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Human heart valves: advanced physiology and structure- function characterization for tissue engineering applications

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NOMENCLATURE

AB	Alcian Blue
AC	Against Curvature
AML	Anterior Mitral Leaflet
APL	Anterior Pulmonary Leaflet
ATL	Anterior Tricuspid Leaflet
ATV	Atrioventricular valve
AV	Aortic valve
CD	Circumferential Direction
CT	Chordae Tendineae
CVD	Cardiovascular diseases
ECM	Extracellular matrix
FBD	Fibers bundle Diameter
GAGs	Glycosaminoglycans
GBD	Global Burden of Diseases, Injuries, and Risk Factors
H&E	Hematoxylin and eosin
HV	Heart Valve
LCL	Left Coronary Leaflet
LMIC	Low and middle-income countries
LPL	Anterior Tricuspid Leaflet
MT	Masson's Trichrome
MV	Mitral valve
NCL	Non-Coronary Leaflet
OI	Orientation Index
PBS	Dulbecco's Phosphate Buffered Saline
PML	Posterior Mitral Leaflet
PR	Picrosirius Red
PTL	Posterior Tricuspid Leaflet
PV	Pulmonary Valve
RCL	Right Coronary Leaflet
RD	Radial Direction
RPL	Right Pulmonary Leaflet
SEM	Scanning Electron Microscopy

SLV Semilunar valve
STL Septal Tricuspid Valve
TEHV Tissue Engineering Heart Valve
TV Tricuspid Valve
VVG Verhoeff Van Gieson
WC With Curvature

ABSTRACT

The four human heart valves (HVs) exhibit substantial physiology, reflected in their structural and mechanical properties. HV diseases remain among the most challenging clinical issues in cardiovascular medicine. The mechanical and structural properties of native human HVs, such as tensile and flexural stiffness, anisotropy, or even simpler factors such as leaflet thickness are largely neglected in the design of commercially available prosthetic devices. Furthermore, these properties are frequently poorly characterized, with data primarily derived from animal or cadaveric tissues. This gap in knowledge in valve physiology and prosthetic devices has increasingly made human cardiovascular tissue characterization a priority in biomechanics and tissue engineering.

The purpose of this study is to fully characterize human HVs structure at both organ and tissue scales and to compare these measurements with equivalent data obtained from porcine and ovine tissue. To achieve this aim, fresh human samples were obtained from heart-transplanted patients and tested within 24 hours from the tissue explant. At the organ scale, thickness topology for the mitral, aortic, tricuspid, and pulmonary (MV, AV, TV, PV) HVs was measured and compared. Quantitative histology and collagen bundle diameter were analyzed and compared at the microscopic scale.

In order to measure collagen fiber bundle diameter a novel decellularization protocol, specific for HV leaflets, was developed. Scanning electron microscopy (SEM) analysis was subsequently performed on the decellularized leaflets, enabling quantification of fiber angle distribution and diameter using a modified, custom-made algorithm in conjunction with ImageJ software.

Functional analyses were conducted through biaxial mechanical tests and three-point bending tests. The bending rigidity (EI) of native valve leaflets corroborated the biaxial response, showing that the MV and TV displayed greater bending rigidity (EI) than the outflow tract valves, the AV, and the PV.

This study highlights structural and functional differences in fresh human HVs. Establishing a detailed database of HV structural and functional parameters is critical for advancing valve engineering and incorporating biomimicry into the design of leaflet, supra-, and sub-valvular

apparatuses. This comprehensive resource will aid in advancing the biomimetic-engineered tissue paradigm by providing detailed descriptions of HV structural and biomechanical parameters that can be replicated using current or future material processing technologies.

1. INTRODUCTION

The four human HVs (Fig.1), two atrioventricular (ATV) - MV and TV- and two semilunar (SLV) - AV and PV - exhibit non-negligible differences under physiological conditions, which are reflected in a variety of structural and mechanical properties [1].

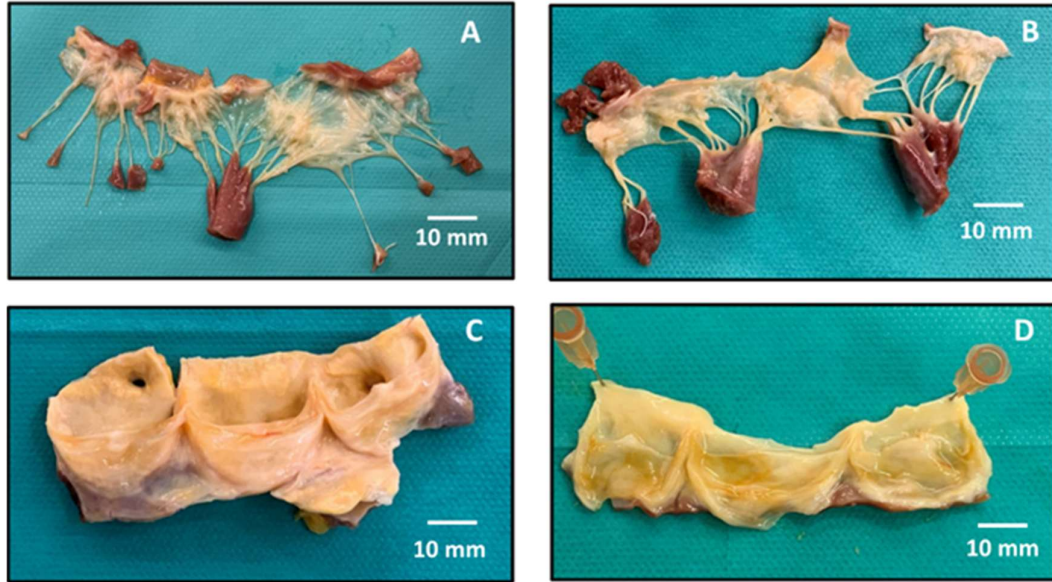


Figure 1. Human heart valve gross examination. The four dissected human heart valves: mitral (A) and tricuspid (B) valves with their chordal apparatus, aortic (C), and pulmonary valves (D).

The incidence of cardiovascular diseases affecting HVs is rising, leading to an increasing clinical demand for engineered valves. Treating HV diseases is among the most challenging clinical problems in cardiovascular medicine [2].

Two primary types of prosthetic valves are utilized for valve replacement procedures: mechanical and biological. Mechanical valves offer the advantage of durability but require long-term anticoagulation therapy to prevent the increased risk of thrombosis [3]. On the other hand, bioprosthetic valves do not necessitate ongoing anticoagulation treatment. Nonetheless, their long-term performance and durability remain plagued by failure modes, leaflet tears, and calcified degeneration, with the latter risk increased in younger patients. A primary drawback of these two implant modalities is that they fail to accommodate somatic development or structural remodeling after implantation [4]. This poses a considerable challenge for pediatric patients, as the development of their cardiac valves must be viewed in conjunction with their physical growing process. Furthermore, current devices often fail to account for the unique properties of native tissue.

Over the past two decades, the field of cardiovascular tissue engineering has advanced significantly and is attempting to address the problems associated with conventional valve replacement by dedicating significant resources to overcoming the current clinical challenges [5]. One promising approach involves polyurethane HVs, which have the potential to overcome the shortcomings of currently available prosthetic HVs [6-8].

Research on the characterization of human HVs remains limited, primarily due to the difficulty of obtaining healthy, fresh human heart samples and other technical barriers associated with sample extraction and analysis [3]. For instance, leaflet topography of human HVs, collagen fibers diameter or fibers network porosity, and bending mechanical properties have never been reported [9]. This reliance on non-human data is increasingly seen as a limitation, potentially undermining the effectiveness of clinical applications, such as functional prostheses, vascular grafts, and heart support devices [3]. For example, the effect of leaflet thickness on native HVs is critical for achieving the physiological properties of native tissue, essential for geometric and mechanical balance in engineered cardiovascular tissues [10, 11]. Unfortunately, literature lacks such basic information that is useful for valve prosthesis manufacturing.

1.1.Human heart valves

The atrioventricular valves (ATVs) separate the atria from the ventricles, ensuring unidirectional blood flow [1]. The mitral valve (MV), positioned between the left atrium and the left ventricle, is composed of two leaflets, whereas the tricuspid valve (TV), located on the right side, has three leaflets [2]. These structures play a key role in preventing blood regurgitation by sealing the atrioventricular junction during ventricular contraction [3, 4]. This process is regulated by the papillary muscles and the attached chordae tendineae (CT), thin connective tissue filaments. During ventricular contraction, blood pressure forces the ATV leaflets upward into the closed position, while the CT prevents the valve from being pushed back into the atrium due to blood pressure. SLVs – AV and PV - possess distinct morphological characteristics. Blood return is prevented by the closure of the leaflets, which are shaped in a way that ensures precise functioning (Fig. 2).

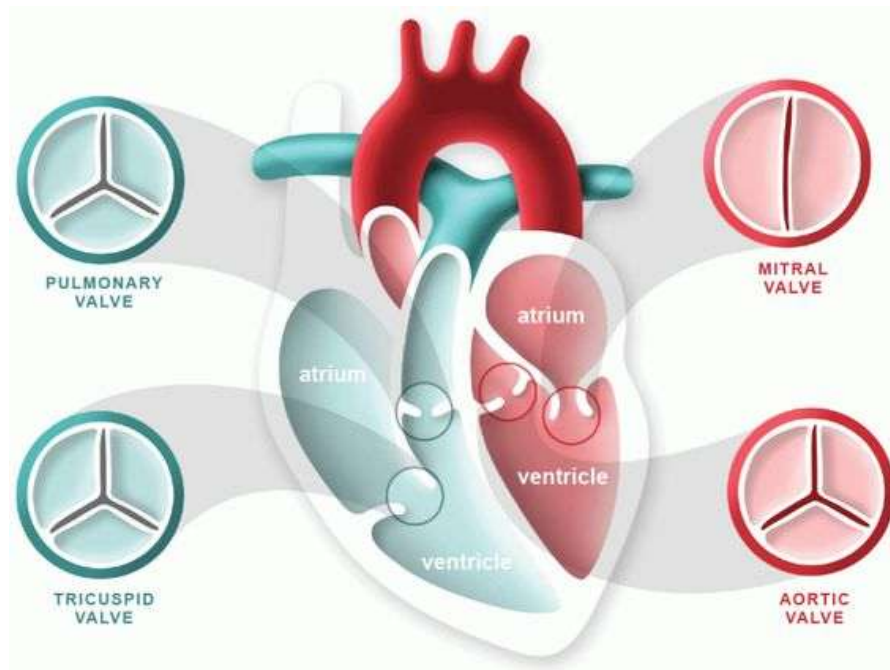


Figure 2. Human heart valves (source: heart foundation)

The opening and closing dynamics of all four valves are controlled by changes in blood pressure gradients. Specifically, the ATVs open during ventricular filling (diastole) and close during ventricular contraction (systole). During the systole, blood is ejected from the ventricles into the arteries through the SLVs, which are open at this stage. The SLVs (Fig. 3), also referred to as "swallow's nest" valves due to their distinctive shape, are comparatively more straightforward in structure than the ATVs.

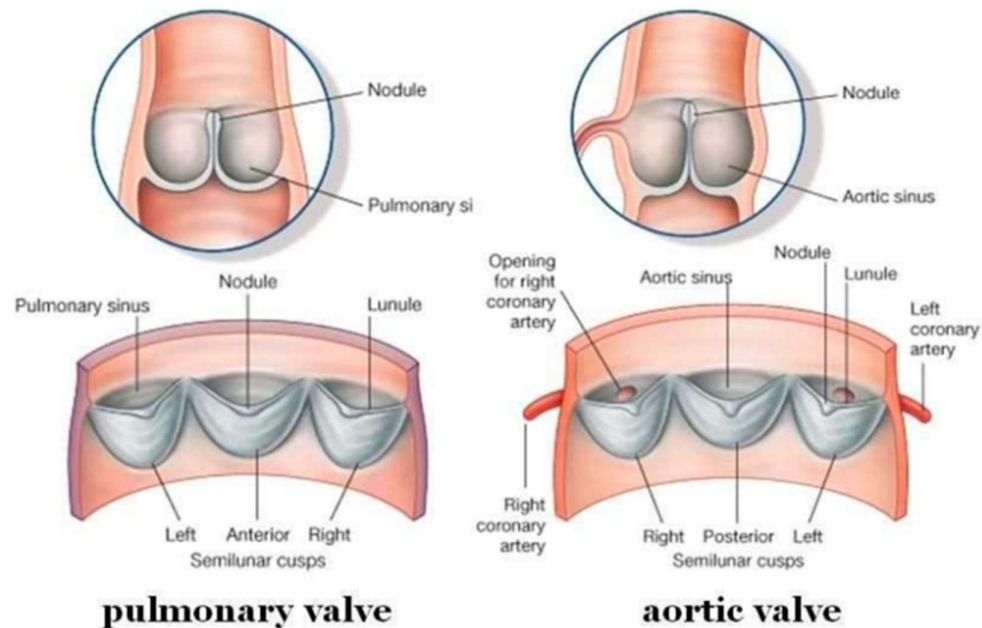


Figure 3. Aortic and Pulmonary valve anatomy

Each SLV consists of three leaflets, or semilunes, attached to a fibrous ring located at the junction between the ventricular outflow tract and the corresponding artery. The SLVs are comprised of a fibrous layer covered on both sides by the endocardium. The ventricular surface forms a contiguous interface with the endocardium, which lines the cardiac cavity. The arterial surface, in contrast, merges with the tunica intima of the corresponding vessel. The fibrous layer exhibits a distinctive nodular thickening at the midpoint of the free margin. This is referred to as the Nodule of Morgagni in the PV and the Nodule of Arantius in the AV.

The arrangements of fibers, cells, and other large molecules in the extracellular matrix (ECM) of a tissue vary according to their specific functional requirements. As a result, the mechanical properties of each tissue are also different [12, 13]. The leaflets of each valve have a specialized ECM that plays a critical role in maintaining proper valve function and giving highly anisotropic properties. Structural features allow for a high degree of flexibility and adaptability in both the SLV and ATV leaflets when they are not under pressure, while also enable to withstand the stretch caused by elevated transvalvular pressure when they are fully closed.

1.1.1. Mitral Valve

The MV (Fig. 4) is located between the left atrium and the left ventricle. Its principal function is to guarantee unidirectional blood flow, thereby preventing retrograde flow into the atrium. The MV is an ATV composed of two leaflets:

- Anterior mitral leaflet (AML) is attached to less than half of the circumference of the fibrous ring surrounding the atrioventricular opening; and it is situated close to the leaflets of the aortic valve.
- Posterior mitral leaflet (PML) is characterized by a broader basis of attachment, and a wider, shorter shape with highly arcuate free edges.

The free edges of both leaflets are anchored to the papillary muscles via CT. These fibrous cords connect the valve leaflets to the ventricular wall through two papillary muscles, anterior and posterior, which guide the CT toward both leaflets. During ventricular contraction, the subsequent rise in intraventricular pressure exerts a force on the MV leaflets, pushing them into a closed position. The CT, together with the contraction of the papillary muscles, stabilizes the valve in its closed configuration, preventing the leaflets from inverting into the atrium.

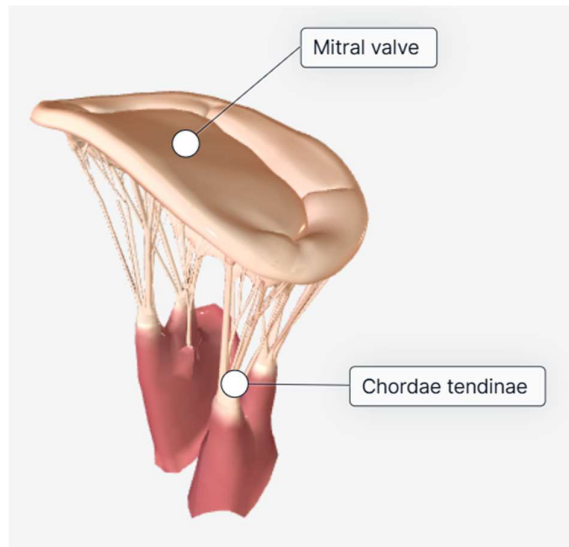


Figure 4. *Mitral Valve Anatomy* (© BioDigital, Inc.)

1.1.2. Tricuspid valve

The TV (Fig. 5) is situated between the right atrium and the right ventricle. The principal function of this valve is to ensure unidirectional blood flow, preventing backflow as the blood is propelled through the heart. The TV is constituted by three leaflets anterior (ATL), posterior (PTL), and septal (STL) which undergo coordinated opening and closing movements with each cardiac cycle, and they are attached to a fibrous ring, the annulus, which is connected to the heart wall. The free edges of the leaflets are anchored to the papillary muscles via the CT. Despite the existence of individual anatomical variations, the papillary muscles of the TV are generally classified as follows:

- **Anterior Papillary Muscle:** The largest and most consistent of the papillary muscles, it originates from the lower end of the septal marginal trabecula and the apex of the ventricle. It anchors the CT attached to both the anterior and posterior leaflets.
- **Posterior Papillary Muscle:** Typically formed from two or three minor bundles, it originates from the inferior posterior aspect of the interventricular septum. It transmits CT to posterior and medial leaflets.
- **Medial or Septal Papillary Muscle:** Deriving from the septal wall, this muscle is typically modest in size and often comprises multiple bundles. Its prominence is less pronounced. The principal function of this structure is to provide CT for the septal leaflet.

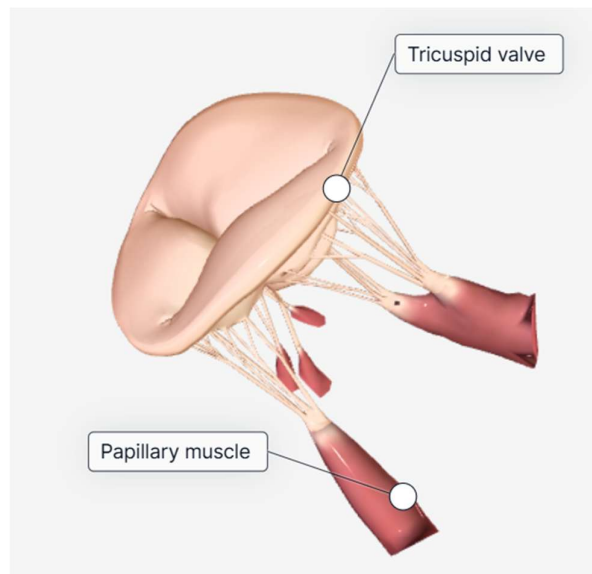


Figure 5. Tricuspid valve anatomy (© BioDigital, Inc.)

1.1.3. Aortic Valve

The AV (Fig.6) is crescent-shaped and is situated between the left ventricle and the aorta and it is anchored to a muscular ring, which connects it to the wall of the heart. Its function is to prevent the backflow of oxygenated blood, thereby ensuring the unidirectional flow of blood through systemic circulation.

The valve is composed of three collagenous semilunes, which are shaped like semicircles:

- Right and Left Coronary Leaflets (RCL – LCL): These are referred to as coronary semilunes because the coronary arteries originate just above them.
- Non-coronary Leaflet (NCL), characterized by the absence of coronary arteries.

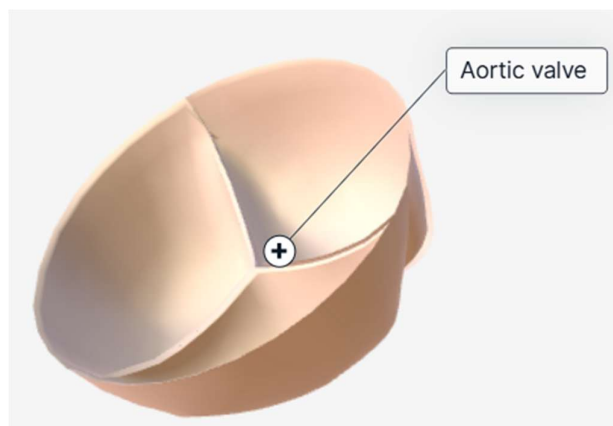


Figure 6. Aortic valve anatomy (© BioDigital, Inc.)

1.1.4. Pulmonary Valve

The PV (Fig. 7) is structurally similar to the AV, also featuring a crescent-shaped configuration. It is located between the right ventricle and the pulmonary artery, anchored to a fibrous ring. Its function is to facilitate the unidirectional flow of deoxygenated blood toward the lungs, where it can be exchanged for oxygen and thus prevent backflow into the ventricle. The PV is composed of three collagenous semilunes:

- Anterior Pulmonary Leaflet (APL), positioned at the front.
- Right and Left Pulmonary Leaflets (RPL, LPL): they are aligned with their counterparts in the aortic valve. These semilunes contain the Nodule of Morgagni which ensures the closure of the pulmonary orifice during ventricular diastole, thereby preventing the regurgitation of blood into the right ventricle.

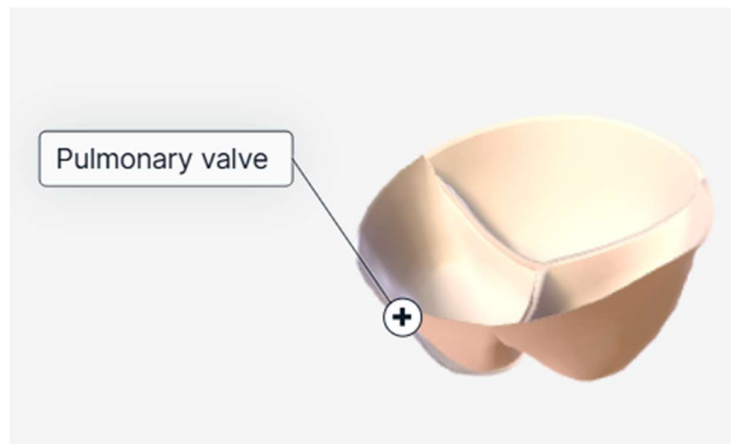


Figure 7. Pulmonary valve anatomy (© BioDigital, Inc.)

1.2. Cardiovascular disease

Cardiovascular diseases (CVD) are a leading cause of mortality worldwide, accounting for an estimated 18 million deaths annually [14]. The prevalence and impact of CVD vary significantly between low- and middle-income countries (LMICs), which collectively account for 80% of all cardiovascular deaths globally, and high-income countries. Several factors contribute to the development of CVD, with the most prevalent conditions disproportionately burdening the fragile healthcare infrastructure and socio-economic frameworks of LMICs, exacerbating the vulnerability of their populations. These include hypertension, ischemic heart disease, and persistent infection-related diseases such as rheumatic heart disease [15]. The Global Burden of Diseases, Injuries, and Risk Factors (GBD) study tracked death and disability trends since 1990. It provided an up-to-date perspective on global, regional, and national cardiovascular health. The GBD study (Fig. 8) also measures the burden of disease attributable to 88 risk factors for cardiovascular disease, focusing on the incidence and prevalence of cardiovascular disease as measured by structured case definitions [16].

The report indicates that CVD persists as the predominant cause of mortality on a global scale, accounting for approximately one-third of all deaths. The established risk factors, including hypertension, tobacco use, elevated cholesterol levels, obesity, and diabetes, continue to exert a significant influence. Additionally, emerging risk factors, such as air pollution, stress, and other environmental and behavioral factors, are increasingly recognized as significant contributors to CVD.

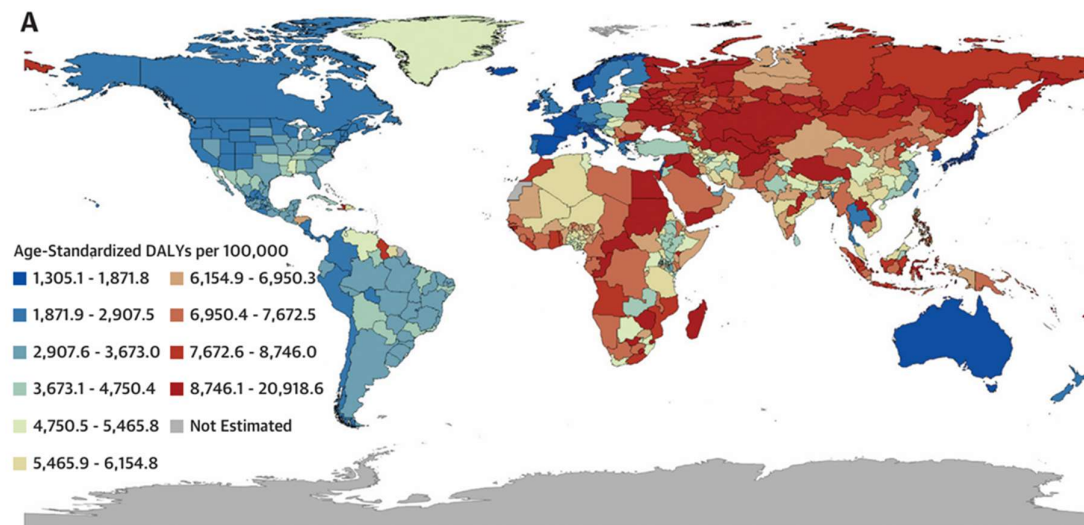


Figure 8. Global Burden of Cardiovascular Diseases and Risks [16]

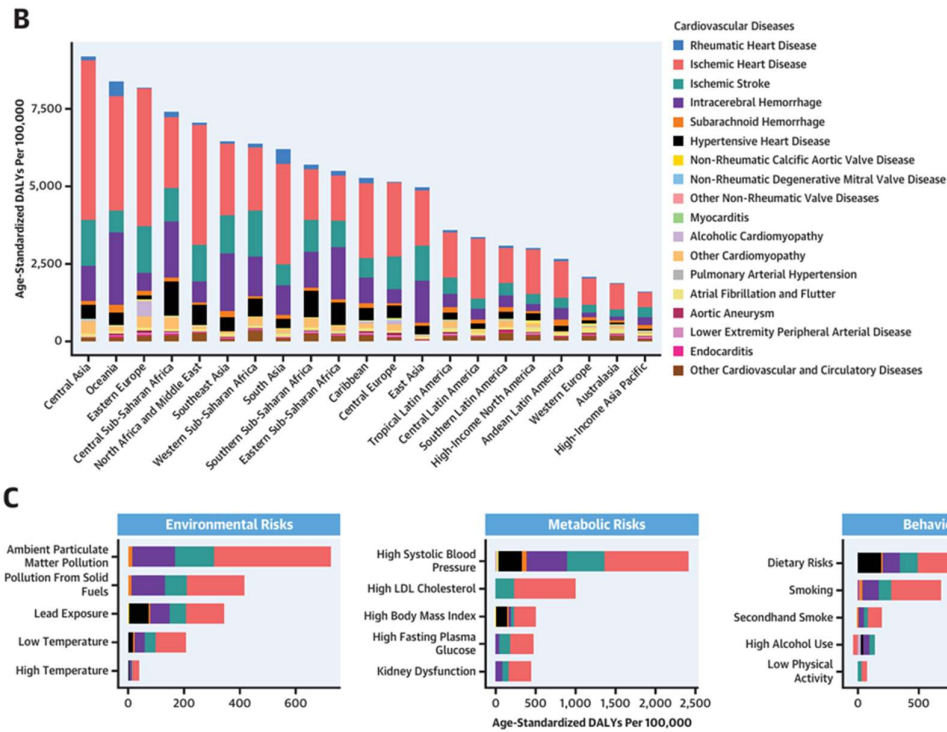


Figure 8. Global Burden of Cardiovascular Diseases and Risks [16]

The number of individuals affected by cardiovascular diseases that target the HVs is on the rise. The precise biological mechanisms that cause such pathological conditions remain largely elusive. Currently, the standard of care for these conditions involves surgical interventions, including valve replacement or repair procedures [17]. However, these approaches are not without considerable limitations. It is estimated that approximately 100,000 cardiac surgeries were conducted in the United States and nearly 300,000 worldwide to address this issue, underscoring the growing burden of cardiovascular diseases affecting HVs [18].

The prevalence of cardiovascular disease may be influenced by underlying heart valve disease (Fig. 9) [2, 16, 19-21]:

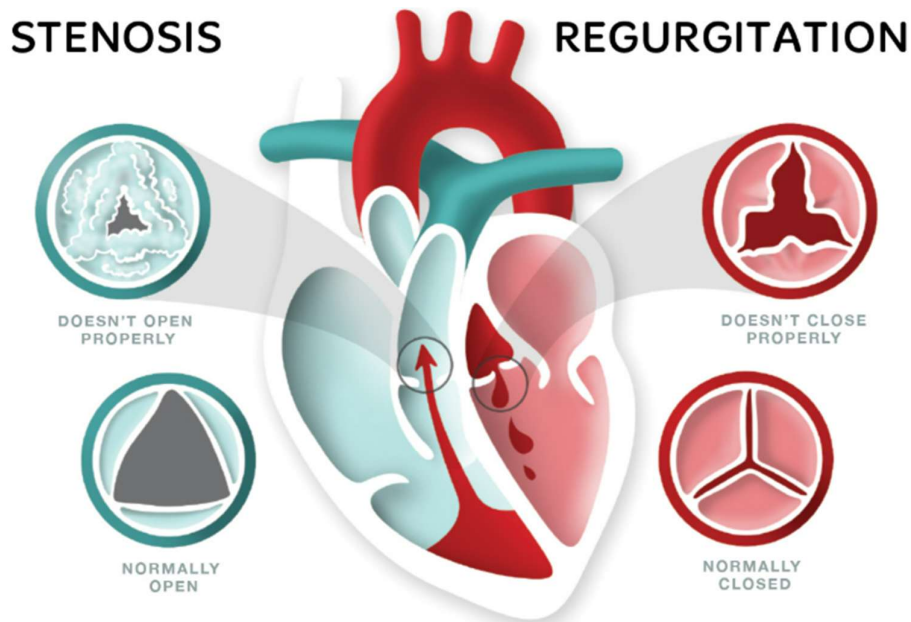


Figure 9. Principal heart valve disorders (source: heart foundation)

- MV: a high prevalence of regurgitation and stenosis characterizes the disease of this valve [22]. Causes include MV prolapse, rheumatic fever, myocardial infarction, or age-related degenerative changes.
- TV: problems such as tricuspid regurgitation are frequently secondary to left-sided heart failure or conditions that increase right ventricular pressure.
- AV: stenosis, a condition marked by narrowing of the valve due to calcification, and aortic regurgitation, a phenomenon characterized by the backward leakage of blood into the left ventricle. Such conditions are frequently associated with advancing age, rheumatic fever, or congenital abnormalities.
- PV: stenosis and pulmonary regurgitation are less common but may result from congenital heart defects or post-surgical complications in congenital heart disease.

In recent years, the field of Tissue Engineered Heart Valves (TEHV) has changed the treatment of cardiovascular diseases, with a particular emphasis on pediatric patients who require tailored approaches to address their unique needs [23-25]. The primary objective is to develop devices that can facilitate physiological adaptation, growth, and repair, thereby advancing the concept of regenerative cardiac care. Significant efforts have been made to develop different types of engineered heart valves as part of the evolution of this medical technology (Fig. 10).

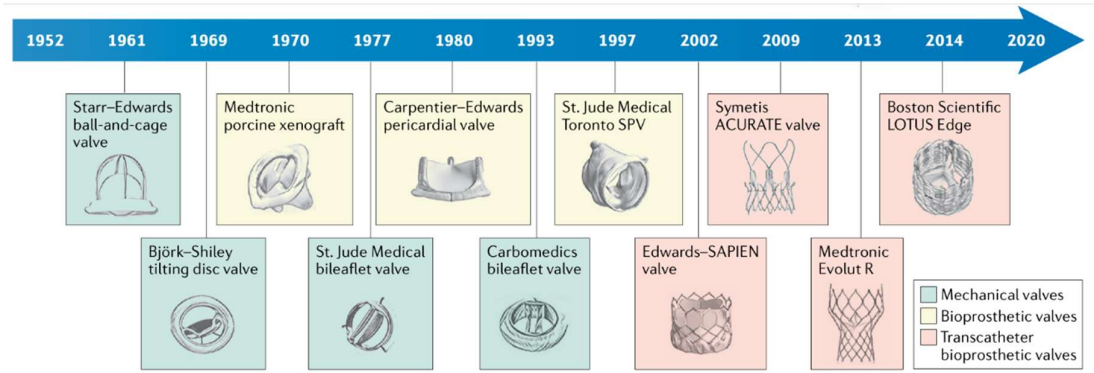


Figure 10. Evolution of heart valve replacement options [26]

The predominant valves employed are mechanical valves and bioprosthetic valves. However, these valves have considerable shortcomings, including the necessity for anticoagulant therapy with the former and the propensity for deterioration due to calcification and degenerative processes with the latter. Research efforts have focused on advancing TEHVs to address the limitations of existing valves while replicating the functional and structural properties of native human HVs [10, 27, 28]. In particular, the new devices must facilitate their integration with the patient's tissue, support cellular growth, and dynamically undergo remodeling in response to physiological stresses [26, 29, 30]. Advances in biomaterials have facilitated the development of innovative HV technologies, which are primarily composed of biodegradable polymers and decellularized matrices employed as scaffolds [31] (Fig. 11). In particular, the scaffolds are cultivated with cells derived from the patient or are designed in a way that attracts cells from within the body. This encourages the natural repair processes that occur in the body and enables regeneration to occur naturally. In order to develop innovative engineered valves that can adapt to the functionality and structure required for longer life, avoid material degradation, and clinical complications, there is a primary need to reproduce the basic characteristics of native human HVs. Furthermore, fundamental support for system optimization may come from advances in bioprinting and computational modeling systems.

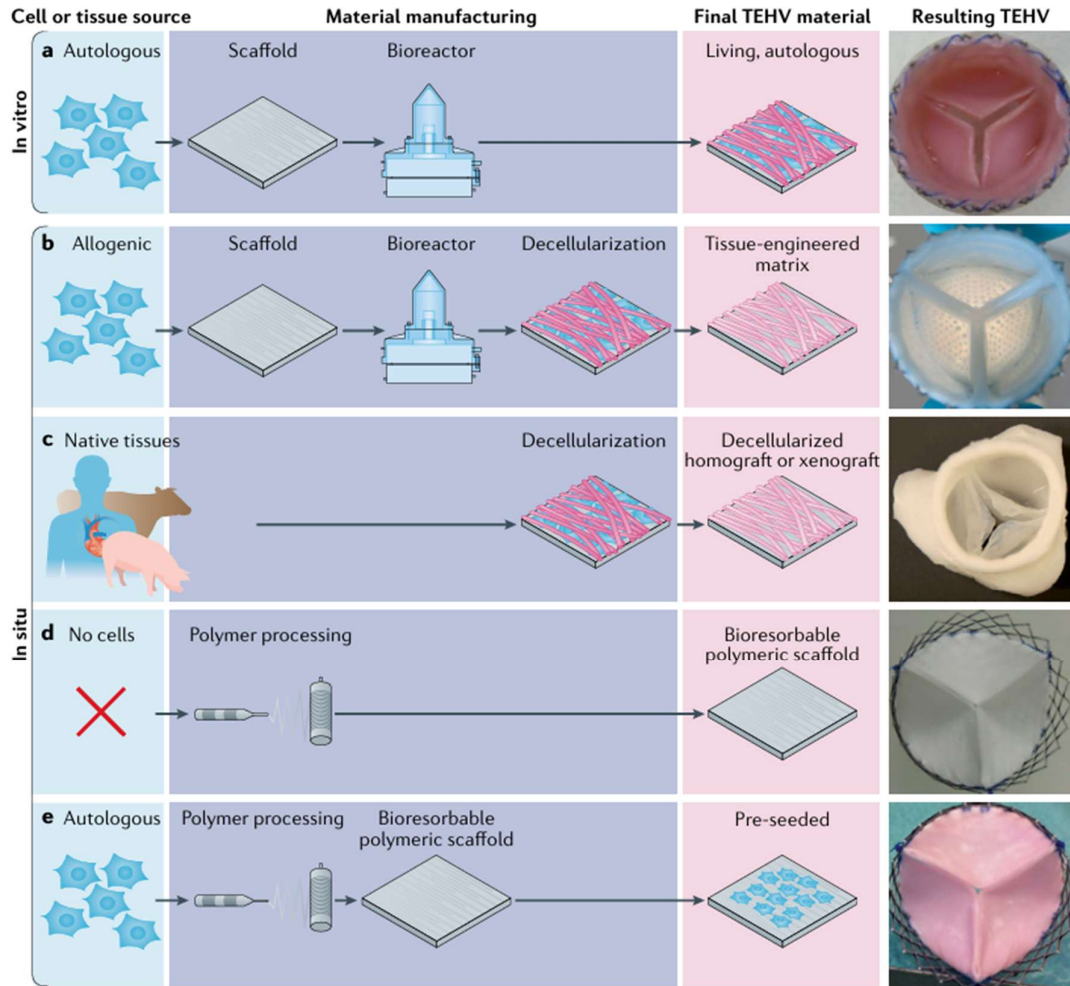


Figure 11. Overview of various tissue-engineered approaches [26]

1.3. Heart Valve Characterization: Human and Animal Models

The characterization of the structural and mechanical properties of biological tissues is of fundamental importance for the comprehension of their physiological behavior and for the design of effective prosthetic devices. Although human heart valve characterization provides insights critical for clinical applications, animal models such as porcine and ovine hearts are extensively used due to their anatomical and biomechanical similarities to human tissues [1, 2]. Porcine heart valves are frequently utilized as models due to their anatomical resemblance to human valves. Alternatively, ovine heart valves are another commonly employed animal model, particularly for pre-clinical testing. While animal models provide valuable insights, they present certain limitations. The absence of a true mitral valve equivalent, for example, in porcine and ovine affects their utility in mitral valve research

[17]. Additionally, species-specific differences in cellular and extracellular matrix composition influence the mechanical behavior of the valves, necessitating careful consideration when extrapolating results to human applications.

The choice of an animal model in cardiovascular tissue engineering depends on the specific goals of the research. The porcine model is particularly valuable when anatomical and physiological similarities to humans are essential, especially in studies involving heart valves, as their structure closely resembles that of humans. Additionally, pigs are commonly used for research on the heart and major blood vessels due to their comparable size. Their cardiovascular system also makes them well-suited for testing medical devices such as stents and pacemakers, as well as for studying treatments for atherosclerosis, given their tendency to develop plaques similar to those observed in humans.

On the other hand, the ovine model is more appropriate for investigations focusing on biomaterials, tissue regeneration, and prosthetic integration, thanks to its pronounced capacity for vascular remodeling and elasticity. These characteristics are particularly relevant in the development of bioengineered heart valves and the assessment of resorbable scaffolds. Furthermore, sheep are frequently used for testing vascular prostheses intended for pediatric applications, as their rapid growth provides an effective model for evaluating how implanted devices adapt over time in a developing organism.

In summary, porcine models are most suitable for research involving cardiovascular devices, heart valves, and atherosclerosis, whereas ovine models are better suited for studies centered on biomaterials, tissue regeneration, and the adaptation of prosthetic implants to growth.

AIMS OF THE PROJECT

An in-depth understanding of native cardiovascular tissue has become a priority in tissue engineering, with the main goal of studying human tissues' mechanical and structural properties. However, the macroscopic and microscopic mechanisms that regulate cardiovascular disease onset are still unclear.

Macro-scale geometric design variables, such as the thickness of the valve leaflets, play a primary role in the structural and geometric optimization of engineered valves [11, 32]. On the other hand, at the microscopic level, the collagen network and fiber scaffold must be considered, given their crucial role in stress distribution. This network also significantly influences cell behavior, such as the processing of the ECM and tissue growth [33]. Moreover, a substantial number of studies have emphasized the importance of determining native valve anisotropy and thus its implications for engineered valves, in terms of mechanobiology and coaptation [28, 34]. A complete perspective on the biomechanics of valves must encompass, in addition to the biaxial properties, the analysis of flexural stiffnesses [35].

The objective of this research is to conduct a comprehensive investigation into physiological processes and the intricate relationship between structure and function within human cardiovascular HVs. The investigation employs a multifaceted methodology that is divided into four principal components, with the objective of conducting a comprehensive and detailed analysis.

- Structural Analysis at the Macroscopic Level: This aspect of the study entails an examination of the large-scale architectural features of cardiovascular tissues, including their overall geometry, surface morphology, and organizational patterns. A comprehensive examination of the tissue at the macro level is crucial for elucidating its physical dimensions and the ways in which they contribute to its biomechanical attributes and functional integration within the cardiovascular system.
- Structural Analysis at the Microscopic Level: This phase examines the intricate, microscopic tissue structures using sophisticated imaging techniques. By examining the cellular organization, extracellular matrix composition, and microstructural arrangements, this phase seeks to identify how microscopic features impact the overall mechanical and biological native tissue.
- In-Plane Mechanics Functional Analysis: This analysis focuses on the in-plane mechanical behavior of biological tissues when subject to a biaxial load, whereby

the material is simultaneously stretched in two perpendicular directions. The biaxial tests conducted in this study allow for the evaluation of crucial mechanical properties, including anisotropy, compliance, and stress-strain relationships within the main plane of the tissue. The aforementioned tests are fundamental to replicate the physiological loading conditions and further understand the tissue's response to multidirectional forces encountered during physiological normal cardiovascular function.

- Out-of-Plane Mechanics Functional Analysis: This analysis examines the flexural mechanical response of the tissue undergoing three-point bending tests. These tests apply a controlled load to the tissue at the central region while it is supported at both ends. The aim is to assess the tissue's flexural properties, specifically its stiffness, bending strength, and deformation behavior. This step is fundamental to an understanding of the tissue's performance in response to out-of-plane forces, which have relevance in scenarios such as bending and the dynamic motion of tissues within the cardiovascular system.

The combination of these four analyses provides a comprehensive framework for investigating the relationship between structure and function in human cardiovascular tissues. The insights gained from this approach are intended to inform the design of biomimetic materials and engineered tissues that replicate the native properties of cardiovascular structures, with the ultimate goal of advancing regenerative medicine and tissue engineering.

2. MATERIALS AND METHODS

2.1.Samples

The research is part of the European Research Council Grant Consolidator “Engineering the mitral valve: bioinspired control of structure and function for enhanced in-vivo performance-BIOMITRAL”, Principal Investigator Prof. Antonio D’Amore, in collaboration with the Fondazione Ri.MED.

Samples for structural characterization were obtained from 22 heart transplant patients at the Mediterranean Institute for Transplantation and Highly Specialized Therapies (ISMETT). All patients provided informed consent before undergoing surgical procedures, and the study was reviewed and approved by the local ethical committee (IRRB/07/21). The Center of Organ Recovery & Education (CORE) provided 8 human hearts (Fig. 12) for functional characterization purposes. Before analysis, the HVs were immersed in Dulbecco's Phosphate-Buffered Saline (PBS) at room temperature.



Figure 12. Human Heart from CORE – Male, 65 years old. Destinated for Mechanical characterization

The nomenclature presented in Fig. 13 and Table 1 was used as a reference to analyze each heart valve leaflet.

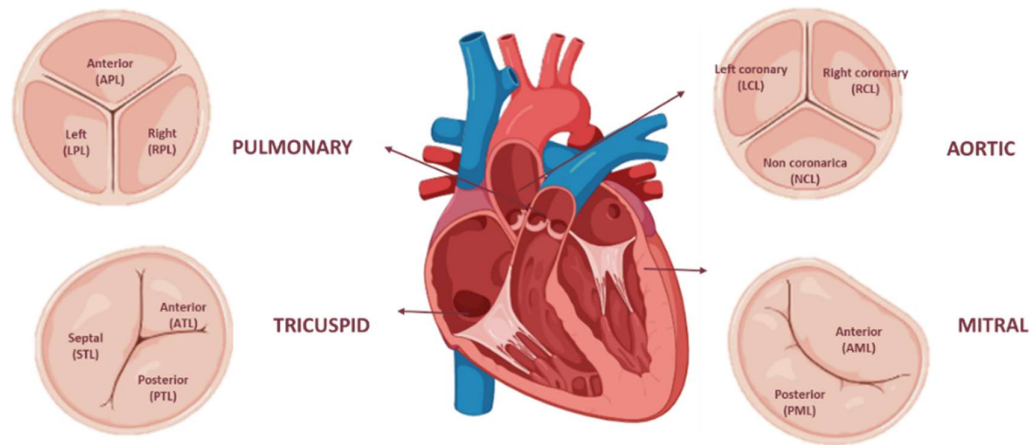


Figure 13. Heart valve leaflets nomenclature (*app.biorender.com*)

Table 1. Heart valve leaflets nomenclature

Valve	Leaflet	Nomenclature
Mitral (MV)	Anterior	AML
	Posterior	PML
Aortic (AV)	Non-coronary	NCL
	Left coronary	LCL
	Right coronary	RCL
Tricuspid (TV)	Septal	STL
	Anterior	ATL
	Posterior	PTL
Pulmonary (PV)	Anterior	APL
	Right	RPL
	Left	LPL

The 30 human heart samples included patients ranging in age from 42 to 68 years, comprising both male and female individuals with a body weight within the range of 47-129 kg. The causes of transplantation or death include cardiomyopathies (of the dilated, hypertrophic, or postpartum variety), ventricular dysfunction, anoxia, and cerebrovascular events (i.e., stroke). A considerable number of specimens demonstrate valvular regurgitation, specifically mitral or tricuspid, which is frequently associated with advanced heart failure or ventricular dilatation. This is a crucial aspect to consider when studying the properties of HVs. The most significant cases include ST-Elevation Myocardial Infarction (STEMI), cardiac arrest, anoxia with mild valve regurgitation, and cerebrovascular stroke (Appendix - 1).

2.2. Leaflet thickness measurements

The native leaflets were dissected from the valves and thickness was measured at fifteen points with a dial gauge (Mitutoyo, Fig. 14) that spanned from the free edge to the belly and commissural regions per each leaflet (Fig. 15) in order to obtain an accurate representation [36]. To minimize experimental error due to the valve leaflet wet content, each measurement was performed for 5 minutes following the positioning of the leaflet on the gauge.



Figure 14. Dial gauge Mitutoyo used for leaflet thickness measurements

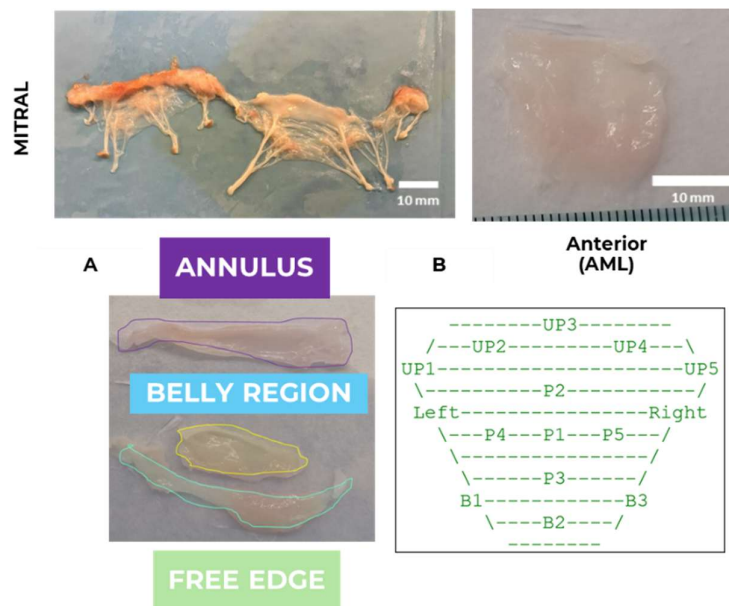


Figure 15. Heart Valve Leaflets Regions example. A) Leaflet regions. B) Leaflet's 15 positions for thickness measurements

Two primary directions are discernible in each leaflet: the radial (RD) and the circumferential (CD) (Fig. 16). These directions are defined by the orientation of the tissue fibers and the predominant mechanical properties of the leaflets, including their elasticity, strength, and durability, which persist throughout the cardiac cycle. The RD extends from the annulus towards the free edge of the leaflet and contains the elastic fibers that enable the recovery of shape following deformation due to the movement of the leaflet. Conversely, the direction of the CD is parallel to the valve ring and is the predominant arrangement of collagen fibers that provide substantial resistance against the pressures generated by the blood pressure during the opening and closing of the valves.

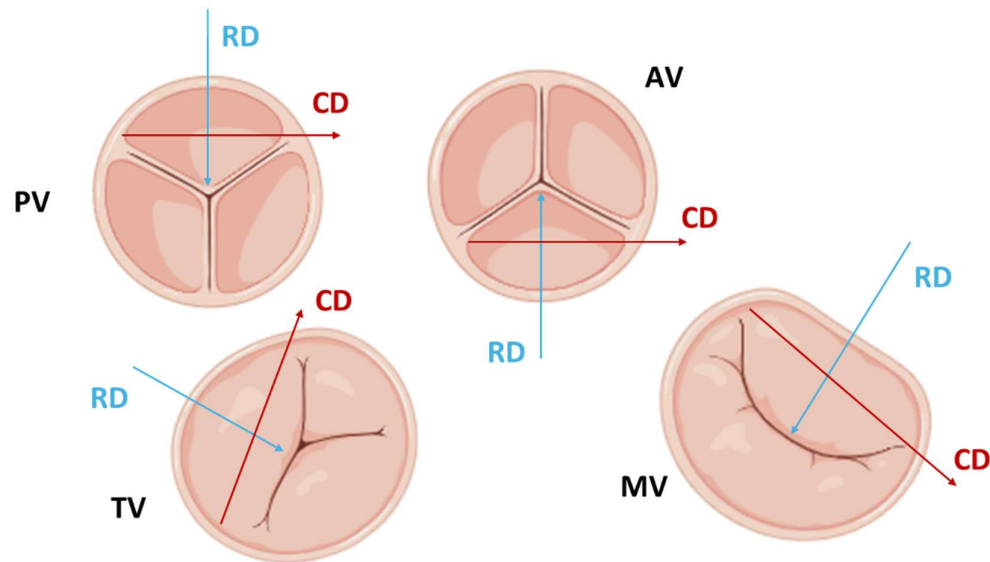


Figure 16. Radial and Circumferential directions per each leaflet of the HVs

2.3.Histological analysis

The structural features of the SLV and ATV leaflets permit flexibility and elasticity when the valves are not under load, while simultaneously providing resistance to extension during periods of high transvalvular pressure when the valves are fully closed.

Two distinct distributions of the layers that constitute the valves have been identified (Fig. 17).

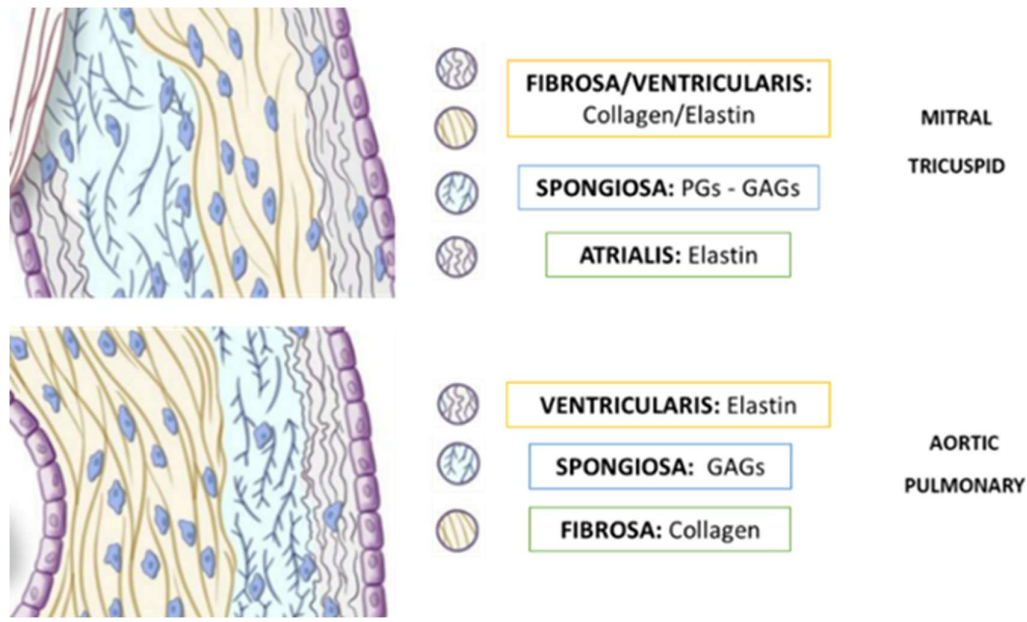


Figure 17. The layer configuration in human HV leaflets [37]

It is noteworthy that the MV and TV are formed in a specific sequential order, originating from the ventricle and extending to the atrium, comprising three distinct layers. The first layer is the Fibrosa/Ventricularis layer, which contains a high concentration of elastin and collagen. The second layer is the Spongiosa layer, characterized by a high glycosaminoglycan (GAG) content concentration. The third layer is the Atrialis layer, which is distinguished by its high elastin content. AV and PV comprise multiple layers, ordered from the aorta/pulmonary vein side to the ventricle. These include the Fibrosa, Spongiosa, and Ventricularis layers. The composition and distribution of collagen, elastin, and GAGs in the three-layered structure of the valve leaflets, along with their viscoelastic properties, are thought to play a significant role in the optimal functioning of the valve and its long-term durability.

A histological assessment of human heart valve leaflets was conducted using several staining techniques (Appendix – 2). Hematoxylin and Eosin (H&E) were employed to assess the general morphology of the extracellular matrix (ECM) of the leaflets, while Masson's Trichrome (MT) and Picrosirius Red (PR) were used to identify collagen fiber architecture. Finally, Alcian Blue (AB) was utilized to detect GAGs.

2.4. Decellularization process

Decellularized heart valves represent a novel class of medical devices employed for the replacement of damaged or diseased HVs [38]. The decellularization process entails the removal of cellular components while ensuring the preservation of the tissue's ECM, which is crucial for tissue engineering applications such as the fabrication of valve replacements. These valves offer several advantages over traditional techniques, including a reduction in invasiveness, accelerated recovery periods, and a diminished risk of complications, including rejection and inflammation. Furthermore, due to their biocompatibility, they integrate more effectively into the human body and ensure a more natural valve function. The most common types are derived from human donor aortic or pulmonary valves and continue to represent a promising solution in the treatment of HV disease [39, 40].

The objective of this study was to develop a decellularization protocol that could be applied to human HVs in order to quantify collagen fiber diameter and fiber bundle diameter. A comprehensive review of the extant literature [41-44], was conducted, and a novel decellularization protocol was subsequently evaluated on human HVs (n=4 leaflets per valve). This investigation revealed that the most efficacious approach for achieving the desired outcome is through the utilization of a combination of chemical, enzymatic, and physical methods during the decellularization process. This approach incorporates established techniques and includes enzymatic and detergent elements, as detailed in Table 2. Additionally, each solution comprised 0.2 g of EDTA, a molecule that can form an annular molecular complex, which retains and isolates a central metal ion, thereby facilitating the separation of cells from the protein substrate.

Table 2. Decellularization solution components

	Property	Impact
Triton x-100	Nonionic detergent	Destroys the DNA-protein, lipid-lipid, and protein-lipid interaction, leaving the protein-protein bond intact
Sodium dodecyl sulfate (SDS)	Ionic detergent	Moves the cellular component out of the tissue satisfactorily, causing a decrease in GAGs concentration and a loss of collagen integrity
Trypsin	Enzymatic	Breaks peptide bonds

Following their placement into the solutions, the leaflets were agitated using the Innova incubator at a rate of 300 rpm and a temperature of 37°C. This process was repeated for two

cycles of decellularization, with each cycle lasting a total of 48 hours. A PBS rinse was performed between each cycle, along with mechanical stress (Appendix - 3).

The algorithm to characterize the fiber distribution is capable of analyzing the complete topology of fiber networks present within planar tissues and scaffolds. The methodology commences with the processing of high-resolution digital images, which is instrumental in identifying and segmenting fibers within the structure. The algorithm calculates the local angular orientation (θ_c) of the fibers using mathematical techniques, including the Fourier transform and orientation-based filtering methods. Subsequent to this, quantitative metrics are calculated, including the orientation distribution function, which captures the prevalence of specific fiber directions. This approach provides a detailed map of the fiber network, enabling the correlation of structural and mechanical properties or the design of engineered scaffolds that mimic the native tissue's characteristics. The algorithm generated the fiber orientation index (OI), which is a widely used measure of fiber alignment, and has a value of OI=1 for perfect parallel fibers and OI=0.5 for isotropic arrangement (Fig. 18-19).

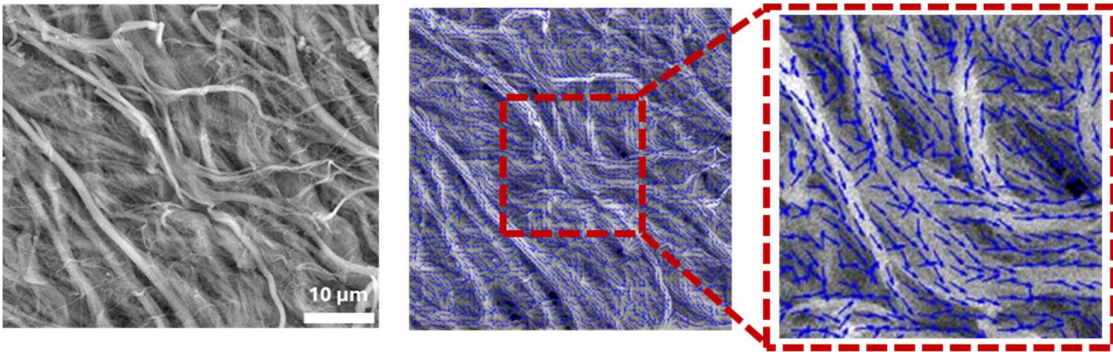


Figure 18. SEM analysis on decellularized Human AML using an algorithm modified by D'Amore et al. [45]

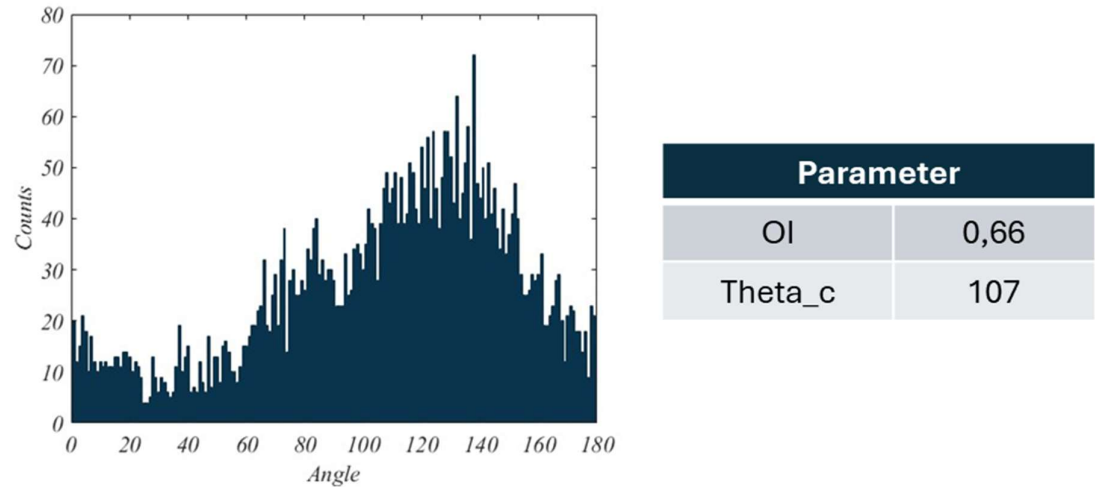


Figure 19. Example of quantitative analysis of fiber alignment (θ_c) and OI of decellularized tissues

2.5. Biaxial mechanical tests

Biaxial tests are fundamental to characterizing anisotropic properties and investigating layer interactions under physiological and pathological loading conditions. These tests provide an accurate representation of the functionality of the valves and closely replicate the multiaxial loading conditions experienced by the leaflets throughout the cardiac cycle [46]. This methodology facilitates a more profound comprehension of the dynamics associated with valve closure, thereby assisting in the prevention of pathological conditions such as prolapse or valvular degeneration [47].

The biaxial device consists of an X-shaped basin, two load cells, two motors, and axes. It is designed to provide a mechanical load on a square sample of material in two directions (normal to each side of the square, Fig. 20). A Lagrangian equi-stress control protocol with a maximum load of 400 kPa was adopted to induce physiologically relevant strain levels.

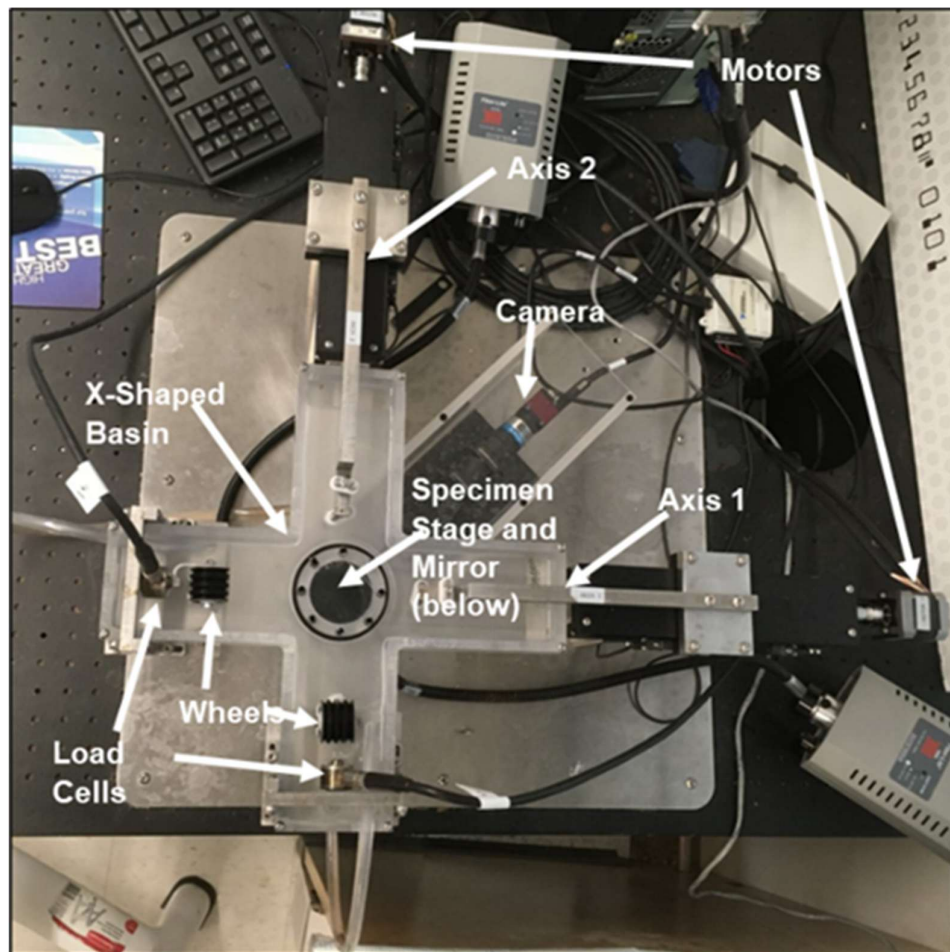


Figure 20. Custom-made biaxial device

Samples of 10 x 10 mm ($n=5$ human hearts, 29 leaflets) were obtained from the belly region leaflet of each leaflet. The Starrett thickness dial indicator was employed to ascertain the dimensions of the specimens in question. This was done at five points, four of which were located at the corners and one in the center of the samples. The resulting values were then averaged, thus providing the mean thickness value. The samples were affixed to the apparatus via a series of hooks and wires. Four hooks were affixed along the edge of the sample on each side, resulting in a total of 16 hooks. Subsequently, four polypropylene markers were positioned in a squared area at the center of the sample and utilized to assess the deformation gradient tensor via super glue (Fig. 21). The preconditioning of the samples was then undertaken, followed by a 10-cycle testing period of 15 seconds in PBS at room temperature.

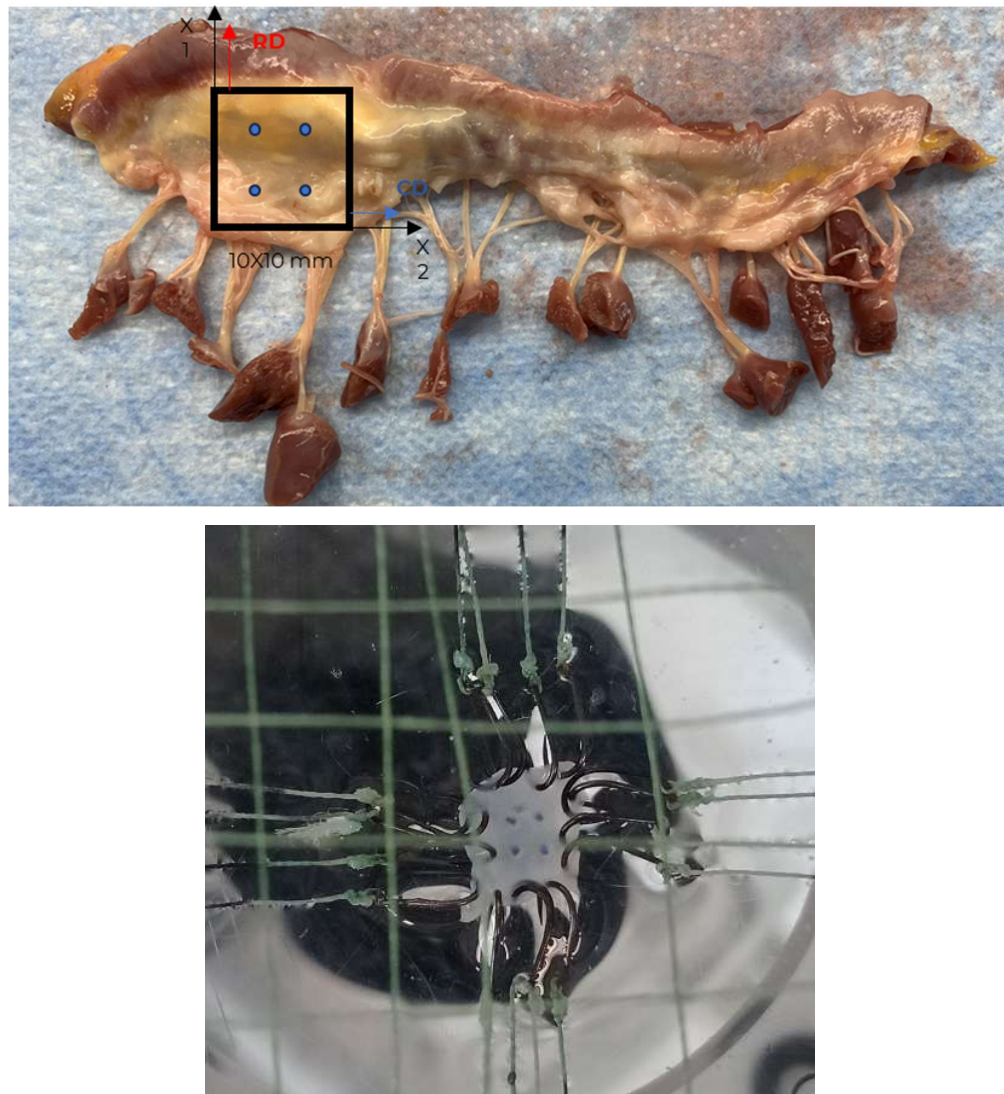


Figure 21. Heart Valve Leaflets before and during the biaxial tests

2.6. Three point bending tests

While biaxial tests are employed to assess tensile behavior in both the RD and CD, flexural tests are essential for investigating out-of-plane properties, including those related to flexural stiffness and resistance to bending. A comprehension of these aspects constitutes an indispensable component of understanding how the leaflets can withstand the bending forces that occur during the cardiac cycle, with a specific reference to the periods of valve opening and closure. A three-point bending apparatus and an experimental protocol specifically developed for heart valve leaflets were used (Fig. 22).

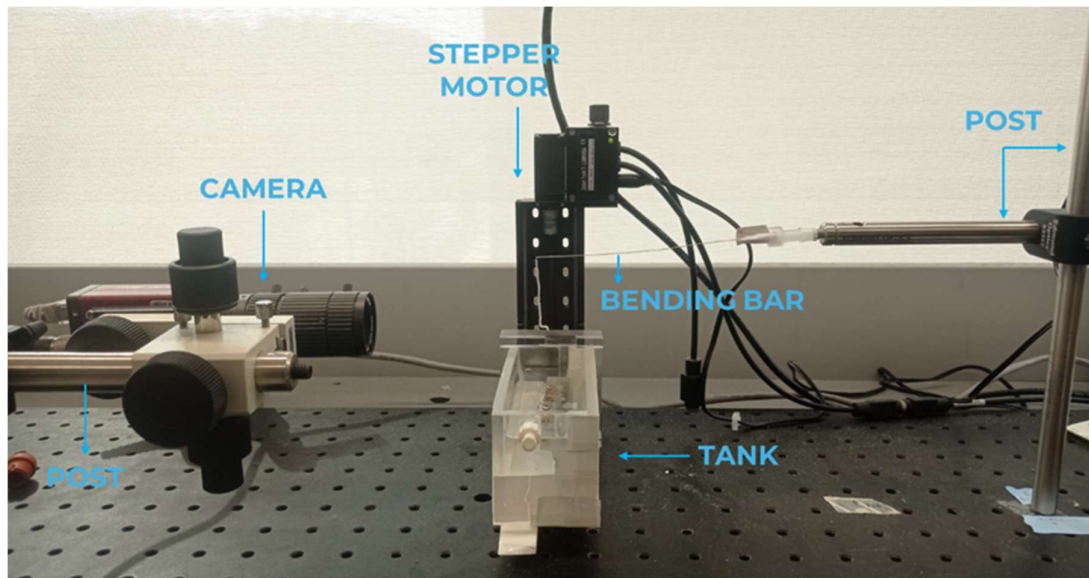


Figure 22. Custom-made bending device

In brief, 12-25 x 2.5 mm sections oriented along the radial direction of the leaflets (n=3 human hearts, 26 leaflets) are dissected from the belly region, and the thickness measurements are taken in three locations (up, middle, and bottom, Fig. 23). Four markers are obtained from a polypropylene wire, and they are attached across the edge of the specimen using super glue.

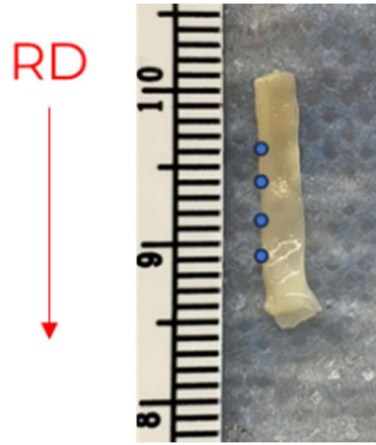


Figure 23. Heart valve leaflet used for three-point bending tests

The marker positions were recorded using a camera and subjected to real-time analysis with a bespoke software program developed in LabVIEW 2010 (National Instruments, Austin, TX). The samples were mounted in a specially constructed holder and submerged in a PBS bath maintained at room temperature. They were then attached to a vertical loading bar with a known stiffness. Tests were performed with and against curvature up to the maximum sample curvature of $\Delta k_{\max} = 0.12 \text{ mm}$ (Fig. 24). The bending modulus, E , and the bending rigidity, EI , were calculated in accordance with the Bernoulli-Euler equation 1:

$$M = EI \Delta k$$

Equation 1. Bernoulli-Euler

Where M is the bending moment, and I is the second moment of inertia.

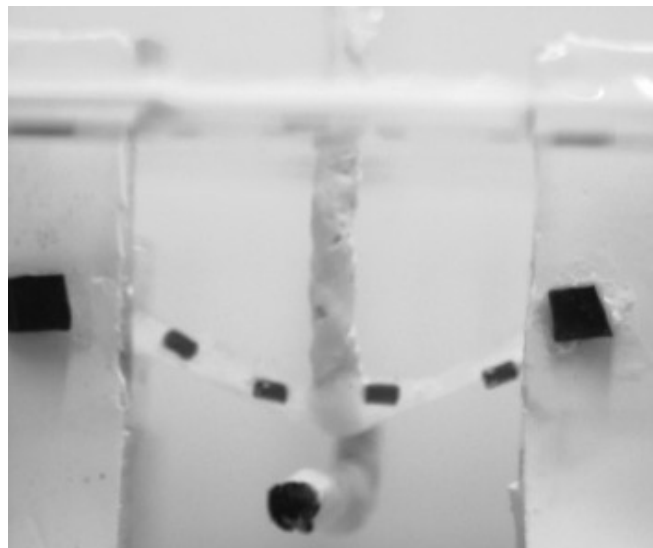


Figure 24. Three-point bending test on the human mitral anterior leaflet

A comprehensive evaluation necessitated the execution of experiments on two distinct surfaces of the leaflets: one that exhibited an inclination with curvature (WC), and another that exhibited an inclination against curvature (AC). In the case of the MV and TV, the collagen-rich Fibrosa/Ventricularis layer, which faces the ventricle, is defined as the AC surface. This is due to the fact that it is the surface that opposes the natural movement of the valve during closure. In the case of the AV, the AC surface is the one facing the outlet towards the aorta, the Ventricularis layer, which is also dense with collagen. This is because it resists valve closure under high pressure. A similar configuration is observed in the PV (Fig. 25).

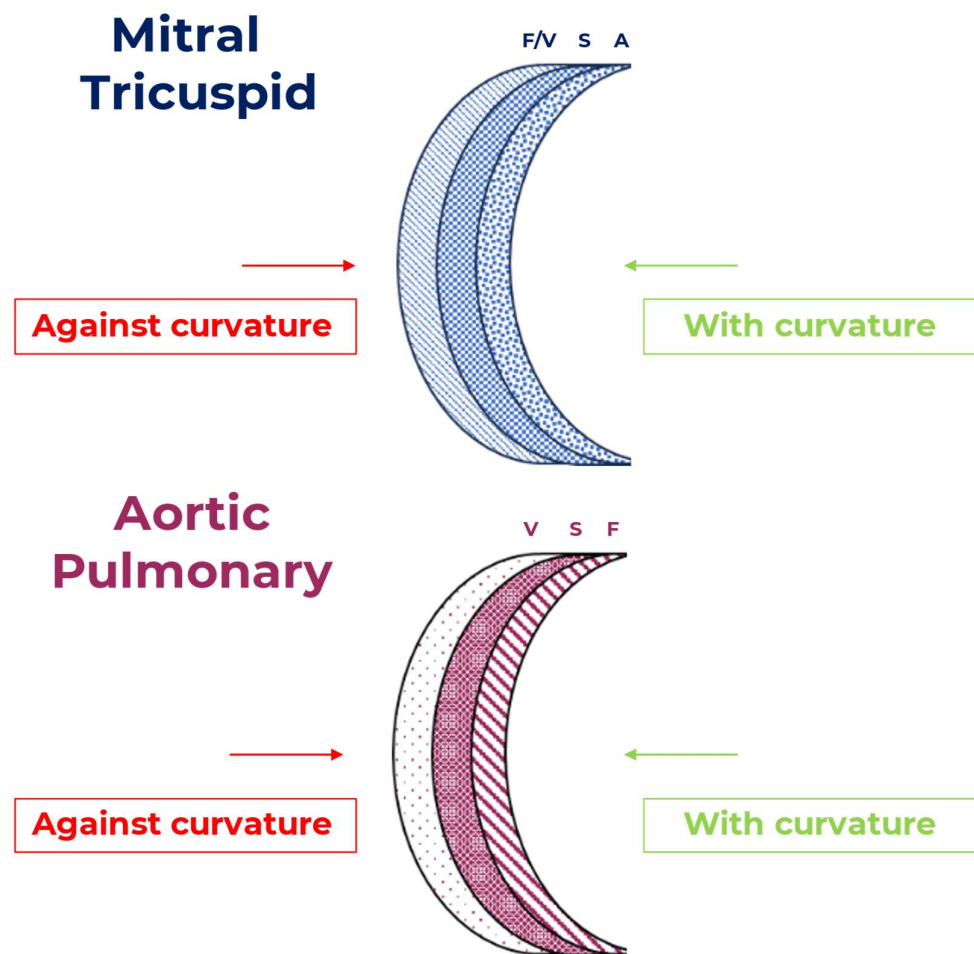


Figure 25. HV's surface curvature

2.7.Statistical analysis

Statistical analyses were conducted utilizing Sigma Plot (Systat Software Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was employed to compare multiple samples. The results obtained are presented as the mean $\pm$ standard deviation of the mean. It is important to note that any differences observed were considered to be statistically significant at $p < 0.05$.

3. RESULTS

3.1. Structural analysis: macroscopic tissue morphologies

3.1.1. Morphological analysis

The results demonstrated variability in the thickness distribution of the valve. Moreover, the MV exhibited a statistically higher mean thickness in comparison to the other valves (Fig. 26, Tab. 3).

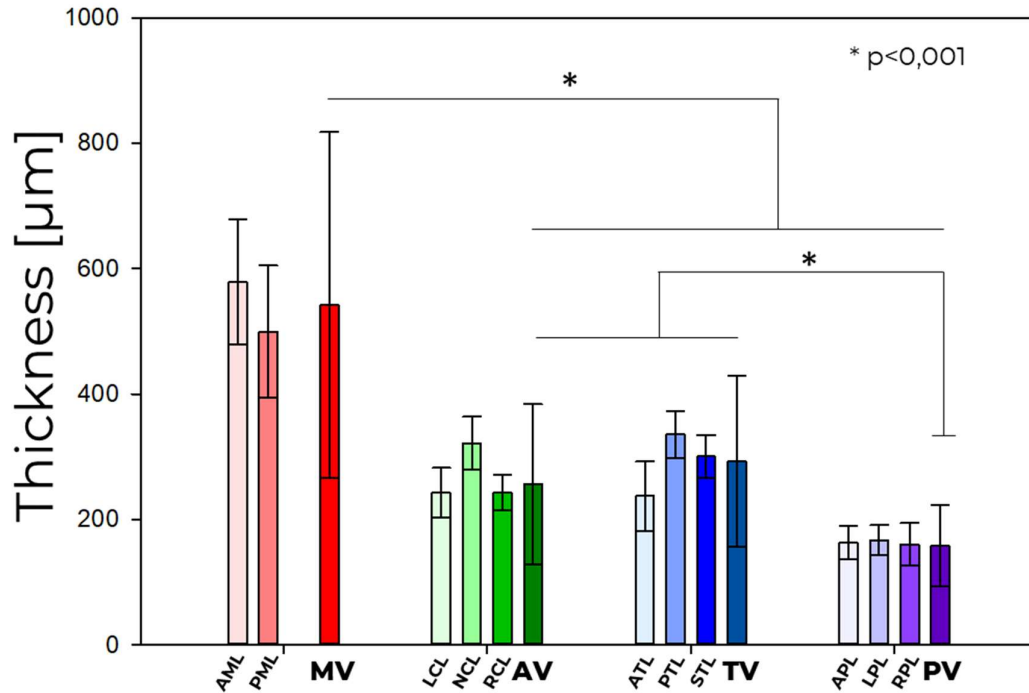


Figure 26. The average thickness of human HVs

The thickness results relationship is reflected in the transvalvular pressure values found in the literature [48]: MVs exhibited higher pressures (120 mmHg) compared to AVs (80 mmHg), TVs (25 mmHg), and PVs (10 mmHg). The area of the leaflets was calculated using ImageJ software, and from the known values of transvalvular pressure for each HV, the transvalvular force was calculated (Tab. 3). The findings (Fig. 27) demonstrate consistency in thickness measurements compared to the transvalvular force measurements.

Table 3. Average thickness, mean area, transvalvular pressure, and transvalvular force of the four HVs.

	MITRAL (n=19)	AORTIC (n=18)	TRICUSPID (n=14)	PULMONARY (n=10)
Average thickness [µm]	584,61 ± 297,41	254,44 ± 135,66	296,41 ± 142,63	157,33 ± 68,51
Mean area [mm ²]	595,01±5,25	192,44±12,02	575,37±51,81	164,92±1,3
Transvalvular pressure [mmHg]	120	80	25	10
Transvalvular force [N]	9,51	2,05	1,91	0,21

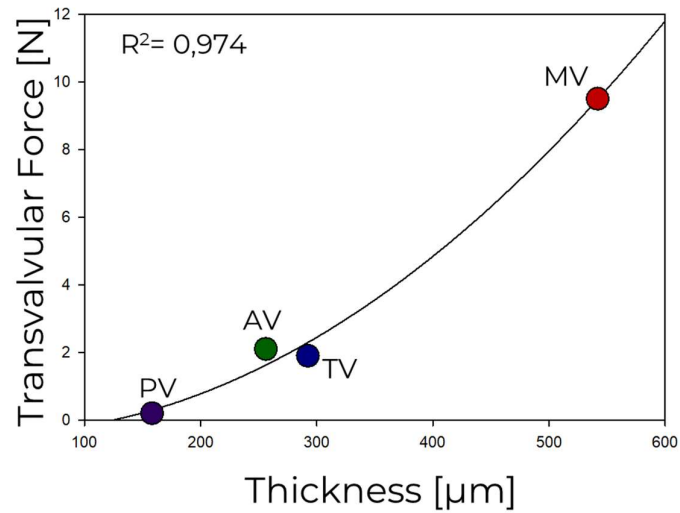


Figure 27. Human HVs thickness VS Transvalvular Force

Moreover, to further characterize the heart valves in terms of surface homogeneity, biquintic numerical interpolation (Matlab, MathWorks, Natick, MA) was used to obtain maps for native valves (Fig. 28) [36]. The thicknesses were measured experimentally at fifteen different locations of the leaflets, as previously described, and at an equivalent number of equally distributed locations around the leaflet edge.

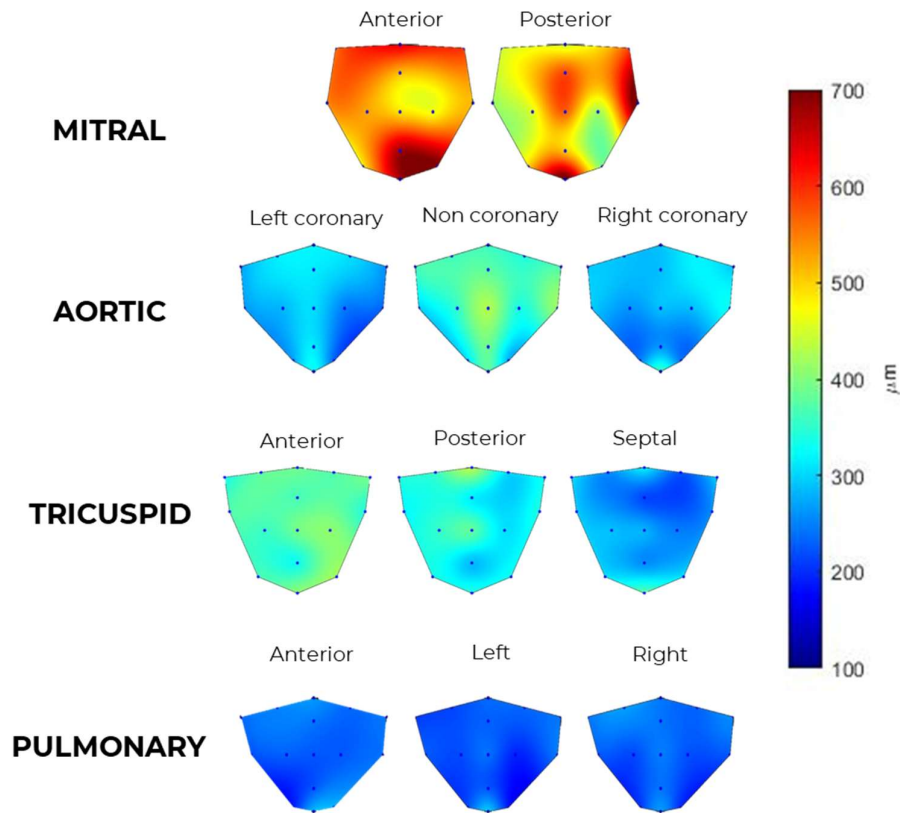


Figure 28. Human HVs thickness topological distribution

Mean valve thickness values were compared with two other species, ovine and porcine using identically testing protocols [36] (Fig. 29-30).

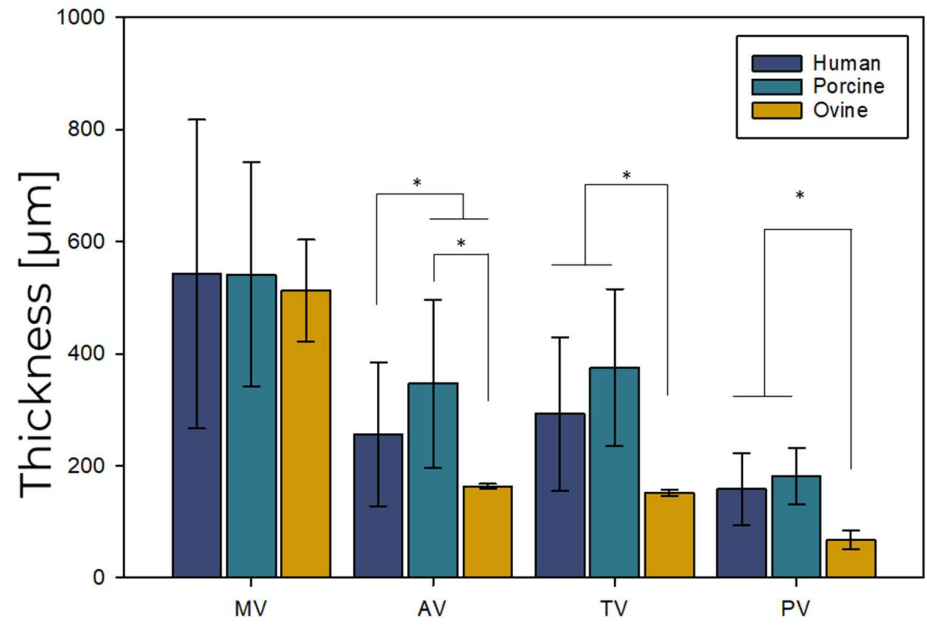


Figure 29. The thickness of HVs oh Human VS. Porcine [36] VS. Ovine

Porcine and ovine models are extensively utilized for the characterization of cardiovascular tissue due to their anatomical, mechanical, and physiological similarities to human tissue.

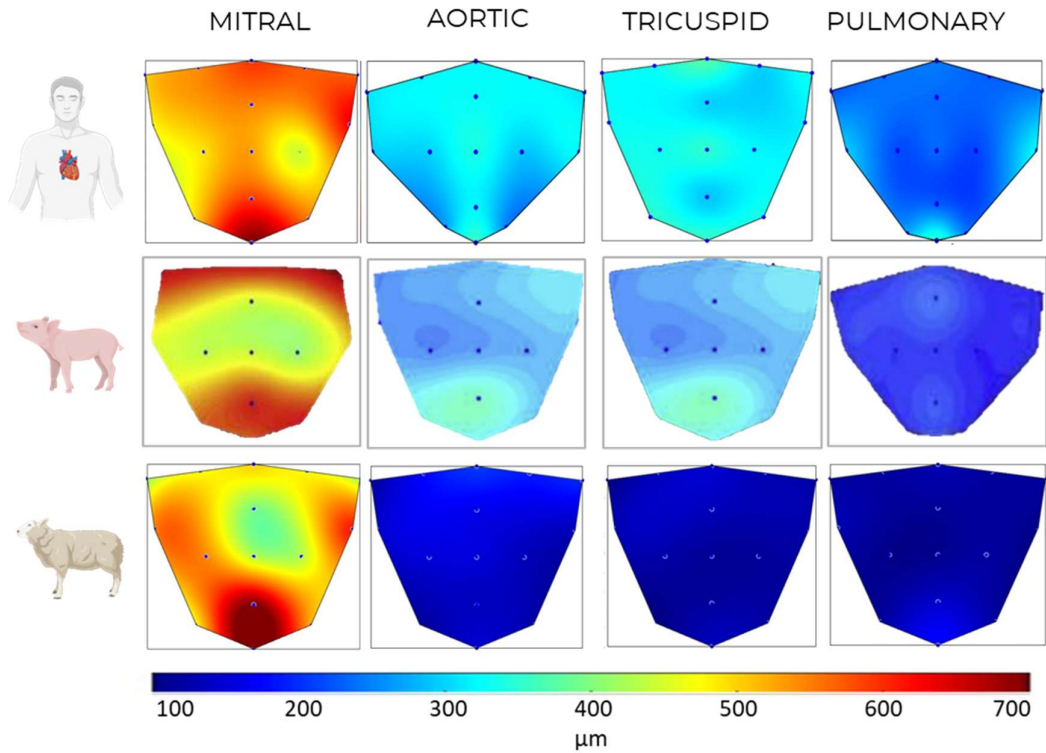


Figure 30. Human VS. Porcine [36] VS. Ovine HV thickness topological distribution

Notably, the anatomical configuration of the HVs and the dimensions of the heart leaflets exhibited by porcine are analogous to those observed in humans. Consequently, they have emerged as optimal subjects for biomechanics research and prosthesis design applications. Conversely, Ovine samples are frequently employed due to their ease of handling and compatibility with in vivo experiments, encompassing tissue regeneration studies and long-term evaluations. The two models permit the simulation of human-like hemodynamic states, offering an equilibrium between biological representativeness and experimental feasibility. A comparison of human and porcine valves reveals no significant differences in thickness measurements. However, ovine valves, which exhibited lower thickness values than the two other species, demonstrated a statistically significant variance.

3.1.2. Histological analysis

As previously mentioned, each HV leaflet has a specialized ECM that plays a critical role in maintaining proper valve function and providing highly anisotropic properties. A detailed microscopic analysis was performed to ascertain the composition of the different layers and the elements that play an important role in optimal valve function and long-term durability (Fig. 31).

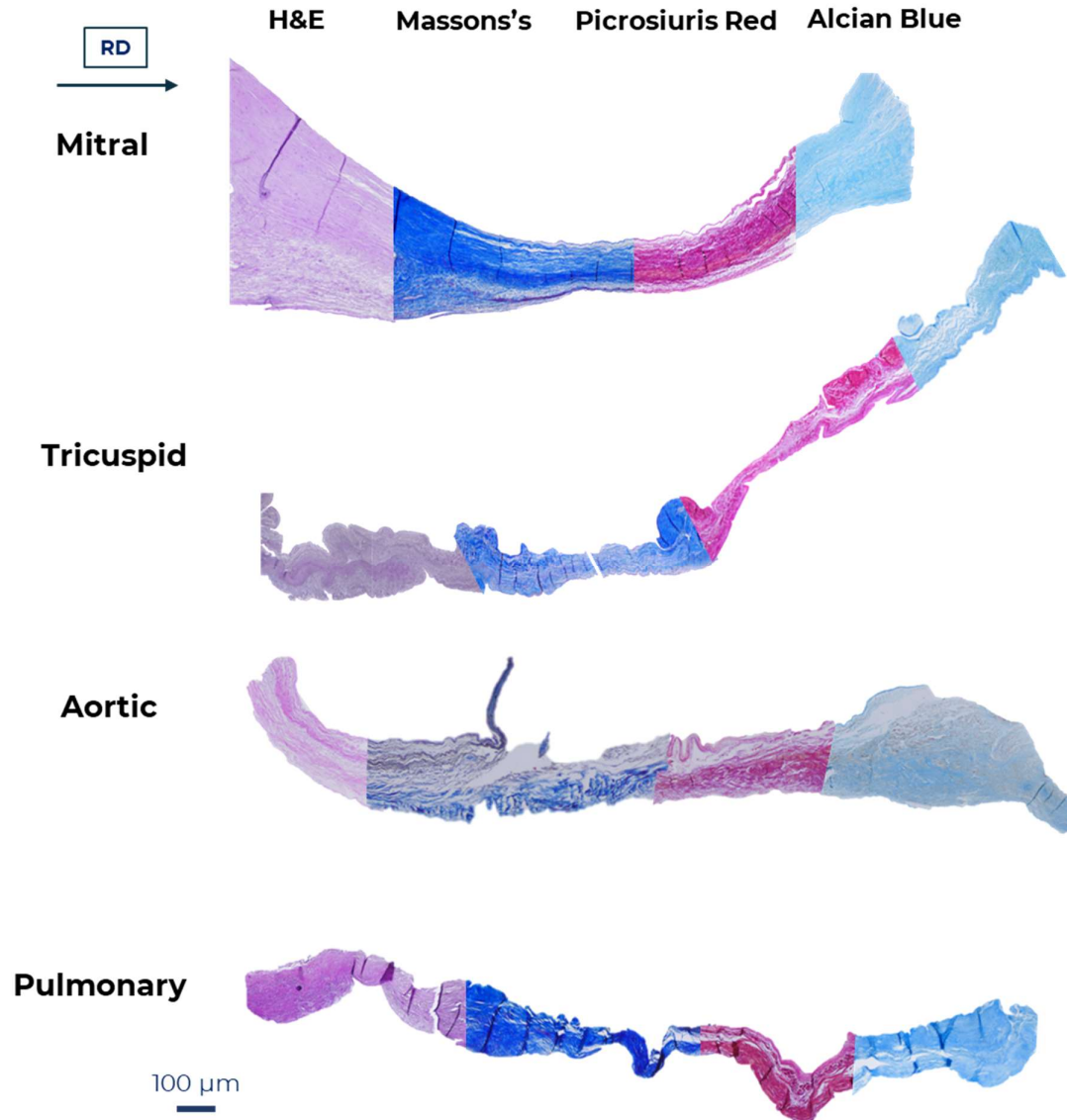


Figure 31. Histological staining technique

- The H&E staining method aims to quantify the cellularity and gross extracellular matrix (ECM) morphology of the samples (Fig. 32). The application of hematoxylin results in the formation of blue/purple stains on cellular components, including

nucleic acids, and cell membranes. Eosin stains red-pink components, including various cell proteins, mitochondrial proteins, and collagen fibers. For the MV and TV, H&E staining revealed thicker leaflets compared to semilunar valves, with well-defined fibrosa, spongiosa, and atrialis layers.

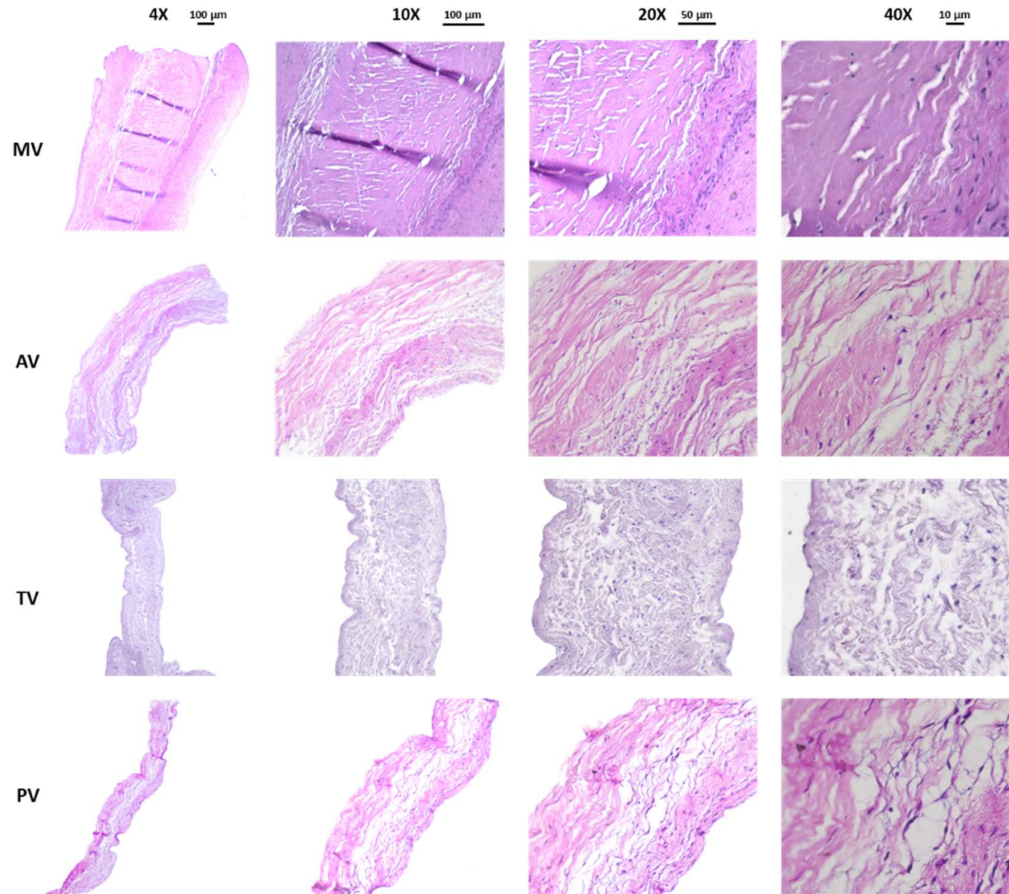


Figure 32. H&E on human HVs

- MT (Fig. 33) is a dye that is utilized to distinguish connective tissues from other structural components. It is capable of imparting blue pigmentation to collagen, a red color to the cytoplasm, and a black coloration to nuclei. MT highlighted a dense collagen network in the fibrosa, indicating high tensile strength in the ATVs.

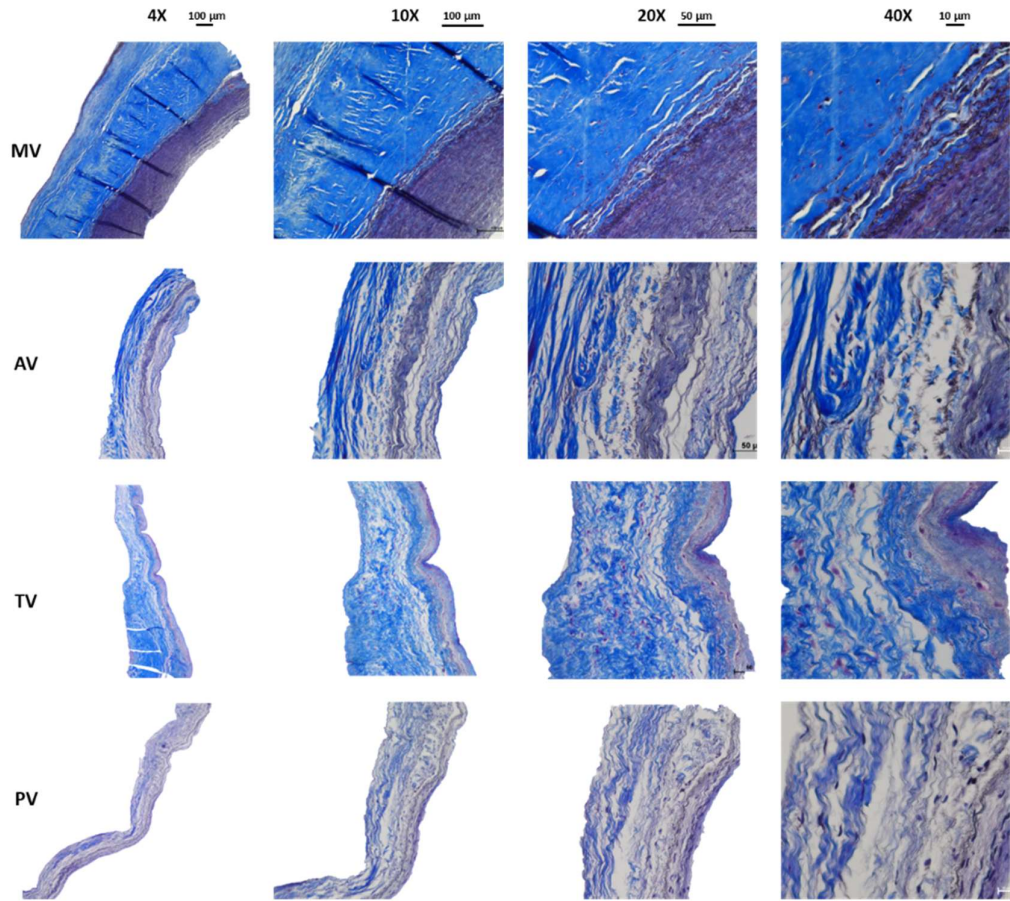


Figure 33. MT on human HVs

- The procedure known as PR (Fig. 34) is a staining technique that is specific to collagen. In this process, Sirius red dye is utilized, which exhibits an affinity for collagen fibers. Through observation under a polarized light microscope, this technique can be used to distinguish between the two types of collagens (types I and III). The technique demonstrated highly aligned collagen fibers, crucial for maintaining leaflet stiffness.

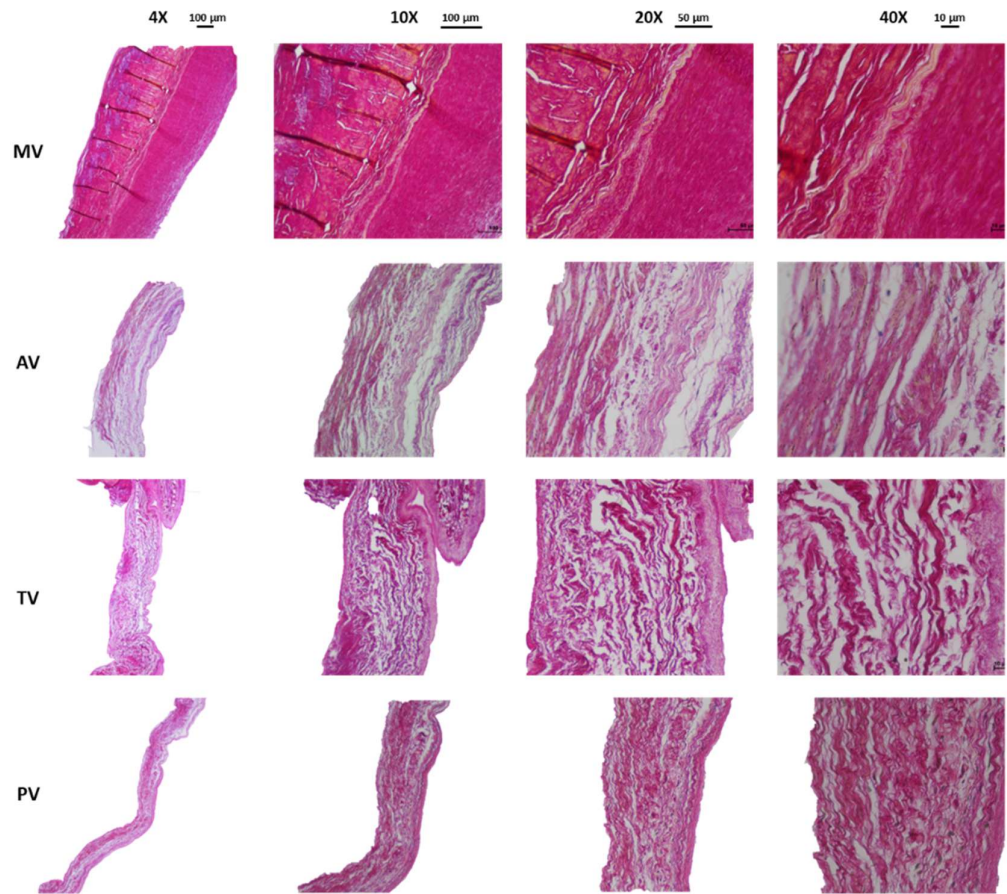


Figure 34. PR on human HVs

- AB (Fig. 35) is a staining method that highlights nuclei in pink and GAGs in blue, making it particularly useful for studying cartilage and tissues with a high mucous component. It is also useful for identifying changes in tissues with mucous secretion. In the SLVs, Alcian Blue staining indicated lower GAG content compared to ATVs, consistent with their role in rapid opening and closing.

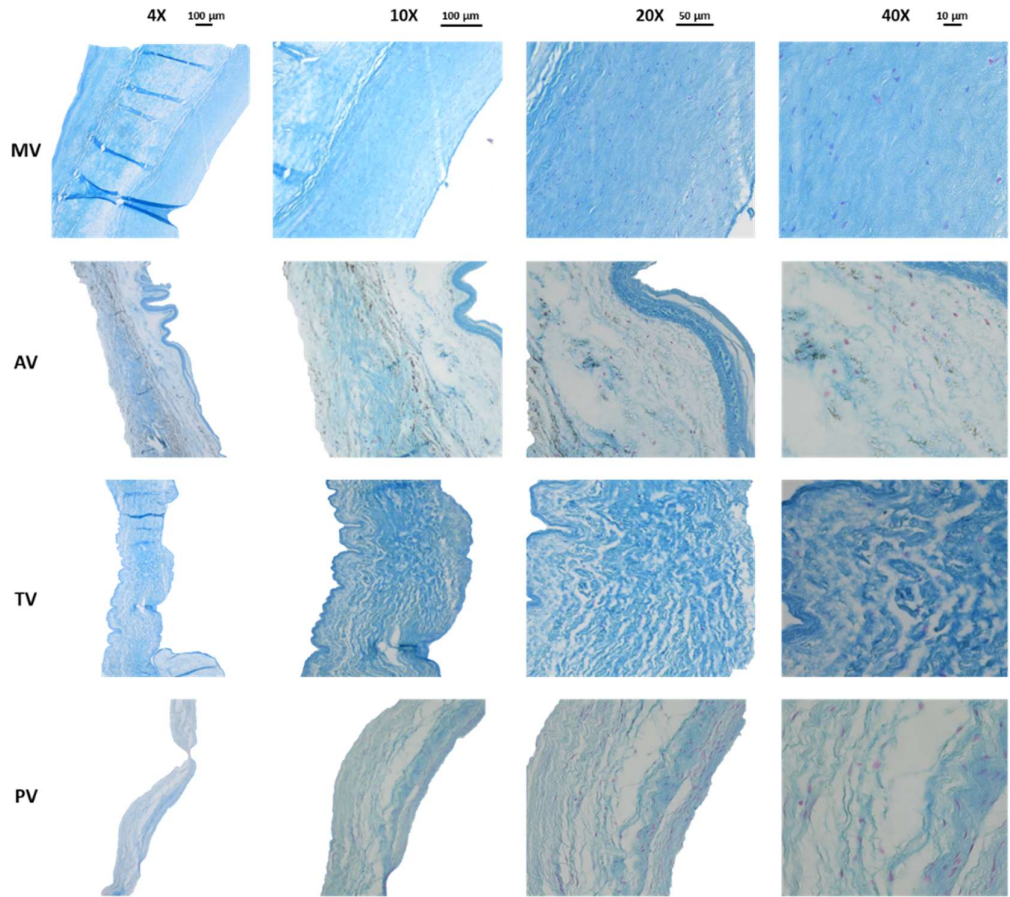


Figure 35. AB on human HVs

The Nikon optical microscope was utilized for the visualization of specimens and the acquisition of histological images. Image processing and analysis were conducted using the NIS-Elements software suited to ensure precise and detailed examination of histological features.

To conclude, Collagen quantity in leaflet cross-sections was estimated by digital image processing executed by implementing custom-made software developed in Matlab (Fig. 36). This outcome is noteworthy and is reflected in the thickness values analyzed above.

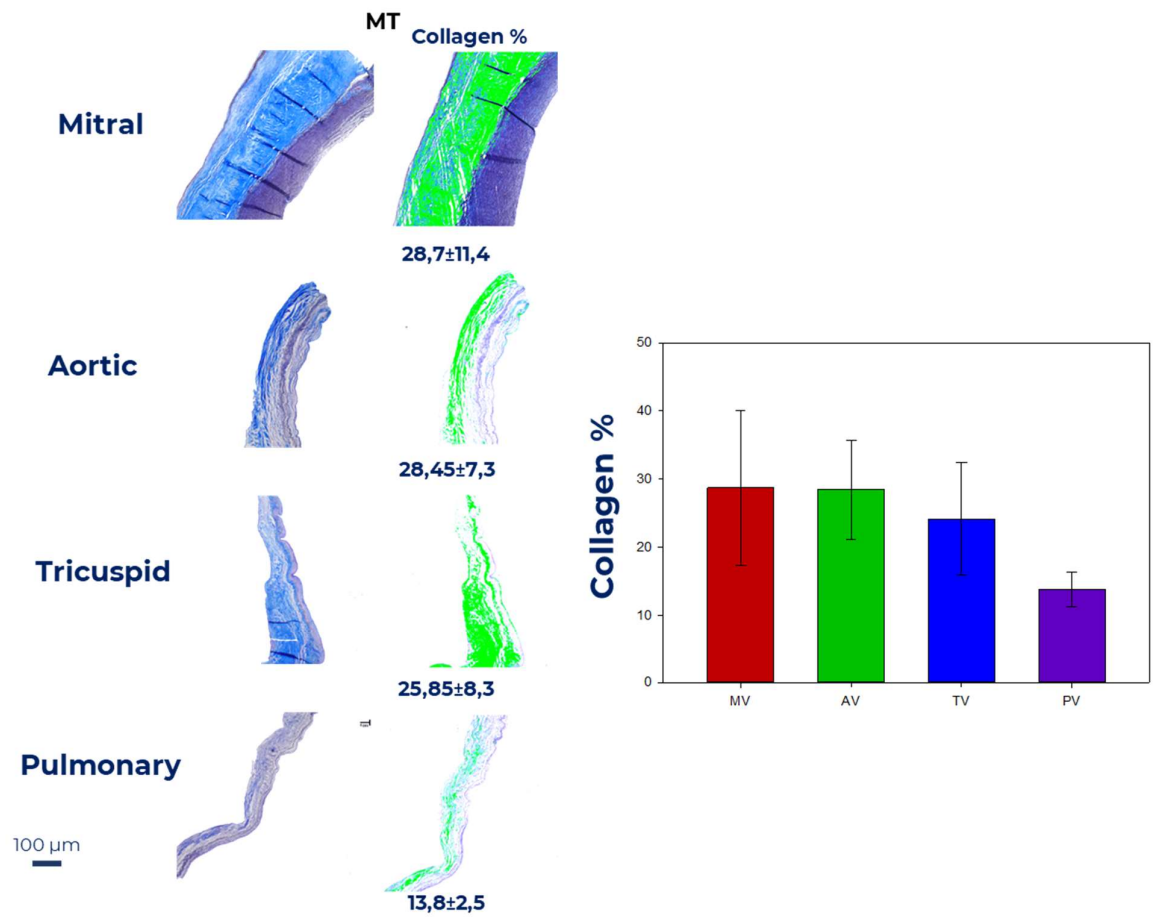


Figure 36. Human HVs collagen quantification

3.2. Structural analysis: microscopic level

3.2.1. SEM analysis

Following a 48-hour decellularization process, the structural integrity of the leaflets was visually intact. Decellularized human HV leaflets were analyzed using scanning electron microscopy (SEM) and histological analysis confirmed successful cell removal (Fig. 37). In this way, it was observed that the decellularization protocol effectively balanced the removal of cells with the preservation of the mechanical and structural properties of the ECM. In addition, the optimal decellularization protocol exhibited a substantial reduction in leaflet thickness in comparison to native tissue (Fig. 38). This substantial occurs subsequent to decellularization is chiefly attributable to the depletion of cellular components, which results in a reduction in the overall volume of the tissue.

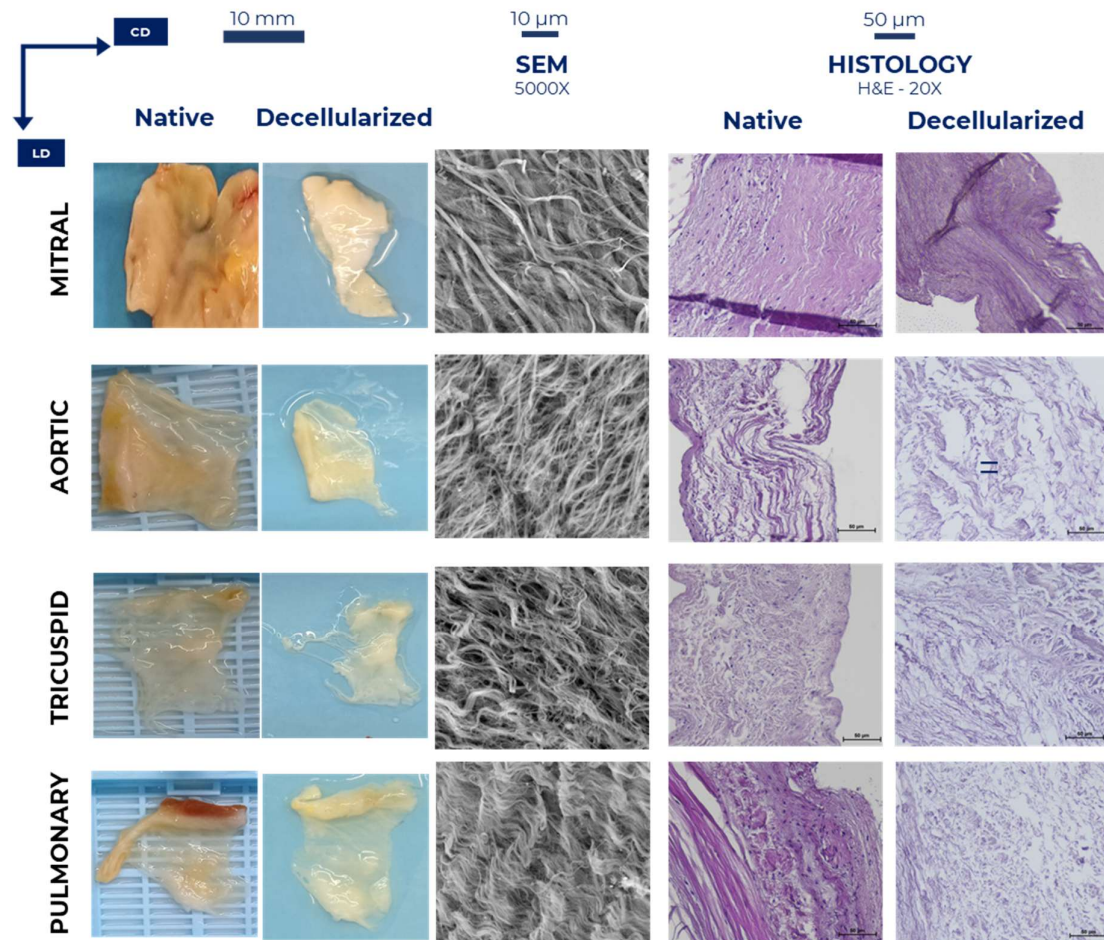


Figure 37. Decellularized Human HV - Surface analysis

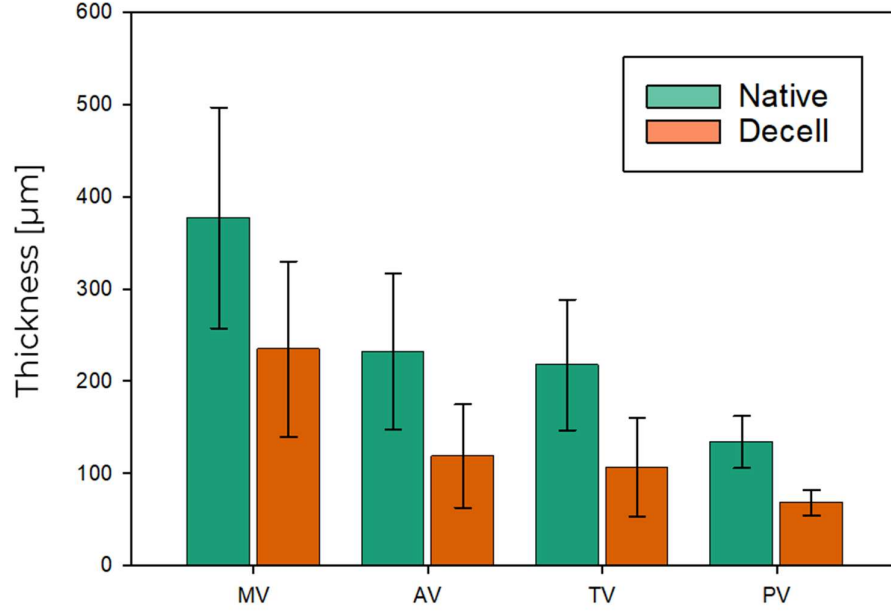


Figure 38. The average thickness of native VS decellularized leaflets

3.2.2. Fiber network analysis

Fiber angle distribution was quantified using an algorithm adapted by D'Amore et al. [45, 49] based on Chaudhuri et al. [50] and Karlon et al. [51] algorithm for quantifying structural parameters. The algorithm employs a finite element approach to model the mechanical behavior of elastomeric fibrous biomaterials. Starting at the microscale, individual fibers are represented as structural components with specific mechanical properties, accounting for their intrinsic elasticity and fiber-to-fiber interactions. These properties are then upscaled to predict the macroscopic behavior of the material. The model incorporates geometric constraints and nonlinear constitutive relationships to capture the tissue's heterogeneity and anisotropy. An iterative method is used to solve differential equations governing deformation and load response. This framework enables precise predictions of mechanical responses under various loading conditions, supporting the design and optimization of biomaterials for medical applications.

The OI confirms an anisotropic structure of the valves, and the level of fiber alignment doesn't give a statistical difference between the valves (Fig. 39).

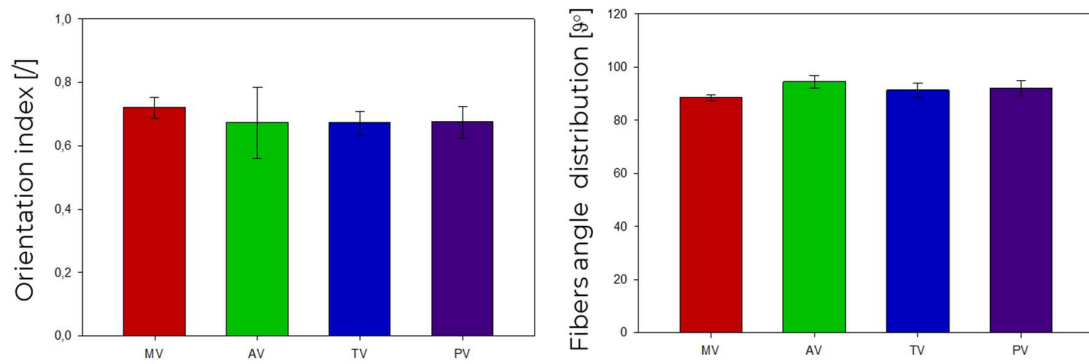


Figure 39. Individual HVs fiber orientation and distribution

The diameter of the collagen fibers was determined by utilizing the ImageJ analysis software. The analysis revealed that the MT and TV displayed significantly large fiber bundle diameters when compared to the AV and PV (Fig. 40).

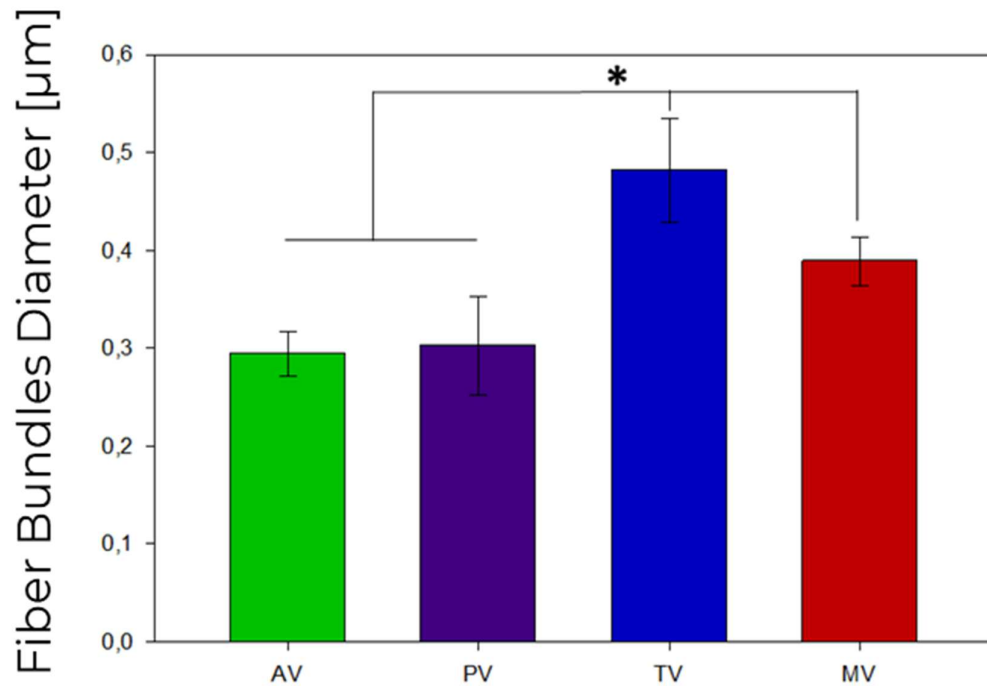


Figure 40. Human HVs Fiber bundles Diameter

A further study was carried out by differentiating the different zones (annulus, belly region, free edge) of each leaflet and then analyzing the differences per zone and valve in terms of OI, the distribution of fiber angles, and the diameter of fiber bundles. The two main surfaces of the leaflets were distinguished as shown in Figures 41-42.

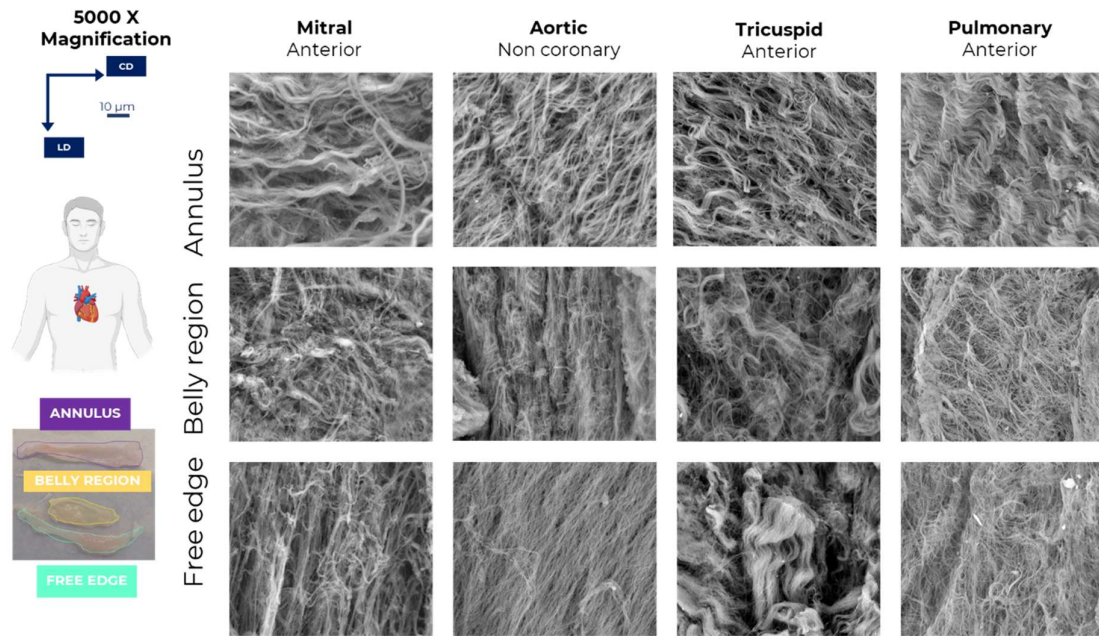


Figure 41. SEM analysis on different leaflet areas

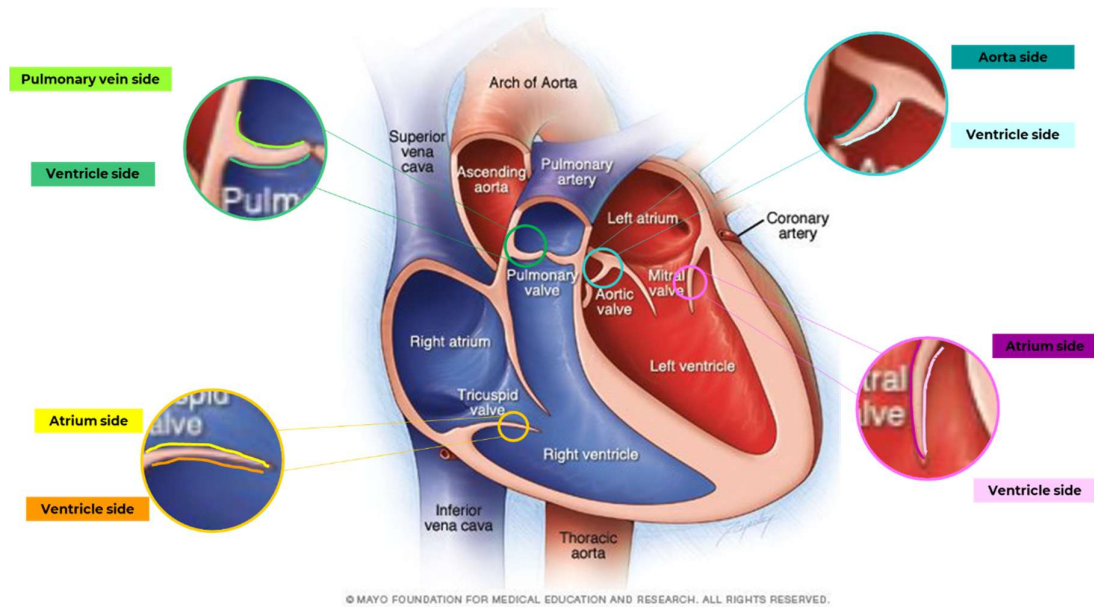


Figure 42. The different surfaces of the HV leaflets

The results demonstrate a striking uniformity between the various regions and surfaces, with negligible variations proving to be inconsequential for all the parameters analyzed. This finding suggests that the structural properties of the leaflets are designed to ensure biomechanical consistency across all regions, presumably to optimize their ability to respond to constant loads and deformations during the valve cycle. The angular fiber distribution, diameter, and orientation demonstrate comparable values across the various regions, thereby substantiating the hypothesis of homogeneous structural behavior (Fig. 43).

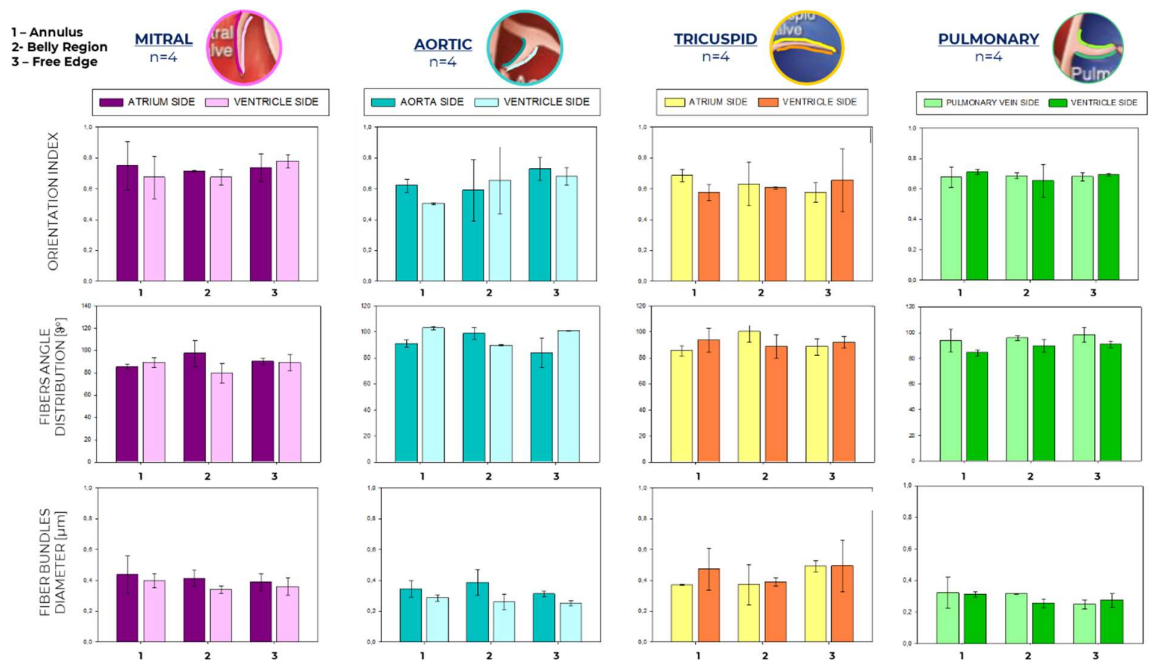


Figure 43. HV leaflets analysis per area

As was the case with the thicknesses, the values of the fiber bundle diameter were compared between species, and it was observed that human valves had the highest values compared to porcine and ovine (Fig. 44).

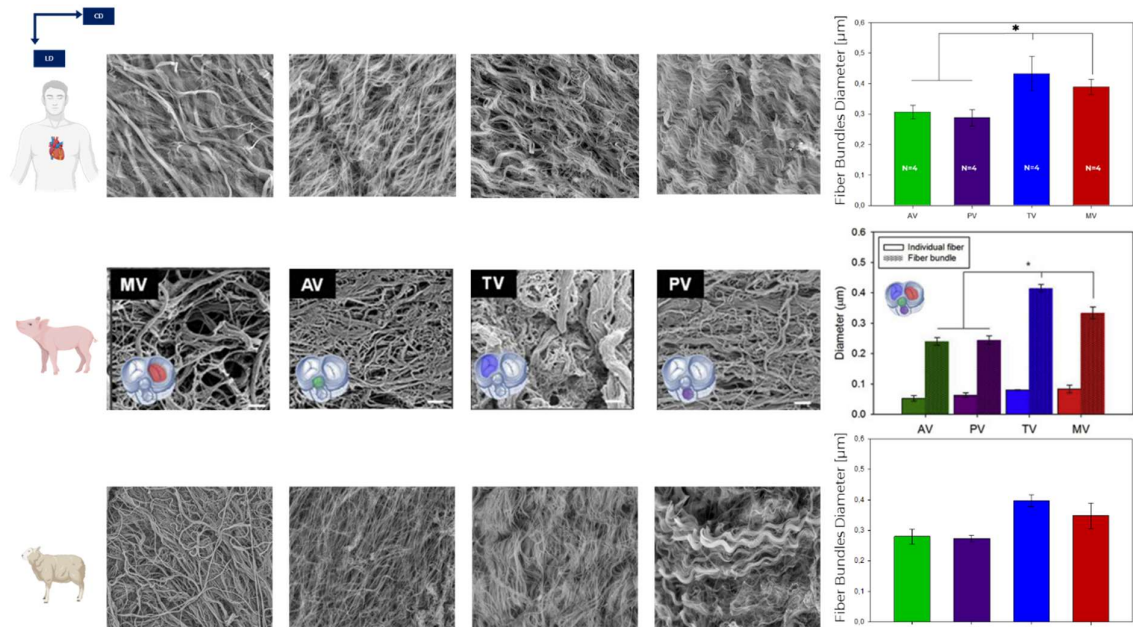


Figure 44. Fiber bundles Diameter Human VS Porcine [36] VS Ovine

3.3.Functional analysis: in-plane mechanics

The data processing was executed by implementing custom-made software developed in Matlab. This software utilizes the free-float position of the markers subsequent to preconditioning as a reference point. When compared to the SLVs, the ATVs exhibited greater stiffness along the RD (Fig. 45). The ATV HVs mechanical response reflects the different coaptation mechanisms driven by the action of the CT. In contrast, outflow tract valves rely on the sinus shape and higher leaflet anisotropy to properly close and prevent backflow.

Anisotropic behavior is particularly evident in human leaflets, which show a very pronounced stiffness in the CD (Fig. 46), hardly deforming, while in the RD, they are more flexible. Human valve leaflets are the most rigid in both the circumferential and radial directions. This suggests a strongly organized and dense tissue structure, probably due to the higher age of the donors and the calcification observed. While the findings provide valuable insights into the valvular structure and calcification processes, it is important to consider the representativity of the studied population. The patients analyzed may not fully reflect a healthy adult population, as factors such as age, comorbidities, and lifestyle choices (e.g., diet, smoking, physical activity) could influence valvular remodeling and calcification patterns. Future studies should include a control group of strictly healthy individuals to better isolate normal baseline characteristics. Additionally, potential sex-based differences in valvular composition and disease progression warrant further investigation.

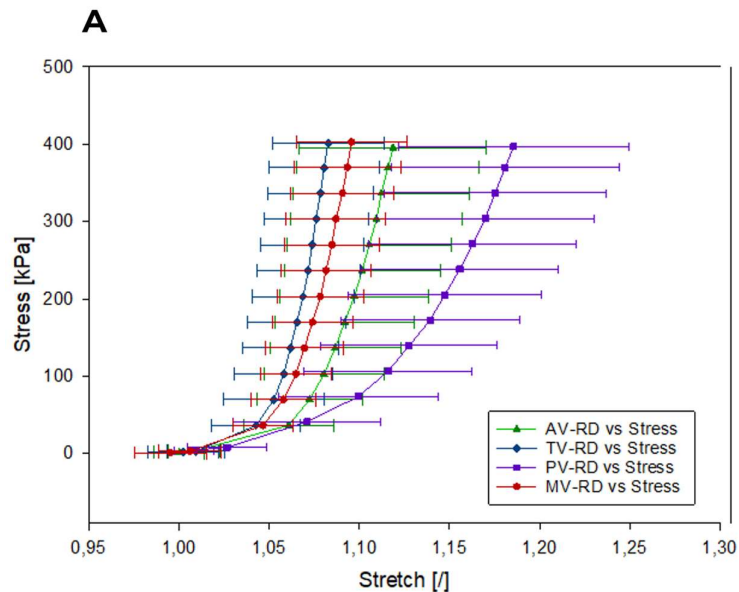


Figure 45. Stress-strain curves for each valve leaflet were generated using the equi-stress protocol in the radial direction

(A)

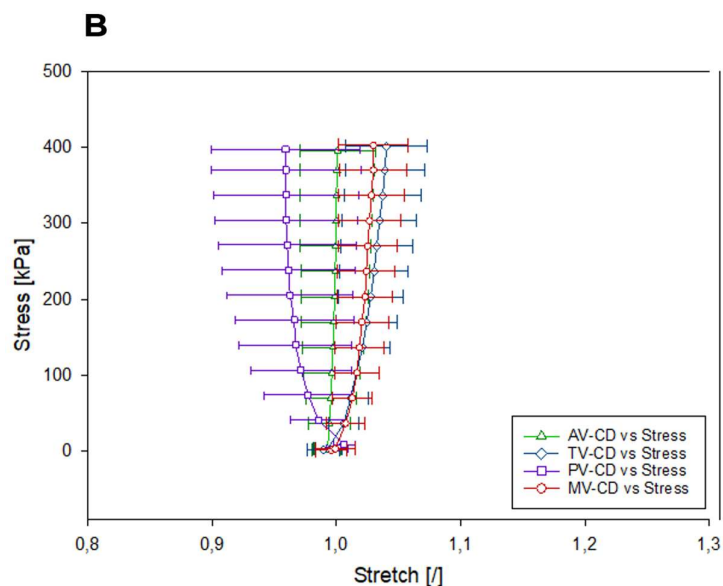


Figure 46. Stress-strain curves for each valve leaflet were generated using the equi-stress protocol in the circumferential directions (B)

An examination of the behavior exhibited by each valve has resulted in the following findings (Fig. 47-50):

- MV: it is evident that RD behavior demonstrates a greater degree of stiffness in comparison to the CD. The RD curve exhibits a higher stress level for a given stretch compared to the CD curve. Conversely, the CD behavior demonstrates enhanced compliance, as evidenced by a flatter curve at the initial stage.

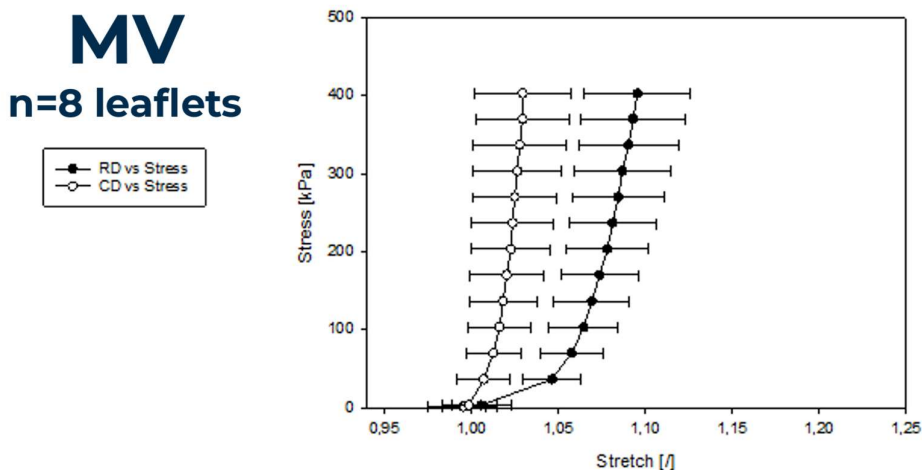


Figure 47. MV stress-strain curves in RD and CD

- The TV displays marked similarities with the MV in terms of anisotropic behavior, which manifests as increased stiffness in the RD. This is

accompanied by a greater dispersion of data, as evidenced by wider error bars, indicative of heightened inter-individual or leaflet variability.

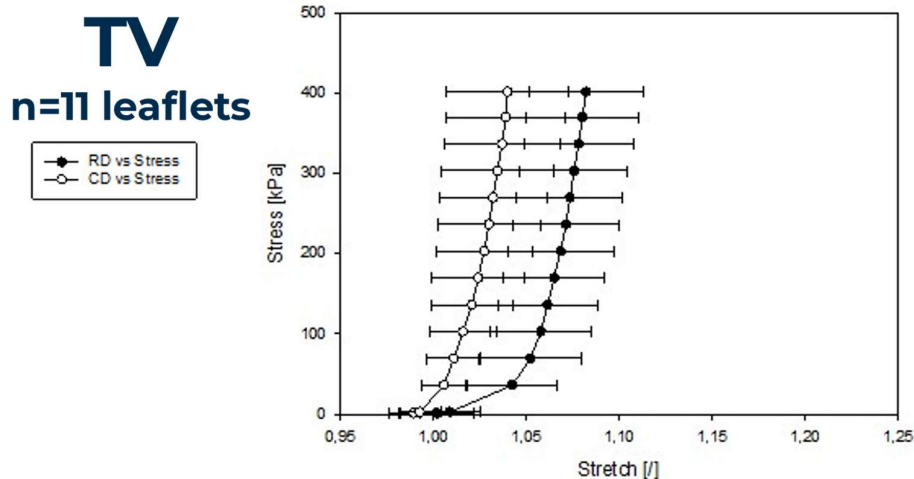


Figure 48. TV stress-strain curves in RD and CD

- The AV exhibited marked stiffness in both directions. The curves demonstrate reduced anisotropic behavior in comparison to the MV and TV, indicating enhanced structural homogeneity between the RD and CD. Initial compliance is reduced. The tissue is less deformable at low-stress values, probably in order to resist the high-pressure load during the cardiac cycle, or because of the nature of pathological tissues.

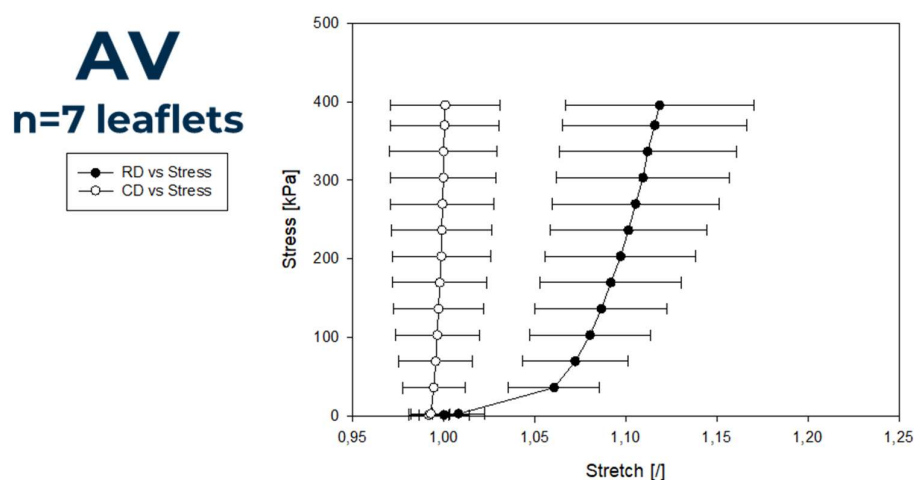


Figure 49. AV stress-strain curves in RD and CD

- The PV exhibits a significant disparity between the RD and the CD. This last manifests remarkably high compliance (exhibiting remarkably flat curves), while RD exhibits considerable stiffness. The observed behavior deviates from the standard physiological range, with the CD stretch diminishing below

1.0. The presence of substantial variability in PV is attributable to the limited number of samples ($n = 3$), suggesting that further investigation is required to refine understanding.

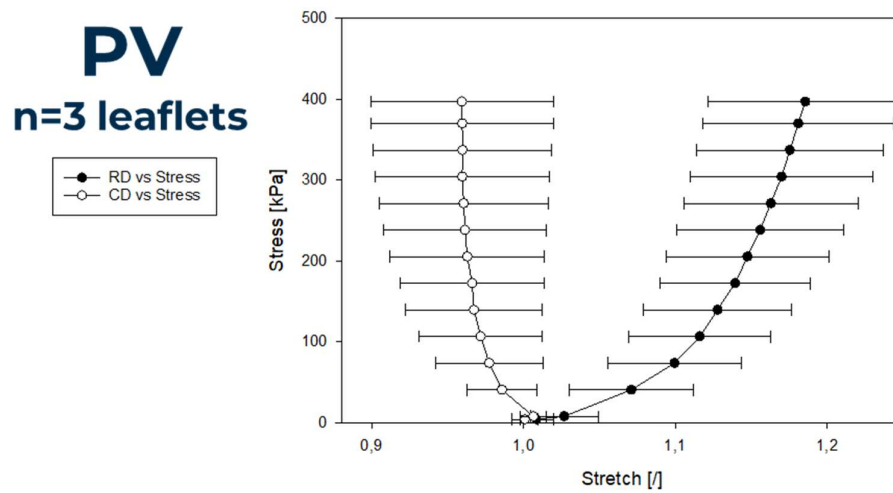


Figure 50. PV stress-strain curves in RD and CD

3.4.Functional analysis: out-of-plane mechanics

The deformation of leaflets is characterized by the concurrent occurrence of bending and tensioning, exhibiting complex behaviors. The three-point bending method facilitates the emulation of dynamic loading conditions, thus offering a more comprehensive understanding of the mechanical behavior of valve tissue. In the case of all HVs, the surface facing the direction of high-pressure flow is characterized by greater rigidity. This phenomenon is particularly evident in the case of ATV, where the AC surface is the ventricular one. It should be noted that these surfaces demonstrate the highest values of Young's modulus (Fig. 51) with a curvature limit of $\Delta k_{max} = 0.12$ mm. The WC surface is more elastic and flexible because it has a different structure (Fig. 52).

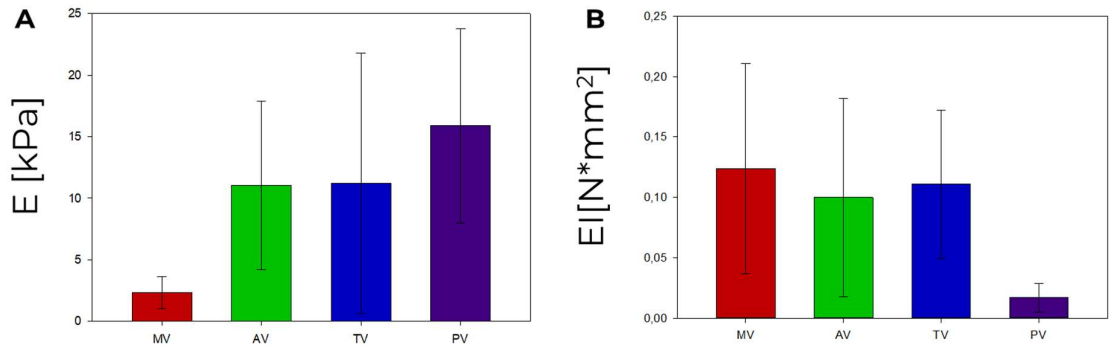


Figure 51. HVs results after the three pointing bending tests in the surface AC

A) Bending Elastic Moduli B) Bending Rigidity

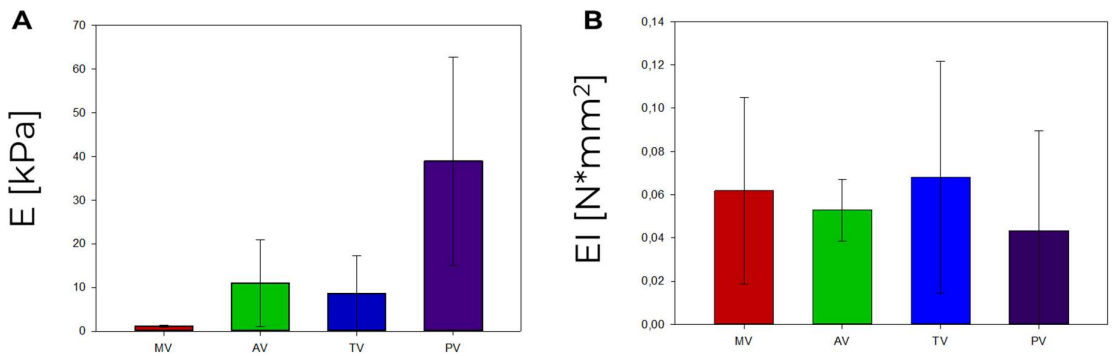


Figure 52. HVs results after the three pointing bending tests in the surface WC

A) bending Elastic Moduli; B) Bending Rigidity

4. DISCUSSION

This study utilized advanced methodologies to analyze human heart valve tissues, with the objective of characterizing their structural and mechanical properties. This analysis contributed significantly to the advancement of knowledge regarding their biomechanical functions. Notably, fresh human tissue was utilized, a significant advancement that overcomes the limitations of previous studies predominantly conducted on animal models or frozen cadaveric specimens [1]. The use of fresh tissue ensured the preservation of the original structural and mechanical properties, thereby enhancing the accuracy of the collected data and their future applicability in the design of valve prostheses for clinical use. The research findings provide a comprehensive characterization of the thickness of the leaflets of the four human HVs. The samples were obtained from fresh explanted specimens within eight hours of explantation. This is in distinction to the study by Pham et al. [1] which analyzed human samples from frozen or cadaver tissues, and reported increased thickness of the MV compared to the other. However, this study improves upon their work by integrating transvalvular force measurements, offering a more comprehensive understanding of the structure-function relationship in heart valves. The results showed that the average thickness of the MV is significantly greater than that of the other valves. This observation was interpreted as a functional adaptation that enabled the valve to withstand higher hemodynamic loads. This finding aligns with the observations of Pham et al. who suggested that the higher-pressure conditions present in the left heart may contribute to the increased thickness of its leaflets. These results significantly contribute to the validation of numerical models and the design of prosthetic devices that better mimic physiological conditions [52]. Porcine leaflets thickness is comparable to that of human valves, making this species a particularly suitable animal model for studies requiring high structural representativeness, such as prosthetic valve design and validation of biomechanical models. On the other hand, probably due to physiological adaptations related to different hemodynamic conditions or intracardiac pressures, ovine valve leaflets have significantly lower thickness than both human and porcine valve leaflets. It is therefore fundamental to select the most suitable animal model in accordance with the specific objectives of the study.

A multitude of research papers focus separately on valve thickness or transvalvular forces [3, 10, 48, 53], the present study integrates these two variables, thus providing a new perspective on the relationship between HV structure and function. This approach provides

a more comprehensive understanding of the role that thickness plays in mechanical strength and stress distribution during the cardiac cycle.

Histological images obtained using H&E, MT, PR, and AB staining techniques allowed a clear distinction of the layers and major components of the MV, AV, TV, and PV. Collagen quantification, performed using an algorithm on MT images at 4x magnification, showed values of 28.7% in the MV, 28.45% in the AV 25.85% in the TV, and 13.8% in the PV. These results are consistent with previous studies conducted in animal models and merit further investigation to ensure full compliance with the extant literature [54-57]. Data from Mega et al. [58] confirmed morphological differences such as lower fibrous density in the pulmonary leaflets. Additionally, the analysis of collagen distribution conducted with PR could be further investigated to compare the results with the Rich and Whittaker studies [59], which distinguish between type I and type III collagen fibers. Finally, the histological analysis approach used overlaps with those described by Cui et al. [60], who studied the morphology and organization of the HV leaflets using advanced techniques, and Schoen [61], who highlights the link between ECM composition and valve functionality.

The decellularization protocol employed in this study successfully maintained the structural integrity of the heart valve leaflets, as suggested by histological analysis and surface analysis of the samples using SEM. The efficacy of the detergents applied in removing cells was confirmed, validating the idea that the combination of chemical, enzymatic, and mechanical approaches on the tissue is paramount to achieve a complete decellularized tissue [19, 62, 63]. The present findings are in alignment with those of the studies conducted by Brown, Cramer, and Badylack [64, 65], which demonstrated that the preservation of the structure of the ECM is indispensable for the maintenance of the mechanical and biological characteristics of decellularized scaffolds. The application of the fiber alignment analysis algorithm, based on the D'Amore algorithm [49], enabled the characterization of fiber networks in detail, thereby unveiling anisotropic and homogeneous behavior in various zones of leaflets, including the annulus, the belly region, and the free edge zone. It's been revealed also that the fibers were more uniform across the various zones of the leaflets, which is important for the functionality of the leaflets during the continuous stress of the heart cycle. Consequently, this validates the necessity for the design of a heart valve to be anchored on these parameters. Furthermore, the results of the present study showed a larger diameter of collagen fiber bundles in human valves compared to porcine or ovine valves, consistent with the reports of Liao et al. [39] and Gong et al. [66], who observed interspecies differences in HVs structure and biomechanical strength. The majority of studies have

focused on animal decellularized tissues of animal origin [62, 66, 67], but the present work is innovative in that it provides an in-depth and comprehensive structural analysis of fresh human samples, which is something that has not been explored before.

Biaxial tests have revealed significant anisotropic properties within HVs, particularly regarding increased tissue stiffness in the CD relative to the RD, confirming the anisotropic nature of valve tissue. This observation, consistent with Liao et al. [39], supports the hypothesis that atrioventricular valves develop increased stiffness to withstand high transvalvular forces. However, the analysis of semilunar valves revealed greater compliance in the radial direction, suggesting an adaptive mechanism to blood flow variations that has not been adequately described in the existing literature. This heightened radial stiffness is particularly evident in ATVs, underscoring heart valves' distinct functional roles [47, 68, 69]. The mechanical response of the ATVs exhibited greater radial stiffness in comparison to that of the SLVs. This phenomenon can be attributed to the presence of the CT, which plays a crucial role in the coaptation mechanism of the MVs and TVs [70]. An observation of particular interest is that of the PV, which is subject to significantly lower pressure loads than other valves. The increased compliance of this valve in the radial direction may indicate a functional adaptation of the valve to support a physiological load and a reduced stress rate. The native tissues may exhibit a reduction in tensile stress or a loss of mechanical relaxation when there is a partial disassembly of collagen fibers and a decrease in the density of structural components in the preferred orientation of these fibers, which is referred to as CD [46]. Following periods of high stress, a contraction of the tissue is observed, and this results in a reorganization of the fiber constituents. This process consequently leads to the occurrence of structural anisotropy.

Biaxial mechanical tests demonstrated that the complex organization of human tissue is reflected in the anisotropic behavior of heart valve leaflets. These exhibited marked differences when compared to those observed in animal models and engineered tissues. In addition, an increase in mitral tissue stiffness with patient age was documented by Pham and Sun, thereby highlighting how the mechanical response of valves is influenced by factors such as age and the use of frozen and/or pathological tissue [1, 71, 72]. Frozen valve tissues exhibit significant alterations in structure and mechanics, including a loss of elasticity and changes in collagen microstructure, and these alterations can compromise the biomechanical response of the tissues [1, 47]. Conversely, fresh tissues maintain the integrity of native properties, including proper anisotropy and stiffness, thereby providing more representative data for prosthesis design and advanced biomechanical studies. These observations

underscore the necessity of employing fresh tissue to ensure the acquisition of more reliable measurements. Such studies are instrumental in modeling the properties of human tissues and developing advanced engineered solutions [34, 65, 68, 72]. The present study is distinguished by the analysis carried out on fresh specimens, which has provided detailed characterization of the mechanical properties of heart valves. The interaction between stiffness, direction of loading, and structural organization represents significant advances in the field, offering a comprehensive understanding of the biomechanical behavior of heart tissue.

Finally, the three-point bending tests facilitated the evaluation of the bending stiffness, thereby enabling the measurements of the out-of-plane properties of human HVs. Furthermore, the tests allowed additional characterization, achieved by distinguishing the surfaces of each leaflet under the test. As demonstrated in the relevant literature, the AC surface, when subjected to elevated levels of flow and, consequently, increased pressure, exhibits higher stiffness. This enhanced rigidity is hypothesized to be caused by a more compact and organized arrangement of collagen fibers. It is important to note that the interactions that take place between the layers present on the valve leaflet surfaces are responsible for a significant mechanical response. These interactions provide essential mechanical support, ensuring the valve leaflet maintains its structural integrity and resists degradation throughout the cardiac cycle, thereby ensuring effective distribution of loads and reduction of localized stress [73-75]. Additionally, the fiber's orientation influences the bending stiffness and mechanical response, thereby enhancing the resistance to loads due to native tissue anisotropy present in native tissues [35, 76, 77]. Moreover, the values of stiffness are consistent with those found for the OI (Fig. 38). Consistent with biaxial mechanical testing, the ATVs exhibited greater flexural stiffness (EI) compared to SLVs. This increased stiffness likely reflects the structural adaptation of the atrioventricular valves to withstand the high-pressure gradients across the atrioventricular valve connection (Fig. 50-51). This observation aligns with the measurements obtained from the quantification of the valve fiber bundle diameters (Fig. 39), confirming the structural differences between the atrioventricular and semilunar valves.

5. LIMITATIONS

Despite the valuable insights gained from this study, several limitations must be acknowledged. Firstly, the sample size may not be large enough to fully represent the variability in heart valve characteristics across a broader population, potentially introducing selection bias. Secondly, sex-based differences in valvular composition and disease progression were not fully addressed in this study. Given that male and female hearts exhibit distinct remodeling patterns, future research should include sex-stratified analyses to enhance the clinical applicability of the findings. It is therefore crucial that these limitations are recognised, as they are pivotal in refining methodologies and ensuring that future studies provide more comprehensive and translationally relevant data.

6. FUTURE DEVELOPMENTS

The results of this work provided data that can be applied directly to the design of valve prostheses intended for use in clinical practice. One of the most promising future directions in this field is the integration of the collected biomechanical data into computational models with the aim of optimizing prosthetic valve designs. By incorporating precise measurements of anisotropy, collagen fiber alignment, and regional stiffness variations, finite element simulations can more accurately predict prosthetic valve performance under physiological conditions, improving durability and reducing the risk of complications such as leaflet tearing or calcification. Structural characterization will be advanced through the quantification of parameters such as network porosity measured directly on human tissue. This study indicates a necessity for more personalized valve designs, as current prosthetic valves frequently exhibit standardized geometries and mechanical properties. However, the observed disparities between atrioventricular and semilunar valves suggest the requirement for a tailored approach. Patient-specific prostheses, conceptualized using comprehensive biomechanical data, have the potential to deliver enhanced hemodynamic performance and prolonged durability, particularly in pediatric and young adult patients who necessitate dynamic, growth-adaptive solutions. The findings of this study are particularly pertinent for tissue-engineered heart valves (TEHVs), where the scaffold materials and fiber architectures could be designed to more closely mimic the native structural and mechanical behavior described in this study. Moreover, a comparison of human, porcine, and ovine valves underscores the significance of opting for suitable animal models in the context of preclinical testing. The parallels between human and porcine valve mechanics underscore the pertinence of porcine models for research into prosthetic valves. Conversely, ovine models may offer more effective avenues for investigating remodelling and regenerative capabilities. These observations have the potential to guide regulatory agencies and medical device manufacturers in refining their validation protocols with a view to enhancing the translational success of new heart valve technologies.

The optimized decellularization protocol could also be applied to assess the mechanical properties of tissues after the procedure, ensuring that their structural integrity and functional responses are preserved.

In addition, the study of the cellular proteome could contribute to the elucidation of the molecular mechanisms behind the disease, such as calcific aortic stenosis, mitral valve prolapse, and congenital valve malformations, thanks to advances in technologies that allow

the comprehensive identification and measurement of proteins in complex biological systems. This will enable correlations between microscopic histoarchitecture, biochemical composition, and large-scale biomechanical properties of human HVs.

7. CONCLUSIONS

This thesis aimed to provide a comprehensive structural and functional characterization of human heart valves, focusing on both native and decellularized tissues. By integrating histological, biomechanical, and imaging analyses, the study examined key aspects such as leaflet thickness, extracellular matrix composition, and mechanical behavior under physiological conditions. By utilizing fresh human tissues and applying innovative decellularization protocols, this work overcomes the limitations of prior studies based on animal models and cadaveric specimens. The integration of advanced biomechanical analyses and computational modeling further enhances the understanding of valve function and durability.

In conclusion, this research has yielded substantial findings that significantly contribute to the fields of regenerative medicine and biomedical engineering, offering novel prospects both clinically and industrially. From a clinical standpoint, the results of this study can be used to design prostheses to meet the specific needs of pediatric and adult patients. This is achieved by leveraging insights into the unique structural and functional properties of native human tissues, enabling the development of biomimetic and patient-specific solutions. Moreover, the integration of biomechanical analysis and decellularization techniques provides a robust foundation for the development of optimized scaffolds for recellularization, consequently enhancing the efficacy of regenerative therapies. From an industrial perspective, collecting mechanical and structural parameters serves as a significant benchmark for innovation in the field, and also as a foundation for the design and fabrication of advanced biomaterials, including hybrid polymers and nanocomposites. These biomaterials have been developed to enhance the durability and functionality of prosthetic devices. Concurrently, collecting HVs structural and mechanical data provides a valuable foundation for computational models, such as finite element analysis (FEA) and fluid dynamic models. These models facilitate the optimization of prosthetic valve designs in a preliminary phase, thereby reducing the development timeline and expenses. This integrated approach, combining the strengths of scientific research and technological development, holds significant potential for enhancing the available biomedical solutions in the market. By integrating fundamental biomechanics with clinical applications, this research initiative has the potential to pave the way for the development of more durable, biomimetic, patient-specific heart valve solutions. Ultimately, the goal is to improve long-term outcomes and quality of life for patients requiring valve replacement.

APPENDIX

1. Human heart overview

C	Human heart (MM/DD/YY)	AGE	GENDER	WEIGHT	CAUSE OF TRANSPLANT/DEATH	SUPPLIER	Valve
		year		[kg]			
1	HH012722	65	Female	70	Severe left ventricular dysfunction in the context of severe dilation, with proper operation of the mitral valve prosthesis.	ISMETT	AV TV PV
2	HH030422	51	Male	77	Orthoptic heart transplant in dilated ischemic cardiomyopathy.	ISMETT	MV AV TV PV
3	HH042722	64	Female	75	Non-structural hypertrophic cardiomyopathy, limiting pattern.	ISMETT	MV AV TV PV
4	HH052622					ISMETT	MV
5	HH053022	48	Female	47	Hypertrophic cardiomyopathy with left ventricular dysfunction. Advanced heart failure	ISMETT	MV AV TV PV
6	HH061022					ISMETT	MV
7	HH092022					POLICLINICO	MV
8	HH092722	46	Female	67	Post-partum dilated cardiomyopathy with progressive reduced systolic function.	ISMETT	MV AV PV
9	HH113022	64	Male	86	Advanced heart failure in post- ischemic dilatory heart disease with severe left ventricular systolic malfunction.	ISMETT	MV AV TV PV
10	HH012023					ISMETT	MV
11	HH021523	42	Male	75	Advanced heart failure	ISMETT	MV AV TV

							PV
12	HH032923	55	Male	78	Advanced heart failure	ISMETT	MV
							AV
							TV
							PV
13	HH061423	43	Female	49	Post-partum dilated cardiomyopathy with progressive reduced systolic function.	ISMETT	MV
							AV
							PV
14	HH071423	62	Male	85	Advanced heart failure. Post ischemic cardiomyopathy	ISMETT	MV
							AV
							PV
15	HH082923	57	Male	79	dilated cardiomyopathy	ISMETT	MV
							AV
							PV
16	HH083023	57	Male	82	Hypertrophic cardiomyopathy with left ventricular dysfunction. Advanced heart failure	ISMETT	MV
							AV
							TV
							PV
17	HH091923	52	Male	67	Advanced heart failure. Post myocarditis	ISMETT	MV
							AV
18	HH020724	53	Male	83	Advanced heart failure in post-ischemic dilatory heart disease with severe left ventricular systolic malfunction.	ISMETT	MV
							AV
19	HH031324	62	Female	70	dilated cardiomyopathy	ISMETT	MV
							AV

20	HH031424	51	Female	70	Hypertrophic cardiomyopathy . Advanced heart failure	ISMETT	MV
							AV
21	HH031824					ISMETT	MV
							AV
							TV
22	HH100224	35	Female	69	Advanced heart failure. Post myocarditis	ISMETT	MV
							AV
							TV
							PV
23	HH092424	65	Male	86	Head trauma	PITTSBURGH	MV
							AV
							TV
							PV
24	HH092524	55	Female	105	ANOXIA Mild mitral regurgitation	PITTSBURGH	MV
							AV
							TV
							PV
25	HH100224	60	Female	120	ANOXIA	PITTSBURGH	MV
							AV
							TV
							PV
26	HH100524	53	Male	129	CEREBROVASCULAR/STROKE Mild tricuspid regurgitation	PITTSBURGH	MV
							AV
							TV
							PV
27	HH101524	65	Female	108	CEREBROVASCULAR/STROKE	PITTSBURGH	MV
							AV
							TV
							PV
28	HH101924	68	Female	62	ANOXIA Mild tricuspid regurgitation	PITTSBURGH	MV
							AV
							TV
							PV
29	HH110124	62	Female	102	ANOXIA	PITTSBURGH	MV

							AV
							TV
							PV
30	HH111224	48	Male	97	CARDIAC ARREST D/T STEMI Mitral and tricuspid regurgitation	PITTSBURGH	MV
							AV
							TV
							PV

2. Histological protocols

Each sample was subjected to a preliminary fixation process, wherein it was placed at 4°C overnight in a 10% neutral buffered formalin solution. Subsequently, the sample is washed with a PBS solution and stored at 4°C. Subsequently, the procedure entails the dehydration of the sample through the utilization of ethanol at varying concentration percentages (50%, 75%, 90%, and 100%). Then, the sample is first placed in xylene and then subjected to the embedding procedure using liquid paraffin at 65°C.

Once the paraffin block has been prepared, the sample can be sectioned. The standard thickness for each leaflet along the cross-section is 7 µm. The application of histological stains was conducted using kits, with each stain preceded by a hydration procedure and followed by a dehydration and mounting process.

- The Haematoxylin and eosin (H&E) staining procedure was conducted using a Haematoxylin solution (HEMG1-OT-500, Histo-line Laboratories) and an eosin solution (1%, 01EOIY101000, Histo-line Laboratories).
- Masson's trichrome procedure was then carried out using Masson's trichrome kit (01MST100T, Histo-line Laboratories).
- The Picro-Sirius Red for collagen kit (01PSR100T, Histo-Line Laboratories) was also employed.
- Alcian Blue staining was performed using Alcian Blue pH 2.5 Kit (01AB25100T, Histo-line Laboratories).

3. Decellularization process

The HV were stored at -80°C until they were required for submission to the decellularization process. The leaflets from each valve were defrosted at 4°C into a PBS solution, and subsequently underwent the following procedure, which is optimized for a sample of 0,100g:

Duration	Solution/Procedure
30 minutes	PBS 1%, twice, 15 minutes
30 minutes	100ml MilliQwater+0,2g EDTA+0,1g Trypsin
30 minutes	PBS 1%, twice 15 minutes
24 hours	100ml MilliQwater+0.1g SDS+0.2g EDTA
10 times	Mechanical Stress
30 minutes	PBS 1%, twice 15 minutes
24 hours	99ml MilliQwater+1ml TRITON+0.2g EDTA
10 times	Mechanical Stress
30 minutes	PBS 1%, twice 15 minutes

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BRIEF SCIENTIFIC CV

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3. 7th TERMIS WORLS CONGRESS – June 2024 – Poster session “Structural characterization of fresh human heart valves for tissue engineering application”, **P. Caruso**, M. Di Giuseppe, L. M. De Mohac, F. Cosentino, B. Zuccarello, M. Pilato, G. Raffa, W. Wagner, A. D'Amore
4. 52° Convegno Nazionale della Società Scientifica Italiana di Progettazione Meccanica e Costruzione di Macchine (AIAS) – September 2023 - "Caratterizzazione strutturale di valvole cardiache umane fresche per applicazioni di ingegneria tissutale”, **P. Caruso**, M. Di Giuseppe, L. M. De Mohac, F. Cosentino, B. Zuccarello, M. Pilato, G. Raffa, W. Wagner, A. D'Amore
5. Giornata studio Gruppo di Lavoro AIAS Biomeccanica – May 2023 – “Advanced physiology and structure-function characterization of human cardiovascular tissues for tissue engineering applications”, **P. Caruso**, M. Di Giuseppe, L. M. De Mohac, F. Cosentino, B. Zuccarello, A. D'Amore
6. 22nd McGowan Institute Scientific Retreat – March 2023 – “Structural characterization of fresh human heart valves for tissue engineering application”, **P. Caruso**, M. Di Giuseppe, L. M. De Mohac, F. Cosentino, B. Zuccarello, M. Pilato, G. Raffa, W. Wagner, A. D'Amore
7. Ri.MED Retreat - December 2022 – “Structural characterization of fresh human heart valves for tissue engineering application”, **P. Caruso**, M. Di Giuseppe, L. M. De Mohac, F. Cosentino, B. Zuccarello, M. Pilato, G. Raffa, W. Wagner, A. D'Amore

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