteoglycan structure

PGs are formed when GAGs are added to a protein core through a covalent linkage (Figure 2). During PG synthesis, a protein core moves from the endoplasmic reticulum of a cell to the Golgi apparatus, where GAGs are then added to the protein core [55]. PGs can be found in intracellular organelles, on the cell surface, and in the extracellular matrix (ECM) [59]. PGs found in the ECM can be divided into three categories: PGs found within the basement membrane, hyalactans or PGs that interact with HA and lectins, and small leucine-rich PGs (SLRPs) or PGs that contain a leucine motif and have considerably low molecular weights. These PGs can be further classified by the type of protein backbone they contain, as well as the amount, type, and sulfation pattern of the GAGs that are attached to the backbone. More than thirty PGs have been characterized [61]. For example, well-characterized PGs that exist in cardiovascular tissue include decorin, biglycan, and versican. Decorin and biglycan have a core protein

size of 40 kDa and are a part of the SLRP family of PGs. They contain CS and DS GAG chains [61]. Versican is a large, chondroitin sulfate proteoglycan. It interacts with HA, and therefore is a hyalectan PG [62]. Other significant PGs in mammalian tissues include perlecan—a basement membrane protein that contains HS and CS, aggrecan—a hyalectan containing CS, and syndecans—a family of cell surface heparan sulfate proteoglycans containing HS and CS [61].

GAGs, and in turn PGs, have a significant role in aortic valve tissue behavior. GAGs have been shown to enhance the viscoelastic properties of the valve leaflets through binding of water molecules [63]. The sulfation and carboxylation on the GAGs make them highly negatively-charged polysaccharides. This negative charge draws in water molecules. Once the tissue becomes hydrated, it acts like a sponge for the valve leaflets. As noted previously, GAGs and PGs are highly abundant in the middle layer of the aortic valve leaflet. One of the main functions of this cushioned layer, the spongiosa, is to provide a barrier between two other layers, the ventricularis and fibrosa, of the valve. This barrier allows for proper shearing between the layers as well as compressibility of the leaflet without compromising the leaflet's overall structural or biological integrity when mechanical stimuli are applied to aortic valve leaflets [63,64]. The mechanical competency that GAGs provide is crucial to the aortic valve leaflets. The aortic valve leaflets serve to ensure unidirectional blood flow from the left ventricle to the aorta. In order to guarantee normal blood flow, the leaflets must open and close properly. Therefore, the flexibility that GAGs provide to the leaflet is crucial to the normal valve's function. In addition, the space that GAGs occupy and form in the matrix serve to organize other molecules within the structure. The structure and hydration that GAGs provide also allow for biological cues to occur within the valve. Moreover, GAGs are known to aid in cell migration, proliferation, act as receptors for signaling molecules, bind growth factors, and serve in the recruitment of various cell types [54].

It is believed that GAGs/PGs likely play an active role in aortic valve tissue disease. Research has shown regional variation of decorin, biglycan, versican and HA in, near, and distal to regions of calcification in diseased aortic valves, suggesting the occurrence of remodeling in the tissue during an unhealthy state [65]. In addition, although the exact causation of calcific aortic valve disease is unknown, it is speculated that it may be due, at least in part, to an inflammatory process [17]. Interestingly, GAGs are thought to play an active role, quite often in the case of cellular injury, in many inflammatory processes for a variety of cell types and have shown to alter in structure and localization in these processes [66]. In addition, some researchers believe that lipid binding due to the unique structure of GAGs may be critical to the accumulation of lipids in calcified aortic valves, a characteristic that is hypothesized to aid in valvular calcification [67]. Although the specific mechanisms underlying calcific aortic valve disease are not quite understood, the complex nature and distinguishable differences of GAGs in both healthy and diseased tissue give rise to the possibility of GAGs being a key factor in valve calcification.

GAGs are very complex disaccharides that highly dictate the behavior of PGs. These polysaccharides are vital in maintaining mechanical, structural, and biological integrity of the aortic valve. Although there is growing interest in further elucidating the role of GAGs in

healthy tissues, the exact role of GAGs in diseased aortic valves needs further investigation, as well.

6. Minor ECM components in heart valves also play significant roles in normal valve function and in pathological states

The extracellular matrix of heart valves contain a number of minor components that perform a variety of functions. They are important in valve development, function, and pathology. The study and further characterization of these minor ECM components not only facilitates the development of targeted therapies but would also aid in the microenvironmental mimicry needed for potential tissue engineering applications.

Vitronectin is a glycoprotein that is approximately 75 kDa in size and is present in both serum and the ECM as an adhesive substrate [68]. It is involved in the inhibition of the complement system [68] and is associated with the regulation of hemostasis [69]. Vitronectin also promotes cellular attachment to ECM and is involved in cellular migration [68]. This glycoprotein, along with fibronectin, is found in moderate amounts in aortic, pulmonary, and mitral valves, localizing around valve endothelial cells (VEC) on the inflow layer [25]. In addition, both fibronectin and vitronectin have been shown to associate with collagen fibers in chordae tendinae [70].

Fibronectin is a dimer glycoprotein which consists of two ~250 kDa subunits and is a component of the extracellular matrix [71]. There are many various isoforms of fibronectin, which is the result of alternative mRNA splicing [71]. In addition to being an insoluble ECM component secreted primarily by fibroblasts, soluble fibronectin is also found in the plasma [71]. Fibronectin acts by binding to integrins, collagens, fibrin, and heparin sulfate proteoglycans [71], which allows it to participate in wound healing [72,73] and act as a critical player in embryogenesis [74]. Although not a major ECM component in heart valves, valve interstitial cells (VIC) secrete fibronectin in response to valve damage, providing a means for cell migration [75].

Additionally, fibronectin, along with osteonectin and periostin, confers stiffness to the fibrosa layer [76]. Periostin is a component of the ECM that acts as a ligand for α -V/ β -3 and α -V/ β -5 integrins and is known to support adhesion and epithelial migration [77]. It is present in the extracellular matrix of several types of tissues and is upregulated in several types of cancers [78]. Recombinant periostin has been shown to promote cardiomyocyte proliferation and angiogenesis after a myocardial infarction [79]. It has been shown previously that periostin plays a role in murine embryonic valve development and remains present in the valves throughout the lifespan even when there is no pathological calcification [80]. A recent study involving chick cardiac development suggests that the presence of periostin in the developing heart may provide a means of organizing other ECM molecules in order to facilitate early epithelial-mesenchymal transition (EMT) [81]. However, the overexpression of periostin and osteopontin can lead to valve calcification.

Osteopontin is a phosphoprotein, meaning that it contains chemically bound phosphoric acid. Originally found in bone, it also contains the arginine-glycine-aspartate (RGD) motif more commonly attributed to fibronectin and is also a constituent of ECM in other tissues [82]. It is secreted by various tissues such as fibroblasts [82] and immune cells, including dendritic cells, macrophages, and neutrophils [83]. Osteopontin is known to interact with various surface receptors that make it a crucial player in bone remodeling [84], wound healing, inflammation, and immune responses [83]. It is also known to be involved in vascular remodeling during endothelial injury [82]. Osteopontin is present in valves calcified as a result of disease as well as in calcified bioprosthetic heart valves [85]. The calcification process of aortic valves closely resembles osteoblast differentiation in regards to expression of genes characteristic of bone formation, such as osteopontin and osteocalcin [86].

Osteocalcin is a small, non-collagenous protein that is considered a late-stage marker for bone formation and is one of a small group of proteins that are osteoblast-specific [87,88]. It is present in general circulation [87] and its capacity for binding hydroxyapatite and calcium suggests that it is largely involved in mineral deposition [88], but it also has recently been shown to act in a hormone-like manner by enhancing insulin secretion [87]. Its traditional role as a product of bone indicates that valve calcification may actually be a result of active bone formation in the valve tissue [86]. This bone formation may be the result of VEGF secretion by endothelial cells during neoangiogenesis occurring in response to inflammation, as seen in rheumatic valve calcification [89]. Additionally, increased serum levels of osteocalcin were shown to be indicative of aortic valve disease in patients [90].

In addition to the matrix proteins, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are also found in heart valves. They assist in tissue development and remodeling and can be used as indicators of disease. It is also believed that the ECM degradation resulting from MMP activity serves to release growth factors bound to ECM components and thus alter the microenvironment chemically, as well as structurally [91]. Calcified leaflets from stenotic valves have been shown to express levels of MMP-2 that are similar to those of normal valves but express higher levels of MMP-3, MMP-9, and TIMP-1 [14]. MMP-1, produced by activated myofibroblasts and macrophages, is also prevalent in calcific aortic valve stenosis and may be related to high TNF- α levels resulting from inflammation [92].

7. The basement membrane supports valve endothelial cells and acts as a barrier between circulating blood and subendothelial components

The basement membrane is a myriad of proteins, proteoglycans, and glycoproteins that not only supply a substrate to anchor the valve endothelial cells, but also has a large array of biological activities that regulate spatial organization, sequester growth factors, modulate angiogenesis and migration, and regulate the diffusion of nutrients through it towards the underlying valve interstitial cells [93]. The major constituents of the basement membrane are laminin, perlecan, collagen type IV and VIII, nidogen, and the glycoprotein SPARC (secreted protein, acidic and rich in cysteine). Each of these constituents play a role in the overall func-

tion of the basement membrane. In addition, MMPs contribute to the biological activity that occurs within the basement membrane. Understanding basement membrane composition and behavior, during both healthy and diseased states of the aortic valve, may lead to a better understanding of calcific aortic valve disease.

Laminins belong to a family of heterotrimeric glycoproteins composed of combinations of α , β , and γ chains that form a cross-like structure averaging between 400-900 kDa in size [94,95]. Laminins play an integral role in the formation of the supportive ECM network. The unique cross-like shape allows laminin molecules to bind with neighboring laminins and ECM via the three short chains, and use the long alpha chain as a cell anchoring site [96]. In addition to their structural contributions to the basement membrane, laminins are essential for proper biological activity. These glycoproteins have been shown to promote cell adhesion, migration, differentiation, and maintenance of cellular phenotype [94,97,98]. Dysfunction in laminin expression has been linked to diseases with improper tissue formation such as muscular dystrophy, epidermolysis bullosa, and various nephritic syndromes [94,99].

Although laminin is not as ubiquitous as collagen, this basement membrane component has been highly investigated as an ECM substrate for *in vitro* cultures. However, this glycoprotein may influence valve cell types differently. *In vivo* and *in vitro* studies have shown that laminin interacts with endothelial and epithelial cells, and can help maintain physiological functionality of the cells [97,100,101]. However, VICs cultured on laminin have been found to support high quantities of calcific nodule formation in the presence of TGF- β , when compared to subendothelial ECM components collagen type I and fibronectin [19,102]. The various regions of laminin protein have been reported to mediate specific cell responses. The G-domains of laminin α chains are associated with heparin binding and cell adhesion, whereas regions along the laminin β chains promote cell differentiation [98,100,101]. The peptide sequence YIGSR from the laminin β -1 chain has been shown to promote endothelial cell adhesion and proliferation, however, it also influences other cell types including smooth muscle and tumor cells [98,101]. VICs cultured on YIGSR were also shown to promote calcific nodule formation, although less than those seeded on fibronectin derived RGDS peptides. However, when the 67-kDa laminin cell receptor was blocked, the YIGSR seeded VIC cultures significantly increased in nodule formation and gene expression for various myogenic and osteogenic markers, suggesting that disruption in laminin binding may be linked to valve calcification [103]. IKVAV, another peptide sequence derived from the laminin α 1-chain, has been linked to promoting angiogenesis, cell migration and spreading [97,98,104]. Though most work with this peptide has been done with endothelial and tumor cells, its ability to promote angiogenesis may also be a future area of interest in studying how angiogenesis mediates valve tissue calcification. Furthermore, laminin influence on cell activity varies between cell types, and may promote VIC activation and tissue calcification in diseased states.

Perlecan (Pln) is one of the more abundant heparan sulfate proteoglycans and is found in several tissues including in the endochondral barrier in bones [105]; however, it is primarily localized in vascular basement membranes. It has a major role in regulating the development of blood vessels, the heart, cartilage, and the nervous system. Physiologically, perlecan plays a prominent role in regulating cellular proliferation, differentiation, organization, and

mediating inflammation [106]. Perlecan derives its functionality from five protein subdomains which share their sequence homology with several other proteins [107]. Domain 1 contains an SEA (Sperm protein, Entek kinase, Agrin) module and three SGD (Ser-Gly-Asp) tripeptide sequences to which three heparan sulfate (HS) glycosaminoglycans attach. These HS can bind and sequester several important growth factors for determining endothelial quiescence in a process known as matricrine signaling. The SEA section is unique to perlecan, and it has no known function other than to influence the O-linked glycosylation of the SGD domain. Interestingly, it has been shown that several factors that determine the activity of these sugar chains vary greatly by the cell source that is producing them [108]. These factors can include the ratio of heparan sulfate to chondroitin sulfate, the length of the chains, and the sulfation level of the chains which all affect how the chains modulate the bioactivity of nearby growth factors. Domain II contains 4 low-density lipoprotein receptor sequences and one immunoglobulin-like repeat. Domain III contains three laminin-like domain modules and eight epidermal growth factor-like repeats. Domain IV, the largest domain, contains many N-CAM-like Ig repeats. Domain V has been demonstrated to be the major cell-binding domain of perlecan due to the laminin and agrin homologies that it contains. Domain V can also be glycosylated, which can contribute along with domain I to the matricrine signaling capabilities of perlecan, which could potentially contribute to the development of CAVD [109].

Matricrine signaling occurs when the ECM modulates cell behavior by controlling the local levels of growth factor concentrations by sequestering or releasing them when the underlying matrix is intact or degraded, respectively [109]. Proteoglycans, like perlecan, and their GAG chains are the major sites for matricrine signaling due to their heparan sulfate and chondroitin sulfate chains electrostatically binding free growth factors. Their role in the pathology of CAVD is widely unexplored despite their presence in normal valves and their increased production in diseased valves [67]. It is known that PGs and GAGs play an integral role in the progression of atherosclerosis via sequestering of inflammatory molecules and lipids [110–113] and mediating angiogenesis into the vessel supplying an entry way for additional inflammatory entities. Both of these factors are seen histologically in CAVD, but their role is merely speculative at this moment.

Collagens in the basement membrane can form lateral, axial, and linear connections with surrounding ECM. Of the basement membrane collagens, collagen IV (COL IV) is the most abundant and essential for network formation. Only found in basement membrane tissues, COL IV molecules are approximately 400 kDa, and composed of two α_1 , and one α_2 [115–117]. COL IV proteins have many biologically active domains that can influence specific cellular responses, as well as have specific affinities to other molecules such as BMP-4, fibronectin, Von Hippel Lindau protein, and factor IX [115,117]. Mapping of COL IV protein reveals 3 major integrin motifs that are located in strategic regions to promote cell activity or protein degradation when activated [115]. During angiogenesis and tumor invasion, COL IV is degraded by MMP-2 and MMP-9 enzymes to allow for cell migration and infiltration into the matrix. Studies have found that the cleavage sites also overlap with many integrin binding domains such as $\alpha_1\beta_1$, resulting in the availability of $\alpha_v\beta_3$ integrin binding sites known to

promote neutrophil binding [115,118]. Collagen IV networks are highly adhesive to all cells types except erythrocytes [115,119]. Furthermore, cell binding has been found to be enhanced in the presence of various ECM molecules such as perlecan, SPARC, and von Willebrand factor (vWF) [115,118,119]. Interestingly, COL IV also has numerous anti-angiogenic domains that are activated after MMP degradation at the non-collagenous (NC) 1 domain, thereby limiting angiogenesis or migration of endothelial and tumor cells [115]. The changes in COL IV bioactivity depending on the domain region and cleavage state can greatly affect the functionality of surrounding cells. Dysfunctional COL IV expression or mutations in the heterotrimer formation have been found to be extremely detrimental and cause matrix disorders such as Goodpasture's syndrome or Alport syndrome [94,115]. Therefore, additional studies should be done to investigate how the highly bioactive COL IV meshworks may promote the onset of calcification in valve tissues.

COL VIII has also been found to play a network forming role, maintaining the sheet-like structure ECM, while sequestering various integrin binding sites and growth factors. COL VIII is smaller than COL IV, and can form tetrahedral and hexagonal assemblies [117,118]. Though work on COL VIII in regards to valve tissues has been limited, vascular basement membrane studies have found that COL VIII plays a large role in interacting with subendothelial cells such as smooth muscle cells and fibroblasts. In vitro, COL VIII promotes fibroblast proliferation and migration [114]. Furthermore, COL VIII may be linked to atherosclerosis, a pathology similar to CAVD, as its expression in cells is upregulated during vessel injury [114,120]. This collagen has even been found to interact with elastic fibers in liver tissues, suggesting it may have a bridging function between the basement membrane components and subendothelial ECM [118]. Therefore, COL VIII could play an integral role in mediating valve interstitial and endothelial cell communication. Recent studies have found after enzyme cleavage at the NC1 domain, the resulting C-terminal fragment known as vastatin will prevent endothelial cell proliferation and induce cell apoptosis [120]. While some work has investigated using vastatin as an anti-angiogenic agent, further studies are needed to elucidate how it may affect the functionality of surrounding cells and ECM, especially in older valve tissue.

Similar to perlecan, nidogen is a 150 kDa glycoprotein that has sequence homologies with other basement membrane proteins. It consists of two amino (G1, G2) and one carboxyl (G3) terminal globular domains that are connected by a rod domain composed primarily of endothelial growth factor repeats [121]. Nidogen binds collagen type IV, perlecan, and laminin. This binding contributes to the hypothesis that nidogen is important in basement membrane assembly; although some recent animal studies have demonstrated that nidogen may not be necessary for basement membrane formation [121]. The role of nidogen in CAVD is unexplored, but it may play a role in maintaining valvular basement membrane functionality by regulating infiltration of inflammatory agents [93].

SPARC positive neovascularisation is a documented histological change in CAVD [122]. Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin, is a small basement membrane protein. It interacts with cells, binds to other members of the basement membrane, growth factors, various proteases, and is found in newly developing neovessels.

Intact SPARC protein inhibits cellular proliferation and has anti-angiogenic activity *in vitro* [123]. However, enzymatic degradation of SPARC can release matricryptic fragments with the KGHK motif that may induce angiogenic activity both *in vitro* and *in vivo* [124]. SPARC has been observed lining blood vessels in early to mid stage calcified valves suggesting the presence of a fully formed basement membrane lining these vessels [125]. However, the presence of the other constituents of the basement membrane is merely speculative at this point as the studies investigating their presence during CAVD have not been completed.

8. Summary

In conclusion, the last several years have witnessed significant acceleration in the number of studies characterizing specific types of extracellular matrix in heart valves, although there is still much to be learned. The basement membrane of heart valves, and its role in regulating valvular endothelial cell function, are particularly understudied. The broad scope of cell-matrix and matrix-matrix interactions within heart valves, and how these are regulated by the local, dynamic signaling environment, is another subject that merits further investigation. We expect that insights gained from these research endeavors will lead to novel treatments for valve diseases in the future.

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