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Decellularization methods for the realization of heart valves pericardium-based

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Author: **Mitrofan Andreea Daniela**

Student ID: 10569000
Advisor: Mantero Sara
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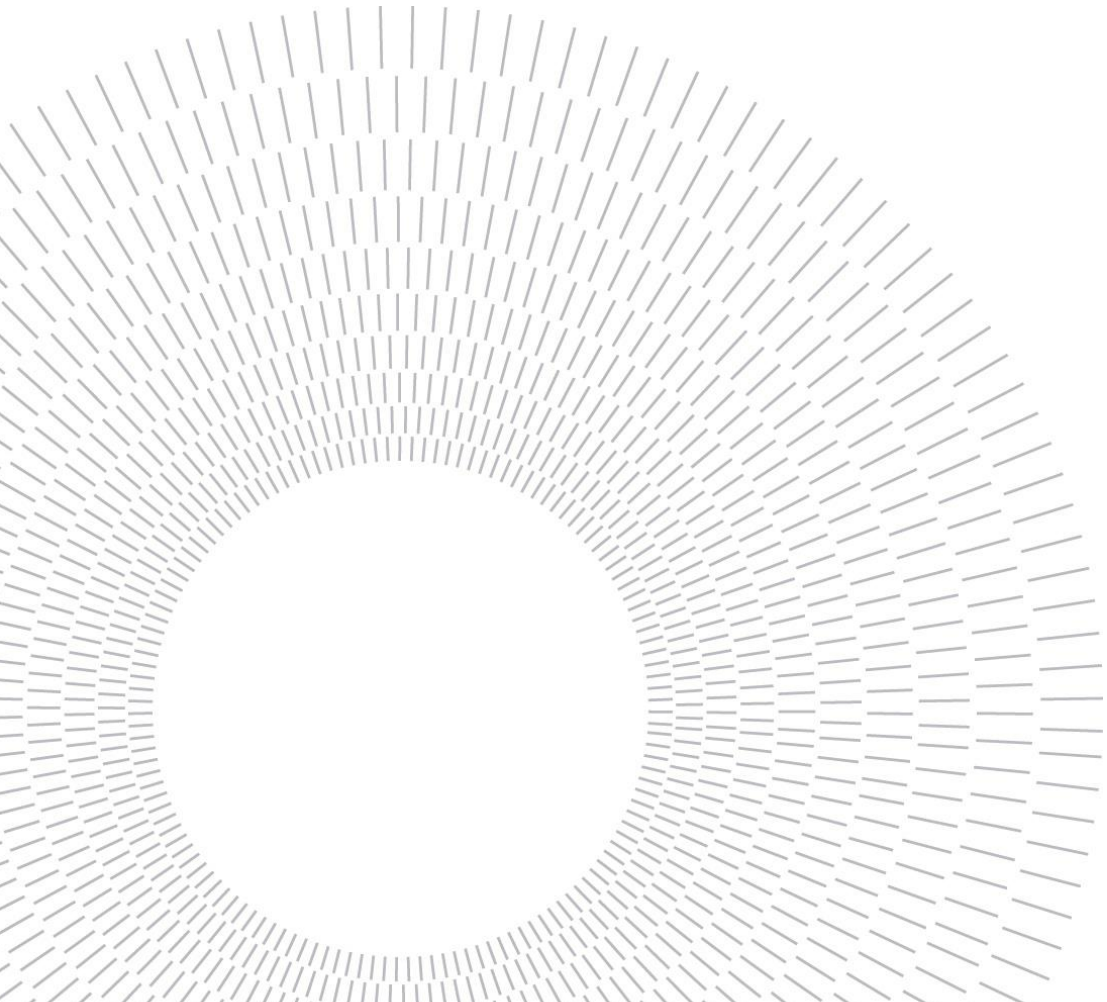

Abstract

Heart valve (HV) disease is among the leading causes of heart failure and death, and as life expectancy has increased in recent years, the incidence of valvulopathies in the elderly has also increased. Current surgical therapy uses mechanical valves or animal-derived biological valve substitutes, but these often lead patients to the need for reintervention because of complications. In the search for new procedures to maximize biological compatibility, decellularization methods have taken on a prominent role. The main goal is to efficiently remove antigenic components while preserving the extracellular matrix (ECM). The aim of this paper was born with the idea of analysing the synergistic effect resulting from the combination of different decellularization agents for the fabrication of aortic valve leaflets from bovine pericardium. Several protocols have been proposed in the literature, however, to date, there is still no standard procedure due to the difficult reproducibility of the experimental set-up and all the factors that contribute to the definition of the effectiveness of the decellularization process. Significant progress has been made in the optimization of protocols and in the study of the synergistic effect resulting from the combination of different detergents, which has led to first excellent results. However, longer follow-ups are needed to determine the durability and viability of the tissue created, and further studies of potential implant calcification are needed.

Abstract in lingua italiana

Le malattie delle valvole cardiache (HV) sono tra le principali cause di insufficienza cardiaca e di morte e poiché l'aspettativa di vita è aumentata negli ultimi anni, è aumentata anche l'incidenza delle valvulopatie negli anziani. L'attuale terapia chirurgica utilizza valvole meccaniche o sostituti valvolari biologici di derivazione animale, ma questi portano spesso i pazienti alla necessità di re-intervento a causa di complicanze. Nella ricerca di nuove procedure per massimizzare la compatibilità biologica, i metodi di decellularizzazione hanno assunto un ruolo rilevante. L'obiettivo principale è quello di rimuovere in modo efficiente i componenti antigenici preservando la matrice extracellulare (ECM). L'obiettivo di questo elaborato nasce con l'idea di analizzare l'effetto sinergico derivante dalla combinazione di diversi agenti di decellularizzazione per la realizzazione di foglietti valvolari aortici a partire da pericardio bovino. In letteratura sono stati proposti diversi protocolli, tuttavia, ad oggi, non esiste ancora una procedura standard a causa della difficile riproducibilità del set-up sperimentale e di tutti i fattori che contribuiscono alla definizione dell'efficacia del processo di decellularizzazione. Progressi significativi sono stati fatti nell'ottimizzazione dei protocolli e nello studio dell'effetto sinergico derivante dalla combinazione di diversi detergenti che ha portato a primi risultati eccellenti. Tuttavia, sono necessari follow-up più lunghi per determinare la durata e la vitalità del tessuto creato e sono necessari ulteriori studi sulla potenziale calcificazione dell'impianto.

Contents

1. Anatomy of the heart and heart valves

The heart is in the chest cavity, between the lungs, in a space called the anterior mediastinum; it lies on the diaphragm that separates it from the abdominal viscera and is protected anteriorly by the sternum and the costal cartilages that act as a shield. It has the shape of an upside-down cone about 12 cm high, and its weight, in an adult individual, is about 200-300 grams. The heart is contained within a double-walled membrane called the pericardium, between the two walls of the membrane, there is a thin layer of fluid that acts as a lubricant and that prevents friction between the heart and the pericardium itself during the cardiac cycle. The heart wall consists of three layers: the epicardium visceral on the surface, composed of a thin epithelial layer that lines the internal cavities and forms the valves; the myocardium, which is the layer muscular who effectively contracts and is internally reinforced by a layer of dense fibrous connective tissue; and more internally from the endocardium, a thin serous membrane that externally lines the heart [1].

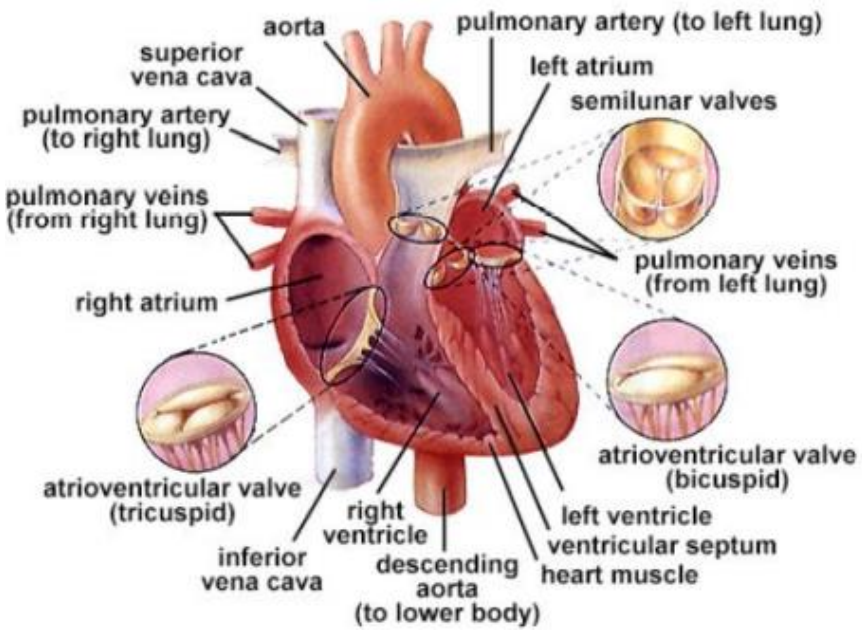

Figure 1.1: Cardiac chambers and valves

1.1 The chambers of the heart

The heart is divided into four chambers, two atria and two ventricles: the left atrium (LA) and the right atrium (RA) are located above the left ventricle (LV) and the right ventricle (RV). The two right chambers communicate via the right atrioventricular orifice, cyclically closed by the tricuspid valve. The two left chambers communicate via the left atrioventricular orifice, cyclically closed by the bicuspid (or mitral) valve. The right heart is completely separated from the left heart through the interatrial septum and the interventricular septum. The right ventricle is connected to the pulmonary artery and is separated from it through the pulmonary valve, consisting of connective flaps. The left ventricle is separated from the aorta by the aortic valve, which has a similar morphology to the pulmonary valve. The right atrium receives both venous (oxygen-poor) blood from the systemic circulation via the upper and lowers vena cava, and waste from the heart itself through the coronary sinus. The blood subsequently passes into the right ventricle and enters the pulmonary circle (small circulation), where it is oxygenated. Oxygen-rich blood is conveyed into the left atrium through the four pulmonary veins, enters the left ventricle and is pumped to the peripheral body districts by oxygenating and nourishing all tissues (large circulation).

1.2 Heart valves

Heart valves can be classified into atrioventricular valves (mitral and tricuspid) and semilunar valves (aortic and pulmonary). The valves allow a unidirectional flow of blood from atria to ventricles and from ventricles to blood vessels. It is defined as the "base" of the heart a fictitious plane that represents the ideal cut to display the four valves. The base of the heart is crossed by the cardiac "skeleton", a fibrous structure of connective tissue containing the annulus of the four valves, which extends to the origin of the aorta and pulmonary artery. The aortic ring is centrally located while the annulus of the other valves sticks to this. The fibrous skeleton remains almost stationary during the contraction of the heart. In addition to anchoring the four valves to the heart, the purpose of this structure is to provide a hook and electrical insulation for the myocardium of the atria and ventricles. The valves consist of thin foils (cusps), flexible and resistant, of fibrous tissue coated with endothelium, firmly anchored to the valve rings. The atrioventricular valves (AV) are connected, through the tendon cords, to papillary muscles that contract together with the ventricular walls and ensure the correct functioning of the valves exerting tension on the free edges towards the inside of the ventricles. The cusps of AV valves consist of the juxtaposition of two lamina fibrosis: one axial foil and a parietal foil. Both sheets have a surface layer, rich

in elastic fibers, and a deep layer with bundles of collagen and mucous tissue. The semilunar valves (SV) are characterized instead by a "passive" movement, dictated by pressure gradients that can be measured between the ventricles and the connected vessels. The valves consist of three cupshaped flaps (cusps) attached to the fibrous valve ring, in addition, there are small pockets, called Valsalva sinuses, in which vortices are formed and they prevent the valve leaflets to adhere to the walls of the vessel during the systole. Due to the reduced aperture, this results in a higher ejection rate compared to AV valves where the opening is wider, in addition, these valves have a faster closure than the AV due to the high-pressure values present in the arteries at the end of the ventricular systole. This means that the SL valves are subjected more to mechanical abrasion. The semilunar valves are formed by three sheets: lamina fibrosa, lamina spongiosa and a lamina ventricularis. The lamina fibrosa faces the vessel and it is mainly composed of type I collagen arranged in a circumferential direction. From a mechanical point of view, it turns out to be the most resistant part, thanks to its thickness and density, and its role is to support the stress imposed by diastolic pressure. The lamina spongiosa, instead, is the central part of the flap and it is composed of a high concentration of glycosaminoglycans GAGs and proteoglycans PGs. The mechanical role of the structure is to mitigate and absorb diastolic stress making bending movements more fluid. It is believed that the loss of GAGs strength can be related to the calcification of the valve leaflets. Finally, the lamina ventricularis faces the ventricle and is composed of elastic fibers and collagen fibers radially oriented, a large amount of fibronectin is also present. The elastin present in this layer allows the maximum opening of the flaps while in diastole allows an increase in dimensions in the radial direction [1].

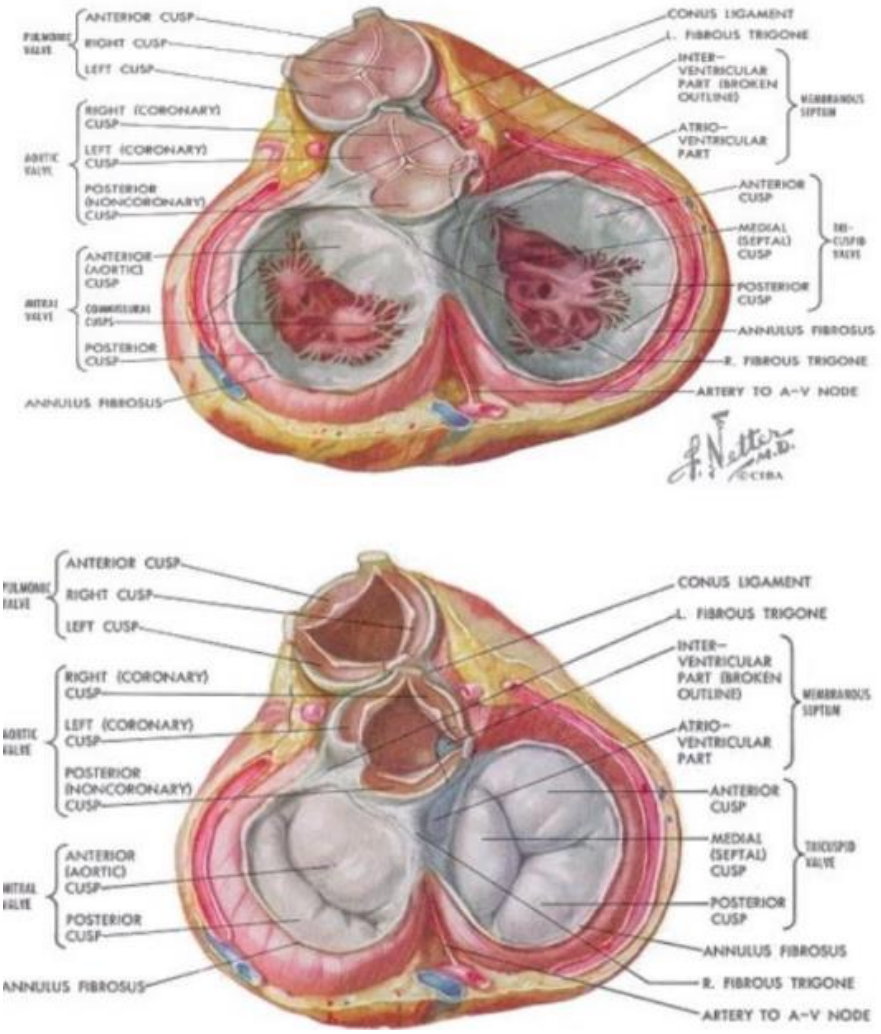

Figure 1.2: Heart valves during the systolic phase (above) and during the diastolic phase (below)

1.3 Heart valve cells

The main cell types in heart valves are interstitial valvular cells (VICs) and valvular endothelial cells (VECs). VECs cover the flaps of the valves and their role is to maintain a non-thrombogenic surface, similarly to the vascular endothelium, but they also play an important role in responding to the stresses to which the flap is subjected, and it is believed that these types of cells regulate the behaviour of the underlying VICs. VECs, unlike vascular endothelial cells, are not arranged longitudinally but circumferentially. Below the VECs there is a continuous basal lamina, composed of

sheets of collagen IV, laminin a, perlecan (proteoglycan) and nidogen (glycoprotein) [2,3]. These components, together with the fibronectin protein, are important for the maintenance of the adhesion and proliferation of VECs [4]. VICs, on the other hand, are a heterogeneous cell population and are responsible for maintaining the valvular structure. VICs include fibroblasts, myofibroblasts, smooth muscle cells (CMS) and mesenchymal stem cells [5]. CMCs and myofibroblasts are localized in the lamina fibrosa, while fibroblasts are segregated in the lamina ventricularis. SMCs' contraction maintains limited intrinsic valve strength and sustains hemodynamic pressures. Myofibroblasts are characterized by important stress fibres associated with the expression of alpha-actin of smooth muscle. Fibroblasts, on the other hand, have synthetic and secretory organelles and play an important role in the regulation of the extracellular matrix. They synthesize collagen, elastin, proteoglycans, fibronectin, growth factors, cytokines but also metalloproteinases (MMPS), which are enzymes able to degrade the matrix, and their inhibitors that implement regulatory and modulatory action in remodelling the ECM to maintain a normal structure and function of the native valve. The degeneration of the aortic valve has been associated with a loss of VICs while their lack of viability in bioprosteses has been correlated with an early dysfunction of the same. [6].

1.4 The aortic valve

The aortic valve interposes between the left ventricle and the proximal tract of the ascending aorta and has the function of ensuring unidirectional blood flow. The functional anatomical context in which it is inserted is the aortic root, consisting of the aortic annulus, Valsalva sinuses, valvular cusps, and sino-tubular junction. The aortic valve is a tricuspid valve composed of three semilunar flaps (right, left and noncoronary) and the annulus. The annulus is a crown-shaped collagen structure that lies at the junction between the aortic valve and the ventricular septum and provides structural support to the valve complex; its diameter varies, in adults, between 24 and 28 mm. The cusps are structures covered with connective tissue, with an average high of 15 mm; at their center, there is a fibrous bulge called "Aranzio nodule". The largest region of the leaflets is called "belly", while the two lines in which the valve leaflets join each other are called commissures; the line that joins the 6 three commissures is called the sino-tubular junction. The sinuses of Valsalva are instead small dilations of the proximal aorta that allow nourishing the heart by spraying the coronary arteries during diastole. At the end of the systolic phase, their presence allows the formation of a swirling flow on the aortic side of the valve; this flow prevents the valve flaps from meeting the aortic wall and ensures the correct closure of the valve. From a histological point of view, the valvular tissue consists of three layers: lamina fibrosa, lamina

spongiosa, lamina ventricularis. This composition is aimed at withstanding loads of stress, shear stress and bending stress to facilitate the passage of blood during the opening phase and to prevent reflux during the closing phase. The commissures, on the other hand, are composed of radially oriented collagen fibers to provide optimal support to the valvular structures, transmitting the efforts from the cusps to the aortic wall. [1]

1.5 Mechanical stress of the aortic valve

The aortic valve, during the cardiac cycle, is subjected to a complex deformation due to the inversion of its flaps from systole to diastole and as a result, it is subjected to large bending loads. [7]. The mechanical stresses to which the valve is subjected can affect its longevity, an excess of them can in fact cause different types of pathologies. Through measurements of stress-strain properties, it is possible to experimentally study the stresses to which the valve leaflets are subjected. Most tissues that contain collagen and elastin have been shown to have anisotropic and non-linear behavior, therefore, since the aortic valve leaflets and the pericardium are composed prevalently of collagen fibers and elastic fibers preferentially oriented in the direction of the prevailing torsion, their behavior will be anisotropic and non-linear. [8]. The visco-elastic behavior of the tissue valve is characterized by three distinct phases [9]. In the first phase there is the involvement of elastin: the flap opposes a small resistance to the imposed elongation, the layer of collagen fibers in the fibrosa give a minimal contribution to the transmission of strength, which is mainly supported by elastic fibers. At this stage, the stress grows linearly with the strain. In the transition phase collagen fibers unwind progressively and align, gradually increasing their contribution to the transmission of force. In the third phase all collagen fibers are elongated and support the load entirely. The ascent of the stress-strain curve is rapid and almost constant and extends beyond the physiological range thanks to the reserve of resistance. The slope of the curve then begins to decrease and a further increase in the load leads the tissue to fracture [10]. Firstly, when the tissue is subjected to a cyclic load it undergoes hysteresis between the loading phase and the unloading phase. This initial period is called pre-conditioning and it is when the adaptation of the internal structure occurs. With subsequent cycles, hysteresis decreases until it can be reached a stable state in which no further changes occur until the routine of the cycle changes.

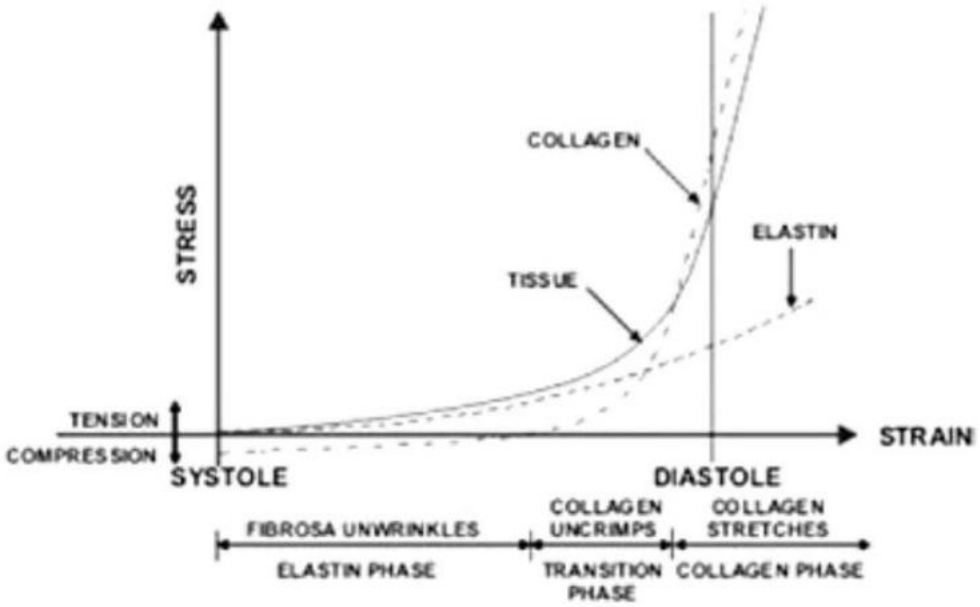

Figure 1.3: Stress-strain graph of aortic valve leaflets

2. Valvular pathologies

Valvular diseases, or valvulopathies, are dysfunctions of the heart valves whose prevalence is clearly growing in the world population, especially in industrialized countries due to the progressive ageing of the population. Valvular diseases can be of two types: congenital or acquired. The first is present at birth and is due to alterations in embryonic development, often also associated with other abnormalities. An example is the bicuspid aortic valve in which there are only two flaps rather than three, this generally causes aortic stenosis. The latter, on the other hand, may be due to structural degeneration of the valves related to age, endocarditis (bacterial infections, often streptococcus or staphylococcus), rheumatic fever, ischemic or large dilation of the ventricle and/or large vessels. Damage caused to the anatomical and functional integrity of the valves can lead to:

- Alterations of the valve surface
- *Stenosis*, that is a reduced opening of the valve due to the stiffening of the valve leaflets which leads to an increase in pressure upstream of the pathological valve and the heart must increase the work produced to let circulate a physiological flow rate. The heart walls then undergo structural changes to increase ventricular systolic pressure. It occurs a thickening of the heart walls (hypertrophy) and a change in the shape of the ventricle that tends to become more spherical. After a certain limit, however, the heart is unable to increase the work produced and therefore heart failure takes over.
- *Insufficiency*, that is incomplete closure of the valve that causes regurgitation of blood in the atrial or ventricular chamber. Imperfect closure is due to anomalies of the flaps or structures connected to them; in the case of the aortic valve, for example, the dilation of the aortic root can cause a displacement of the flaps. In these conditions the regurgitation causes an overload of the ventricle because the ability of the heart to pump blood is reduced, as a result, there is an increase in telediastolic pressure, which reaches the values of the left atrial pressure leading to premature closure of the mitral valve.
- *Composite diseases*, that is, the coexistence of stenosis and valvular insufficiency Aortic stenosis and mitral sufficiency are the most frequent valvular pathologies, and this is due to the fact that the left heart is subject to greater pressure than in the right heart, in which dysfunctions are rarer and generally associated with congenital problems. [11]

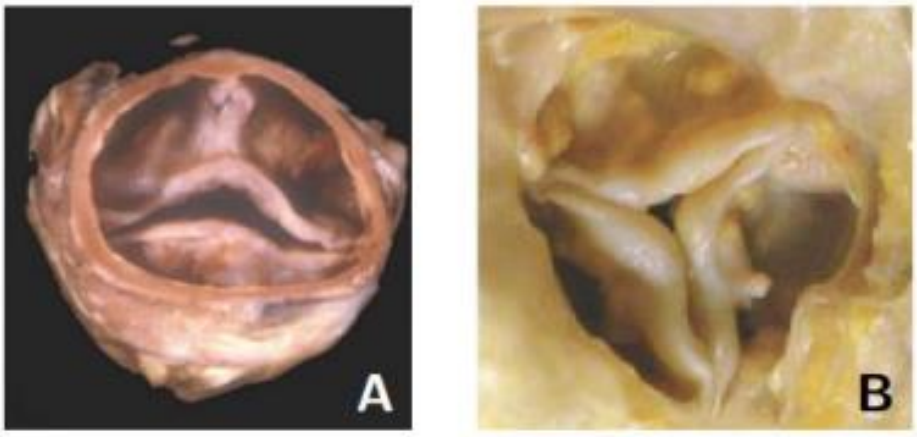

Figure 2.1: Calcific bicuspid aortic valve (Figure A) and senile calcified aortic stenosis (Figure B)

3. Intervention strategies

Aortic valvular stenosis is the most common valvular disease in the adult population and is associated with high mortality if it is severe and symptomatic and it is not treated appropriately [12]. In these cases, the decisive therapy is valve replacement (AVR) with implantation of a mechanical or biological prosthesis. Traditionally, it is an open-heart surgery that involves the use of a heart-lung machine whose task is to pump blood when the heart is stopped. Not all patients, however, are suitable candidates for this type of surgery because of their advanced age and their complex state of health that would imply non-negligible risks. [13]. In recent years, a new minimally invasive valve replacement technique has been developed (Transcatheter Aortic Valve Replacement TAVR) as an alternative to SAVR (Surgical Aortic Valve Replacement) with a transfemoral or transapical approach [14,15]. TAVR has numerous advantages over the traditional technique thanks to the fact that it drastically limits all the risks associated with open-heart surgery, thus decreasing the risk of infection and the post-operative recovery time, and increasing the number of patients who can undergo valve replacement. Several studies have highlighted the sustainability of the intervention with excellent results of valve operation. [16]. As already announced, the types of heart valves that can be used for the replacement of

the pathological valve are two: mechanical or biological. In 1962 the surgeon Dr. Dwight Harken defined the criteria by which valve substitutes should be made, later extended to tissue engineering products:

- The elements of the prosthesis must not be obstructive, and their closure must be quick and complete
- The constituent elements of the prosthesis must be non-thrombogenic materials that last for the life of the recipient
- Prostheses should be remodelled to facilitate tissue homeostasis and repair of any damage
- Prostheses must be reshaped and grown together with the patient.

3.6 Mechanical valves

Mechanical valves are made of synthetic materials, mainly metal and carbon, and consist of three main components: occlusor, housing and sewing ring.

- The occlusor constitutes the mobile part of the prosthesis, it must open and close without interference and adapt to the pressure differences of the blood flow.
- A housing, which supports the occlusor
- A suture ring, which represents the point of conjunction between prostheses and the recipient's tissues.

Mechanical prosthesis open and close thanks to a passive movement that depends on the pressure gradient and the changes in the blood flow. The prosthesis must be designed in a way such that they minimize any turbulence of the flow to reduce the risk of thrombi formation and the appearance of haemolytic phenomena. The movement of the occlusor on housing plays an important role in these terms because it greatly influences blood flow. In the last sixty years increasingly, advanced prostheses have been developed regarding the hemodynamic profile and biocompatibility. Mechanical prostheses can be classified according to their structure in caged-ball (1960), single-tilting (1969) and bileaflet-tilting disk (1970). With the advent of the first heart-lung machine in 1960 it was possible to perform the implantation of the first mitral valve. The prosthesis used (Starr-Edwards) consisted of an occlusor which was a silicon ball, a housing represented by a cage, initially made of stainless steel and later of cobalt- 10 chromium-molybdenum and nickel alloy called Stellite, and a suture ring made of polytetrafluoroethylene PTFE (trade name: Teflon). The valve, however, had several limitations: it turned out to be bulky due to its size that caused interference with the surrounding anatomical structures, the mobile element led to have disturbed fluid dynamics with the formation of vortices since the path of the blood was hindered by the ball and, in addition, the mobile element

produced haemolysis phenomena due to its jerky movement. In 1967 the first oscillating disc control valve (Bjork-Shiley) appeared on the market; it was composed of a disc occlusor that allowed an improvement in fluid dynamics by reducing the distortion of blood flow. The housing was an alloy of Stellite 21, the suture ring was in Teflon, while the disc was in polyoxymethylene (trade name: Delrin), later replaced by pyrolytic carbon (LTI), a material that guarantees a low haemolytic risk, high resistance and excellent hemocompatibility. Subsequent evolutions of the prosthesis include a titanium housing covered with LTI. In 1997 there is the advent of the first bileaflet valves (St. Jude Medical Inc), which represent today the most used mechanical prostheses, having supplanted the previous ones. The occlusor consists of two LTI hemi-discs that ensure minimal interference with the blood flow when opening, approaching physiological conditions. The housing is also in LTI while the suture ring can be made of Dacron, Teflon or polypropylene. Compared to previous prostheses, the hemodynamic characteristics are significantly improved, as well as the size of the valve. One of the main advantages of mechanical valves is their ability to guarantee excellent mechanical performance for a longer duration of time than biological valves minimizing the probability of the need for re-intervention. However, this is not true regarding paediatric patients. One of the requirements for the realization of valve replacement is to guarantee the ability to reshape and grow in response to the physiological growth of the recipient, this request, however, is not met leading to the need for re-intervention for prosthetic replacement. Another big disadvantage of mechanical valves is the demand for a lifetime anticoagulant therapy, not without risks and potentially dangerous for the patient [17].

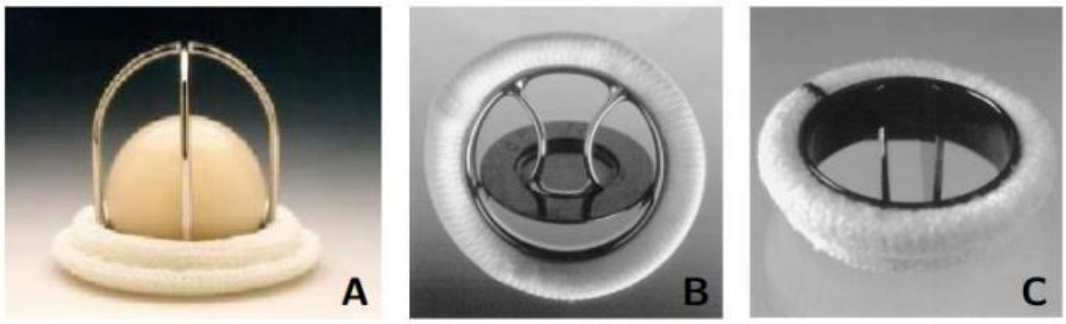

Figure 3.1: (A) Caged-ball prosthesis (Starr-Edwards); (B) Single-Tilting disk prosthesis (BjokorShiley); (C) Bileaflet-tilting disk prosthesis (St.Jude Reagen) [18]

3.7 Biological valves

Biological valves can be of two types:

- *Animal-derived bioprostheses or xenografts*, typically consisting of porcine aortic valves or bovine pericardial tissue treated with glutaraldehyde. Stented prostheses and stentless prostheses can be distinguished. In the stented valves, the tissue is mounted on a support (stent) made of plastic or metal, covered with synthetic material, which acts as a support. The presence of this structure can however determine an obstruction to the flow going to create a transvalvular gradient, moreover, the actual area of the stented prosthesis is lower than the stentless. Stentless prostheses, on the other hand, are anchored directly to the patient's aortic wall without support.
- *Homografts and Autografts*. Homografts are valves derived from a human donor (cadavers) and are of limited availability. Autografts are the patient's pulmonary valve, and these is used to replace the pathological aortic valve while in the pulmonary position a homograft valve will be inserted (Ross intervention) [19].

This type of prosthesis, compared to mechanical ones, have the main advantage of mimicking the structural, morphological, and hemodynamic characteristics typical of native valves, moreover, they have a low thrombogenic power and therefore no anticoagulant therapy is required for life. Xenografts, before implantation, must undergo pre-treatment with glutaraldehyde to preserve and increase the resistance of the tissue to chemical and enzymatic degradation and to reduce its immunogenicity. Therefore, they are not vital valves as the treatments applied to the donated tissue compromise the biological component. Glutaraldehyde is a cross-linking chemical agent highly used in the biomedical field since its introduction in 1969, for the preparation of bioprostheses. Glutaraldehyde has the following roles: it is an efficient sterilizing agent, reduces the antigenicity of ECM proteins and modifies the mechanical properties of the tissue by increasing the stability of collagen fibres. The unreacted glutaraldehyde residues that remain in the matrix can however give rise to several problems, including cytotoxicity, inflammatory response, calcification. In addition, it prevents re-endothelization by hindering tissue integration and ultimately leading to the failure of the prosthesis with the need for re-intervention. It is, therefore, necessary to pay extreme attention to post-fixing detoxification treatments to remove glutaraldehyde residues and thus limit these risks. [20] The two main limitations of biological valves are the possible rejection (in the case of xenografts and homografts) due to the immune response of the recipient and the onset of degenerative processes and calcification due to the treatments applied to the donated tissue. The average life

of biological valves is around 10 - 15 years due to structural degeneration [21] induced mainly by two processes:

- *Calcification* of the valve leaflets, due to a reaction between the organic phosphoric groups of cellular residues after treatment with glutaraldehyde and plasma calcium. This leads to nucleation, that is, to the formation of calcium phosphate minerals, these, in turn, can increase their size by forming mineralized nodules. The crystallization process proceeds quickly according to the concentrations of calcium and phosphate and according to the balance between the accelerators and the mineralization inhibitors. In young patients, in whom calcium-phosphate metabolism is accelerated, calcification is faster. Calcified deposits follow the direction of collagen fibres and accumulate in areas affected by increased mechanical stress, such as commissures, causing stiffening of the valve leaflets [22]. To minimize this problem, several anticalcific agents have been introduced to the market to treat bioprostheses. [23,24]
- *The non-calcific degradation* of the ECM due to fixation with glutaraldehyde that devitalizes the cellular component, thus eliminating the mechanisms of remodelling and repair of ECM damage, and fixes the collagen fibres in a single configuration, preventing any rearrangement of the fibres [25]. For this reason, the mechanical stresses applied to the prosthesis fixed during the opening and close phase cause the breakdown of collagen fibres and the deformation of the cusps. Other factors, however, can influence this process: the damage due to chemical treatment, the decrease in the amount of GAGs and the average thickness of the pericardial leaflets (0.25 mm) lower than that of the native flaps. The small size could decrease the mechanical strength and therefore the life of the system.

However, even homograft tissues that are not chemically treated undergo calcification and degenerative processes. This is because allogeneic tissues, after extraction, are cryopreserved and kept in liquid nitrogen vapours pending use. An analysis carried out with a Multiphoton Imaging technique for the three-dimensional visualization of elastin and collagen showed partial destruction of the ECM, imputable to the freezing induced to the tissue that caused the formation of ice crystals. Several methods of tissue preservation have been developed over the years to minimize ECM damage and optimize the maintenance of structural integrity. [26, 27] The choice of the type of prosthesis for the replacement of the pathological aortic valve depends on several factors, including the choice of the patient, age, the possibility or not of administering an anticoagulation therapy, the risk of valvular deterioration, the desire for

motherhood etc. As far as paediatric patients are concerned, Ross's intervention is generally used, obtaining excellent results. The procedure involves replacing the damaged aortic valve with the patient's own pulmonary valve, while, in the pulmonary position, a homograft is inserted. This choice is dictated by the fact that the pulmonary valve can potentially grow and regenerate thus reducing the risk of re-intervention, in addition, it has a lower risk of endocarditis and a lower mortality rate than other procedures. [28, 29]

3.8 Ozaki Procedure

In 2007 Professor Ozaki together with his colleagues developed an innovative surgical technique for the reconstruction of aortic valve leaflets with three flaps obtained from the autologous pericardium. The tissue used, being the patient's own, does not stimulate the immune response and does not cause a chronic inflammatory reaction. The technique has numerous advantages, including excellent post-operation hemodynamics, no post-operative anticoagulation requirements, absence of foreign body reaction and maintenance of the natural movement of the aortic annulus thanks to the absence of the metal support of the ring that is inserted to support the prosthesis. In addition, autologous tissue exhibits better resistance to infection than a homograft [30]. This is an invasive technique and involves a sternotomy approach for the biopsy of a stretch of the patient's pericardium which, after removing the adipose tissue, is then placed on a plate, and stretched in such a way that it does not suffer restrictions during the subsequent fixation with glutaraldehyde. Fixation is necessary to make the tissue slightly stiffer to facilitate manipulation by the surgeon. A model of the pericardial tract extracted from the patient is then taken to close the defect with a commercial decellularized matrix (submucosa of the porcine small intestine). Subsequently, through an aortotomy procedure, the patient's calcified valve leaflets are removed completely, also thanks to the help of an ultrasound device for decalcification if necessary. The fixed pericardium is placed on a piece of paper for easy handling during cutting and then valve leaflets of appropriate size (35 mm per leaflet) are cut, guide points are placed for each suture point and these are then sutured to the annulus. [31]

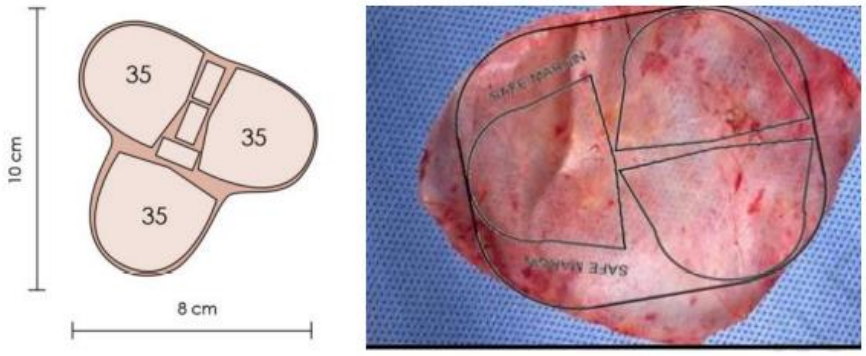

Figure 3.22: Valve leaflets of appropriate size (35 mm per leaflet) [18]

The authors, after performing a retrospective study of 850 patients, reported no subsequent valve replacement. Although the technique has many advantages, several studies are still necessary to ascertain the reliability and structural stability of the valve because the duration is uncertain since the longest follow-up was about 10 years [32]. The second retrospective study by number of patients considered and duration was carried out by the Monzino Cardiology Center, including all patients operated between 2014 and 2020. The conclusion of the medium-term follow-up shows excellent results in terms of mortality, transaortic valve gradients, freedom from major cardiac events related to valvular functioning of 97% and lower occurrence of aortic valve failure. In addition, the large coaptation area of the neo-valve ensures valvular continence even in the event of an aortic root aneurysm. This peculiarity makes the surgical procedure tempting for paediatric patients or for patients who have an aortic dilation. The risk of degeneration of the autologous pericardium, however, must be considered. Several studies, in fact, have shown that the use of glutaraldehyde represents a risk for valve calcification and consequent degeneration. In this type of procedure, the tissue extracted from the patient is kept in solution with glutaraldehyde only for 10 minutes, unlike commercial biological valves that are maintained in solution for longer periods before use. Despite this, the degeneration of autologous tissue is still an open question, and further studies are needed to come to a conclusion. Other critical issues related to the technique are the incompatibility with the transcatheter technique for valve implantation, a longer aortic clamping and cardiopulmonary bypass than traditional AVR. However, the advantages of the procedure are significant in terms of patient's life quality after the operation. It is believed that this surgical technique can be considered as the gold standard for the treatment of pathological aortic valves when conventional repair techniques are not applicable for young and middle-aged patients [33]. Similar considerations have also been achieved by other follow-up studies, such as the one carried out at the West

German Heart and Vascular Centre, University Hospital Essen [34]. The procedure, performed at the Monzino Cardiology Center since 2014, has undergone some changes with respect to the original instructions presented by Professor Ozaki. To minimize the section of the pericardium required for the biopsy, a special template has been created to extract only the material necessary for the construction of the flaps with a small margin of safety to adjust the size at the time of cutting; moreover, some surgical measures have been added to facilitate the procedure from a practical point of view to the surgeon. Since the sizing of the leaflets is a critical aspect of the procedure, surgeons need to learn to master the technique, and this is achieved by performing a training on a 3D printed model of the aortic root that is designed ad hoc starting from patient data extracted from transthoracic echocolordoppler reports and CT chest X-ray for the simulation of the mechanical characteristics of the aorta [35].

4. Introduction to Tissue Engineering

Tissue engineering is the interdisciplinary therapeutic field that aims to meet the medical needs related to tissues and organs by recreating them, engineering them or favouring their repair in case they are damaged, stimulating the self-repair mechanisms of the organism to restore, recreate and improve their original biological functions. This recent field of research was born in response to the growing need for tissues and organs that donations are not able to satisfy, to the insufficiency of the amount of tissue available in the case of auto-transplantation, to the need of avoiding all the rejection problems present in the case of tissue transplants (allotransplants or xenotransplants) and to avoid problems related to viral infections that can be carried by materials of human or animal origin used for grafting in transplants. These limitations are relevant in the case of paediatric patients. In fact, 1% of new-borns have congenital cardiac dysfunction and, in most cases, these involve the aortic or pulmonary valve. Biological and mechanical prostheses, however, have the great disadvantage of not following the physiological growth of patients, so they will need continuous re-interventions for the implantation of increasingly wide valves over the years. [36]. For this reason, tissue engineered products could make a significant contribution. For the realization of the new biological constructs, tissue engineering uses three main elements: cells, scaffolds, and bioreactor. The strategy involves isolating cells from a native source, expanding them in vitro in order to obtain a large number of cells that can subsequently be seeded on a porous scaffold, that is, a three-dimensional structure that acts as a support for tissue formation. The construct, composed of scaffolds and cells, is then inserted into an appropriate bioreactor. The bioreactor is a culture conditioning system through which a sterile environment is maintained, and appropriate stimuli are provided. The tissues obtained in vitro are then grafted into the patient, restoring the compromised functions and, if successful, integrate with the patient's tissues, thus providing a contribution to the treatment of the disease. A relevant importance assumes the knowledge and the study to understand in detail the mechanisms that regulate the proliferation and differentiation of cells and the ways in which the components of the ECM interact with cellular functions. Studies on healthy, pathological, or substituted heart valves have shown that the main factor determining valve lifetime is the ECM, which quantity and quality depend on the viability and functionality of the valve interstitial cells (VIC). [37].

4.1 Cells

In most approaches of cardiovascular tissue engineering the cells to be used for in vitro seeding are collected from tissues of autologous donors, classifiable in differentiated cells and stem cells. The choice of cells plays a decisive role in the engineering of the tissue and to this end it is necessary to consider the availability of the cell type, their proliferative capacity in vitro, phenotypic stability and immunogenicity. Differentiated cells have the advantage of already possessing the specific functional and morphological characteristics of the tissue, as they are specialized cells; however, they have a problem of availability because it is difficult to isolate healthy cells in pathological tissues and there is a risk of damaging the site from which they are extracted. In addition, they have limited proliferative capacity (10-12 steps before senescence) and experience frequent loss of the phenotype. Alternatively, there are stem cells, which in turn can be divided into adult stem cells (ASC) or embryonic stem cells (ESC). In both cases, these are non-specialized undifferentiated cells with unlimited proliferative capacity. From stem cells it is possible to obtain different types of specialized cells if properly stimulated through internal signals (controlled by genes) or external signals (chemicals, mechanical stimuli or physical contact). When they divide, they form in the embryo two stem cells, in the adult a stem cell (to maintain stem memory) and a cell that will specialize (progenitor). Each tissue has its own stem cells, which reside within stem niches, but not all of them are known today; one of the known niches is the one in the bone marrow where the MSC mesenchymal stem cells and the HSC hematopoietic stem cells (from which the blood cells specialize) are located. From the point of view of cellular power, the ESCs, which are extracted from embryos or umbilical cord, are pluripotent, that is, they have the ability to differentiate into all the cell lines that make up the tissues except those that make up the extra-embryonic tissues; the ASCs instead are typically multipotent; therefore, they have the possibility of forming only the cell types of the lines of origin. In fact, even adult stem cells are a resource not to be underestimated, in fact, MSCs have shown remarkable plasticity in a variety of experimental situations if properly stimulated, it has been possible to differentiate mesenchymal cells into nerve cells [38]. Once the cells are found from the tissue of interest, they must be differentiated into the desired cell line and subsequently expanded using standard culture techniques. The cells grown in vitro are seeded on the scaffold after which the construct is inserted into a bioreactor that creates the ideal conditions for cell proliferation and differentiation.

4.2 Bioreactors

Bioreactors are devices that allow biological and/or biochemical processes to be carried out under highly monitored, automated and controlled environmental and operating conditions (pH, temperature, nutrient supply, catabolite removal). The advantages deriving from the use of these systems are the possibility of having a uniform automatic sowing thanks to an imposed flow 17 pattern, the physical conditioning of the construct and an increase in the mass transport of nutrients and gases. Bioreactors also allow reproducibility and minimal dependence on the operator since their operation is based on an automated system, thus allowing to reduce human errors and the risk of contamination by favoring the maintenance of sterility in the environment in which the construct is immersed. The logical scheme of operation of a bioreactor provides a culture environment in which the engineering of the tissue takes place under sterile conditions, a reading and implementation subsystem for conditioning and a control subsystem useful for providing information on the progress of the system. It is also possible to use different types of sensors with the aim of monitoring parameters of interest (CO2, O2, pH). For the realization of a bioreactor, it is necessary to consider the project specifications:

1. The use of cytocompatible materials
2. The materials used must be sterilizable and sterility must be maintained in the culture chamber and in all parts where the cellular medium passes
3. Automation and control of the system
4. The system must be reliable
5. The system must allow the optimization of mass and gas transport
6. The system must allow the conditioning of the fabric and its stimulation
7. GMP Compatibility
8. There must be no cross-contamination
9. The bioreactor must be versatile
10. Small footprint
11. Stand alone

Dynamic culture systems have several advantages over static cells culture. The latter in fact involves a sedimentation by gravity of the drops containing the cells that are deposited on the scaffold and then penetrate the pores by diffusion. The sowing performed, however, is not uniform, with the formation of cellular aggregates onto the scaffold, moreover, it is not very reproducible since it is a manual sowing (operator-dependent). Alternatively, there are dynamic seeding techniques for which it is also possible to make use of a bioreactor. These can be distinguished into dynamic confined

sowing and confined dynamic sowing. For the first type we can have several bioreactor systems including the Mixed Flask and the Rotating-Wall Vassels. The Mixed Flask is a stirring system that puts in motion the medium containing the cells thanks to a rotor, this however generates swirling flows with consequent imposition of high shear forces on the tissue, moreover, the sowing is not very uniform. The Vassels Rotating wall is instead a system that involves that the scaffolds are anchored to a rotating shaft structure in a flask, the flow turns out to be laminar thus producing low shear forces but the sowing is still not uniform due to the low cell migration in the scaffold due to the fact that the fluid threads meet a resistance in crossing the scaffold and therefore turn around it. For confined dynamic sowing, instead, there is a U-Cup bioreactor. This is a U-shaped structure containing a scaffold bound with silicone tubes and connected to an actuation system (syringe pump) that requires a displacement of the fluid in one direction and then in the other by convection. The perfusion of the scaffold appears to be confined with mandatory passage of the medium in the scaffold favouring mass transport and oxygenation, the sowing is uniform and reproducible. In addition, through appropriate shear forces generated by the fluid itself, the mechano-transducers present on the cell membranes are activated, promoting the production of extracellular ECM matrix and cell differentiation. [39]

4.3 Scaffold

The scaffold is the support structure that has the function of guiding the growth and development of functional tissue from a mass of cells, it guides adhesion, proliferation, migration, cell differentiation, provides a temporary biomechanical profile and defines a 3D space for tissue formation. The scaffold must be highly porous with interconnected pores (d=100 um) to allow cell growth, nutrient transport and removal of catabolites, but at the same time the porosity must ensure the right stability of the scaffold. Since the objective of the structure is to provide only initially a mechanical support, this will have to biodegrade with time to make room for the new tissue that is being formed. Degradation products must be non-toxic to the body and the rate of degradation controllable. The surface chemistry of the material composing the scaffold shall be suitable for the adhesion, proliferation, cell differentiation and incorporation of ECM elements, while the mechanical properties of the material shall be like those of the host tissue at the implant site or appropriate for conditioning. The materials that are used for the construction of scaffolds can be divided into two large categories: synthetic materials or natural materials. Synthetic scaffolds are made of biodegradable and bioresorbable polymers that degrade progressively allowing the growth of an ECM indistinguishable from the native one. Several materials have been approved by the Food and Drug Administration (FDA), including the most widely used polyesters.

Synthetic scaffolds have found wide use in different areas of tissue engineering, mainly thanks to the great advantage of being able to obtain scaffolds with a wide range of mechanical properties and a good control of degradation kinetics, acting on chemical composition and processing. They turn out to be, in fact, very versatile materials and reproducible with industrial productions. Despite this, synthetic scaffolds are not suitable for the replacement of valve leaflets due to the stress imposed on the material generating microfractures, accumulation of plasma proteins and mineralization of the structure [40]. Natural type scaffolds, on the other hand, can be distinguished into living materials or non-living materials. The former refers to donor organs or tissues or tissue engineering products. The latter are materials such as collagen, hyaluronic acid, alginate and chitosan. These last materials have problems related to the integration with the host organism and have limiting characteristics from an anatomical and biomechanical point of view, in fact, the use of natural materials does not ensure the reproducibility of mechanical characteristics and the control of biodegradability over time. Both types enjoy a wide use and have proven to be largely functional for the realization of different types of fabric, but despite this they have also shown some limits for which research has constantly worked for the realization of new structures that can perform this function. For these reasons, in particular glutaraldehyde-fixed matrices, have dominated the scope of the heart valves. The scaffolds thus obtained can be:

- Formed by the constituents of the ECM (collagen and fibrin)
- Biologics of non-valvular origin (pericardium)
- Biologics of non-valvular origin (allograft and xenograft).

5. The pericardium as a biomaterial

The aortic valve replacement (AVR) with mechanical or biological prosthesis is the standard treatment for aortic valve stenosis and regurgitation. However, despite the progress in the construction and in the design of the prosthetic valves, these still do not enable full motion of the aortic valve annulus and root, leading to suboptimal hemodynamics compared to that of native aortic valves. For this reason, an alternative is required. The pericardium of different mammals (bovine, porcine, sheep, human) is a type of biological matrix widely used both for surgical and research purposes. This material has excellent handling characteristics and good suture retention, but also an anti-calcification manufacturing process that may improve the outcomes of long-term implantation [41]. The cardiac surgical field is the one that sees its greatest use. In interventions on pediatric patients with congenital cardiomyopathies, the pericardial patch is used to repair the interventricular septum separating the systemic circle from the pulmonary one [42]. The patch can also be used for surgical correction of coronary artery abnormalities and for interventricular septal repair after post-infarction rupture [43]. In the cardiovascular field, the autologous pericardium has been shown to have excellent characteristics for the construction of valvular leaflets. The main advantages are the easy access and the absence of stimulation of the immune response, being autologous tissue. However, the extraction intervention turns out to be invasive, as previously discussed in the section on the Ozaki procedure, with all the consequences of the case, in addition, there can be a post-pericardiotomy inflammatory reaction that alters the tissue properties. Despite these limitations, the elective material for the construction of heart valves (both traditional and percutaneous) is the bovine pericardium because of its flexibility and easy handling. Trileaflet aortic valve reconstruction technique has been proven to be effective and feasible in treating different aortic valve diseases [41]. Halees et al. [44] reported that the overall freedom from reoperation was 47% at 16 years after aortic valve reconstruction with pericardium. Song et al. [45], in 2014, reported excellent early clinical and hemodynamic outcomes after aortic valve reconstruction surgery with bovine pericardium patch CardioCel in pediatric and adult patients. [46]. Evora, et al. reported, instead, the case of a cusp replacement with bovine pericardium that has shown with good ventricular function and minimal structural valve deterioration at a long-term follow-up of 23 years [47]. Given that the aortic root is a complex and dynamic structure composed by streamlined segments in which the size of the leaflets is proportional to the size of the root, dysfunction may occur in case those proportions are lost [48, 49]. Thus, the size of the pericardial leaflets should be proportional to the

size of the root which includes the aortoventricular junction, the sins of Valsalva, the STJ and the aorta. Moreover, the pericardial material should be sufficiently thin with good mechanical characteristics. These valves are built from scratch using certain portions of the pericardium selected from specific areas of the tissue. In fact, it must be considered its intrinsic variability. The tissue is heterogeneous at the histological and biochemical level, probably due to the different physiological stresses to which the different portions of the tissue are subjected [50]. This variability has made the characterization of the mechanical properties of PB an important task for improving its use as a biomaterial. The selected areas must have a high degree of uniformity and orientation in the distribution of collagen fibers and regular mechanical properties. The ideal portions are those with uniform thickness [51]. As reported in the study of [52] there is significant evidence of location-based differences in terms of properties and durability within a single sample of pericardium. In fact, only a limited part of the material showed a homogeneous usable thickness. Only a limited part of the pericardium showed a homogenous and usable thickness. Microscopical observations showed that the integrity of the ECM was not influenced by the decellularization procedure with detergents (0.5% sodiumdesoxycholate/0.5% sodiumdodecylsulfate). Biomechanical properties were determined using uniaxial tensile tests. To assess the long-term durability, patches were tested in a high-cycle system for a duration equaling the stress of three months in-vivo. Commercially available, fixed pericardium patches served as control group. For a long time, it was believed that the bovine pericardium had lower mechanical performance than the porcine valve leaflets for the construction of bioprostheses, however, pericardial prostheses demonstrated a greater opening of the leaflets, completer and more symmetrical with consequent hemodynamic advantages. In addition, the higher percentage of collagen present in the pericardial tissue would result in a longer life of the valve itself [51]. Moreover, structure and rheological properties of bovine pericardium tissue depicted that the material exhibit unique and interesting viscoelastic behavior which give it the ability to go through millions of cycles of opening and closing during the cardiac cycle without any fatigue. A mandatory process to which the tissue to be used for the engineering of heart valves must be subjected is that of decellularization. The cellular component must necessarily be eliminated to avoid a hyperacute rejection, that is a rapid rejection reaction that occurs immediately after the implantation of a xenograft. Currently, in bioprostheses, the neutralization of the species-specific antigens that cause this reaction occurs through glutaraldehyde fixation. Hyperacute rejection is caused by the presence of an epitope known as α-1-3 galactose (α-Gal), which is present on the surface of almost all mammalian cells except human ones and some monkeys. These organisms develop anti-α-gal antibodies in response to their intestinal bacterial flora, and these antibodies also react with the antigen present in the

implanted xenogenic tissues or organs and damage the endothelial lining of blood vessels leading to the formation of clots resulting in blockage of circulation, hypoxia, and necrosis. In some commercially available bioprostheses, the epitope α-Gal' has been identified despite fixation in glutaraldehyde and has been shown to stimulate the SI response of patients in whom the prosthesis was implanted. Whether the induction of an immune-specific response against this antigen may contribute to valvular degeneration has not yet been elucidated. Despite this it is clearly desirable to avoid any reaction against the implanted valve. However, some decellularization protocols studied seem to be able to eliminate the α-gal antigen [53,54] and for this reason they constitute promising protocols for the creation of engineered heart valves. Regardless of using autologous pericardium or bovine pericardium, pericardium calcification is a main cause for concern and determines the freedom from reoperation over the long-term. Glutaraldehyde treatment can provide more resistance against retraction and degeneration and can maintain the intrinsic pliability of the pericardial tissue. pericardium treated with glutaraldehyde has the advantages of sterility, good biocompatibility, no tissue rejection, and convenient material acquisition, ensuring the survival and growth of tissue cells, the residual aldehyde agent can cause degeneration of the pericardium tissue and sterile necrosis. Moreover, the pericardium calcifies easily and loses its growth ability, especially in the high-pressure intraluminal area of the aortic valve. [55, 56]. the remaining unrinsed free glutaraldehyde easily combines with calcium to cause calcification, ultimately leading to tissue fatigue and loss of the cytoplasmic matrix. In conclusion, at present, the mid- and long-term results in patients undergoing trileaflet aortic valve reconstruction with bovine pericardium are encouraging but further long-term results should be evaluated and followed up for at least 15-20 years to better asses their durability and function [45].

6. Decellularization

6.1 Introduction

Over the last few years, the development of the processes of decellularization of organs and tissues has led to the introduction of a valid alternative to the use of traditional scaffolds: the use of extracellular matrix of human or animal origin. Several studies have shown the importance of ECM in guiding the differentiation of progenitor stem cells towards the specific tissue phenotype required. The extracellular matrix is a complex structural entity formed by the set of macromolecules collagen, elastin, proteoglycans, and structural glycoproteins. It structurally stabilizes tissues, thanks to the relative rigidity of its wide-meshed reticular structure and regulates the development, migration, proliferation, shape, and function of cells that are in close contact with it. The stress and tension induced by the extracellular matrix regulate not only cellular activities and phenotype in development but also later, during tissue repair and regeneration processes. Scaffolds used in tissue engineering aim to recreate its structure and function. The clinical success of such scaffolds is strongly related to the choice of the material: it must be capable of transmitting biochemical and biophysical signals. Allogenic and Xenogenic antigens present in the tissue implanted can be recognized in the host as foreign and thus they induce immune rejection, but the components of the ECM are generally preserved among species, and they do not lead to rejection by the recipient. Extracellular matrix scaffolds are obtained after a process of decellularization of tissue from donors, usually corpses or animal tissues. The goal is to isolate the primary tissue and remove all the cellular components, which turn out to be immunological, through physical, enzymatic, and chemical processes, without causing biological, mechanical or composition alterations of the native extracellular matrix. The advantages of this procedure lie in the fact that the chemical composition turns out to be the correct one present in the ECM of our tissues, the substrate is recognized by the cells, so cell adhesion and viability are favoured. There is a great variety of products derived from the ECM that has been approved by the Food and Drug Administration (FDA) and currently used in the clinical setting. All this shows that decellularization protocols can be applied to clinical use and become available to many patients. There are, however, still several obstacles that need to be overcome. There is a limited availability of donor tissue, a low reproducibility due to individual differences that cannot be bridged, and the decellularization processes are not very standardizable, expensive and the timing of obtaining the product is long. Most of the decellularization techniques used today, in fact, are complex and require

sequential steps in which different substances are used to remove the cellular part; the risk of the use of these agents, however, is to damage the matrix and to leave toxic residues, the mechanical characteristics of the structure could therefore vary after the decellularization process and trigger an inflammatory response due to the release of toxic substances not properly removed [57]. Porosity and pore size are significant parameters for the scaffold functionality given that they define the final mechanical properties. Interconnected networks and open porous are critical elements per cell migration, proliferation, and nutrition in tissue formation therefore researchers have focused their attention on the possibility of making use of decellularized pericardial ECM as regenerative support.

These kinds of scaffolds have been proven to be optimal for cell activities thanks to the capability of the matrix components to provide signalling cues to modulate cell functions. They are a promising alternative to other natural biomaterials, since, beyond being a supportive material, decellularized matrix can be incorporated with bioactive factors facilitating the regenerative action of the scaffold [58].

6.2 Description of decellularization methods

In the literature there are different techniques used for the decellularization of organs and tissues. A distinction is generally made between physical, chemical, and enzymatic methods: in most cases the three methods are combined to obtain a better decellularizing effect. The effectiveness of the protocol depends on the tissue that is decellularized and the type of detergent with which the latter is combined. Depending on the type of procedure, in fact, the effect of the process on the tissue varies. In a decellularization protocol, generally, there are four phases:

1. *Lysis of cell membranes, using physical methods or hyper/hypotonic solutions* to disrupt cellular membrane such that cells release their content. This allows cells exposure to chaotropic agents and provide a path for cellular debris removal. This kind of treatment is not sufficient to guarantee total decellularization and chemical treatments are generally employed to achieve the result.
2. *Separation of the cellular components of the ECM using an enzymatic treatment*. The procedure is used to disrupt intracellular and extracellular bonds. However, it is of critical importance to minimize the disruption such that native mechanical and biological properties can be maintained, therefore, inhibitors are used to counterbalance the action of enzymes. [59.]
3. Solubilization of cytoplasm and nuclear components, using *non-ionic or zwitterionic detergents*

4. Final extraction of cellular residues from the tissue using *ionic detergents*. After that also the residual chemicals must be eliminated to avoid a negative host tissue response.

However, these solutions can degrade or denature the ECM proteins or leave toxic residues. It is therefore necessary to evaluate the combination of the various stages of the process, the choice of detergents and the duration of the washes.

A typical progressive approach would be to start with treatment in a hypotonic or hypertonic solution followed by a mild non-ionic or zwitterionic detergent. If necessary, an enzymatic treatment of tryspin/EDTA can be added prior to the detergent treatment to assist in breaking the bonds between the cell membranes and the ECM. Finally, if these treatments are still inadequate to remove the cellular material, an ionic detergent such as SDS, deoxycholate, or Triton X-200 can be added to the decellularization protocol.

A tissue can be considered as decellularized if in the histological analysis there are no visible nuclei, DNA residuals are < 200 bp and the concentration of DNA is not >50 ng/mg per dry weight.

6.2.1 Physical methods

Physical methods include freezing, sonication, agitation, and direct pressure. The snap freezing method is frequently used for the decellularization of ligamentous and tendinous tissue [60-65] and nerve tissue [66]. The rapid freezing of the tissue leads to the formation of intracellular ice crystals which disrupt the cellular membrane and cause cell lysis. The change of rate temperature must be controlled carefully in order to prevent the ice formation from disrupting also the ECM. This approach must be followed by processes to remove the cellular components from the tissue. Another method is the application of direct pressure to the tissue, but this is effective only for tissue or organs which have not a densely organized ECM (e.g liver, lung). Mechanical agitation and sonication are other options that are employed simultaneously with chemical treatments to facilitate cellular lysis and the removal of cellular debris. Mechanical force can be used to delaminate tissue layers from organs (e.i small intestine and urinary bladder). The efficacy of these methods has been assessed and it has been also proven minimal disruption of the ECM. Mechanical agitation can be produced by a magnetic stir plate, a low-profile roller or an orbital shaker. The optimal magnitude or the sonification frequency to disrupt cells have not been assessed yet, but there is evidence of the effectiveness of a standard ultrasonic cleaner in removing cellular components when a tissue in placed on an orbital shaker. The optimal speed,

length of mechanical agitation and volume of reagent is strictly dependent on the composition, density and volume of the specific tissue considered.

6.2.2 Chemical methods: alkaline and acid treatments

These methods are used in the decellularization protocol to solubilize the cytoplasmatic elements of the cells and to remove nucleic acids (RNA, DNA). To effectively disrupt cell membranes and intracellular organelles acetic acid, peracetic acid (PAA), sulfuric acid, ammonium hydroxide (NH4OH) and hydrochloric acid can be used. However, these solutions can dissociate GAGs from collagen tissue. The effects of PAA treatment on the ECM components are studied extensively. Different tissues are decellularized using PAA at concentrations of roughly 0.10–0.15% (w/v). This treatment is very efficient at removing cellular components from thin ECM structures, like porcine tissues including SIS and layers of the bladder, and simultaneously disinfects the fabric by entering microorganisms and oxidizing microbial enzymes [67,68]. PAA treatment preserves the structure and performance of the many of the expansion factors that are resident within the ECM, including transforming growth factor-b, basic fibroblast protein, and vascular endothelial protein [69, 70]. PAA doesn't appear to possess any adverse effect on the mechanical behaviour of the biologic scaffold [71].

6.2.3 Chemical methods: non-ionic detergents

Non-ionic detergents are used extensively in decellularization protocols due to their relatively mild effects upon tissue structure. Non-ionic detergents disrupt lipid–lipid and lipid–protein interactions but leave protein–protein interactions intact such that proteins can maintain the functional conformation within the tissue after the treatment. [72]. The most studied anionic detergent is Triton X-100. Contrasting results have been reported upon the treatment with this detergent. Complete removal of nuclear elements has been observed after the decellularization of a heart valve with Triton X-100 and the maintenance of valvular structure has been perused after 24h. However, the treatment led to an almost complete loss of GAGs and a decrease in the laminin and fibronectin content has been found [73]. Other studies have shown the inefficacy of Triton X-100 in completely removing cellular components from other tissues as well (tendon, ligaments, blood vessels) after different time exposure [74-76]. Histological staining showed that nuclear material was still present in the tissues after decellularization procedure, and it has been found that the treatment affected the tensile strength of collagen fibres in the tendon. On the contrary no effect on cruciate ligament ACL collagen content has been reported. Mixed results have been found also regarding the GAGs content. In the heart valve a nearly complete removal has been

observed, while no effects on GAGs after decellularization treatment has been reported for ACL. Therefore, these results suggest that the efficacy of the method depends upon the tissue considered and upon the other detergents/methods that are used in the decellularization protocol.

6.2.4 Chemical methods: ionic detergents

Ionic detergents are used to solubilize cytoplasmatic and nuclear cellular membranes. However, they cause denaturation of proteins by disrupting protein-protein interactions [57 repeated]. Sodium Dodecyl Sulfate (SDS), Sodium Deoxycholate and Triton X-200 are the most studied ionic detergents [78-80, 76, 81-83]. It has been proven that SDS effectively remove cellular material, nuclear debrits and cytoplasmatic proteins [75]. Nevertheless, the detergent affects the native tissue structure, and leads to a decrease in the GAG concentration and therefore a loss in collagen integrity. Sodium deoxycholate is effective in removing cellular material as well but, compared to SDS, it causes a greater damage to the tissue structure. It is difficult to assess the single effect of Sodium Deoxycholate since, usually, it is not used alone but in combination with other methods/solutions.

6.2.5 Chemical methods: zwitterionic detergents

Zwitterionic detergents show the properties of both non-ionic and ionic detergents. They tend to denature proteins more than non-ionic detergents. This type of detergents includes 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), sulfobetaine-10 (SB-10) and -16 (SB-16). CHAPS is used for blood vessels decellularization and it has been shown that it preserves the collagen content and the elastin morphology but a notable decrease in the burst pressure and in the maximum stress of arterial tissue has been reported. However, the decrease can be compared to the burst pressure of arteries treated with Triton X-100 and hypotonic/hypertonic solutions [74]. Sulfobetaine-10 (SB-10) and -16 (SB-16) are used, instead, for nerve decellularization. The combined treatments had a less detrimental effect on the structure of the nerve ECM than combined treatments with Triton X-100 and sodium deoxycholate [80, 83].

6.2.6 Chemical methods: Tri(n-butyl) phosphate (TBP)

Tri(n-butyl) phosphate (TBP) is an organic solvent employed to inactivate viruses in blood without affecting the coagulation factor activity. Lately, TBP has been used as a chaotropic agent for the decellularization of ligament grafts and tendons. The approach allows total removal of nuclear debris from tissue, although, the elimination

of the cellular components is not assured. it has been proven that TBP has no effect on the collagen fibres tensile strength in isolated rat tail tendon, but it leads to a decrease in collagen content [75,76]. Overall, TBP seems to be a promising decellularization agent with minimal effect on the mechanical behaviour of the matrix and it is worthy of further investigations.

6.2.7 Hypotonic and hypertonic treatments

To increase detergents' action, hypotonic or hypertonic solutions can be incorporated in the decellularization protocol. The immersion of the tissue in a hypotonic solution (in which solutes concentration is lower than the one in cellular cytoplasm) leads to cellular swelling and subsequent rupture. This process is known as osmotic shock or osmotic lysis. The swelling is induced by water motion from extracellular space to intracellular space. On the contrary, the immersion in hypertonic solution (in which the solute concentration is higher than the one in the cytoplasm) has an opposite effect. The water motion happens from intracellular space to the surrounding extracellular space leading to cellular dehydration and contraction. The result is the cellular death and detachment from the matrix [84]. However, this method cannot guarantee the removal of cellular debris from the tissue and neither the removal of nuclear elements due to their "sticky" nature and tendency to adhere to ECM proteins. Therefore, additional enzymatic or chemical treatments are required to efficiently remove remnants.

6.2.8 Chelating agents

Chelating agents form a ring-shaped molecular complex which strongly binds and isolates a central metal ion. An example of these is EDTA and EGTA. It has been reported that divalent cations, such as Ca2+ and Mg2+, are necessary for cell attachment to collagen and fibronectin at the RGD (Arg- Gly- Asp) sequence [85-88]. These agents bind the divalent cations and therefore facilitate cellular removal from the tissue. Typically, EDTA is used in combination with trypsin.

6.2.9 Enzymatic methods

Enzymatic methods include protease and nucleases digestion and calcium chelating agents [89-91]. One of the most used proteolytic enzymes is **trypsin**. This is a specific enzyme that cleaves peptide bonds on the C-terminal of lysine and arginine if the subsequent residue is not proline [92]. The enzymatic activity is maximum at a temperature of 37° and at a ph of 8. Its mechanism of action can break the bonds between the cells facilitating their detachment from the matrix, thus, creating a more

open structure [93]. Trypsin has been extensively tested and has been shown that it is not capable of completely decellularize the tissue and, moreover, it causes significant damage to the ECM. [94, 71]. Attention must be paid in using this enzyme to avoid excessive damage of the biomechanical structure. **Nucleases** are divided into 2 groups: endonucleases and exonucleases. The former catalyzes the hydrolysis of the internal bonds of the ribonucleotide or deoxynucleotide chain while exonucleases catalyze the hydrolysis of terminal bonds leading to RNA or DNA degradation [95]. Enzymatic treatments efficiency in removing the cellular material from the ECM has been studied for different tissues. Results are conflicting. Efficient removal of cellular components from porcine pulmonary valves after a treatment with 0.05% trypsin/0.02% EDTA for 24 h with agitation [96] has been proven, on the other hand, other studies have showed less efficient decellularization: after treatment with 0.5% trypsin, 0.05% EDTA, 0.02% Gentamicin, 0.02 mg/ml DNase and 20 mg/ ml RNAse-A in Milli-Q water for up to 17 h at 37°C with agitation porcine aortic valve cells were not-viable but removal of cellular components hasn't occurred [73]. Given that nucleic acids seem to act as sites of calcification and seem to contribute to the premature failure of the prosthesis, in decellularization protocols it would be appropriate to use nucleases in combination with other decellularizing agents [97-100]. Protract treatment with trypsin/EDTA leads to ECM structure disruption even if the amount of collagen is not affected [101]. However, a significant reduction in laminin and fibronectin content has been observed. Moreover, a prolonged exposure causes a reduction in elastin amount and GAGs over time contributing to a decrease in tensile strength of up to 50%. Thus, it is important to control the time of exposure to trypsin/EDTA to minimize adverse effects upon the ECM composition and the ultrastructure. It is important to note that numerous proteases can be released from lysed cells and damage the ECM of the scaffold leading to alterations in mechanical/hemodynamic behaviour and in the potential repopulation of the valves. It is therefore necessary to add protease inhibitors to the solutions in which the tissue is inserted. Some inhibitors that can be used are:

- Reversible and competitiveserin-protease antagonists: aprotinin, benzamidine (BA) and phenylmethanesulfonyl (PMSF) [69, 70, 83, 102]
- Cysteine protease inhibitors (alkylane cysteine residues prevent their digestion), such as iodoacetamide (IA) and N-Ethylmaleimide (NEM) [103].

The use of buffer solutions at pH 7-8, the control of temperature and exposure time to cell lysis solutions can also limit the activity of proteases.

6.3 Antibiotics

One concern regarding a prolonged treatment duration is the presence of bacteria and contamination of the material. Therefore, several protocols have included antibiotics such as streptomycin, or amphotericin B and penicillin 76, 82, 104-106]. However, attention must be paid to antibiotic residues in the scaffold after the decellularization procedure because it can increase the complexity of regulatory approval.

6.4 Verification of cell and residual chemicals removal

It should be considered that a method or protocol is dependent upon the tissue considered. In fact, for example, despite identical duration of exposure and greater concentration of trypsin (0.5% vs. 0.05%) it has been found that the treatment was not effective in removing the cellular components from the rat aortic valve but, on the other hand, complete removal of cells from porcine pulmonary valve has been shown [73,96]. The differences in the decellularization results cannot be explained by differences in the protocols, it is more likely that the effectiveness of the decellularization procedure depends on the source of the tissue, its composition, its density, but also other factors. Depending on the tissue studied it is necessary to optimize the protocol such that one can obtain acceptable cellular removal. Several methods are available to assess the efficiency of the removal of cellular components after decellularization procedure (Table 1.2). Electron microscopic methods or polymerise chain reaction (PCR) are also possible but due to their technical complexity and expense are not typically employed to investigate the presence of nuclear or cytoplasmic debris. Moreover, it is mandatory to prove that adhesion proteins, such as fibronectin and laminin, GAGs, growth factors, elastic fibres and collagens are maintained in the ECM because these are required for the infiltration of cells into the matrix. Furthermore, mechanical testing of the ECM is desirable to assess the integrity of the structure. It is also necessary to quantify the presence of residual chemicals in the scaffold to avoid their accumulation in the tissue after the treatment which may be toxic for the host cells that will populate the matrix.

METHODS	
Hematoxylin and Eosin istological staining	Observation of nuclear structures
Masson's Trichome, Movat's Pentachrome, or Safrin O	Examine the presence of various cytoplasmatic and extracellular molecules

Immunohistochemical methods	Inspection for the presence of specific intracellular proteins (e.i. actin and vimentin) [76]
DAPI or Hoechst	Examine the presence of DNA with fluorescent molecules that bind to the AT clusters of DNA [107, 110]
Propidium iodide and PicoGreen assays	Quantitative assay which provides data about the presence of DNA [111]
DNA probe techniques	For tracking the fate of DNA in allografts after implantation or for examining the presence in the decellularized tissue [112].

Table 6.2: Methods available to assess the efficiency of the removal of cellular components after decellularization procedure

6.5 Preservation methods

Clinical use of decellularized bovine pericardium in cardiovascular surgery requires tissue banking until their clinical application, meaning that the development of preservation technologies is mandatory. Preservation methods must not alter tissue properties during long-term storage. The potential techniques that can be employed for this purpose are cryopreservation, vitrification, and freeze-drying. However conclusive investigation on adverse impacts of these procedures on decellularized bovine pericardium (DBP) matrix integrity is still missing. Cryopreservation is the standard method used in most tissue banks, it is based on the principle of water freezing, considering that in a tissue hydration reaches 80% of its mass. The goal of this technique is to ensure high viability and genetic stability of cells and to maintain unaltered the architecture and the structure of ECM during cooling and after thawing. The procedure is conducted using 10% DMSO and slow rate freezing (1°C/min) to allow storage at an ultra-low temperature [79, 80]. It should be considered that induced cryoinjury to cells and ECM due to ice formation might happen, this depends on the amount of free water present in the cells and in the tissue and it depends also on their ability to crystallize during the cooling process [113]. Conflicting results are reported

in the literature regarding the effects of this preservation method. Gerson et al. studies using two photon-laser scanning confocal imaging have shown no evidence of structural damage in cryopreserved allograft valves [75]. On the contrary, a significant loss of autofluorescence was proven with similar microscopic imaging in conventionally cryopreserved porcine heart valves [76]. Moreover, Brockbank et al. found that the rapid deterioration that has been observed in some cryopreserved allograft heart valves is in part due to interstitial ice formation [114,115]. To overcome these drawbacks an alternative ice-free method has been proposed, known as vitrification [116,117]. It is a simple and cheap procedure, and it is performed using vitrification solution (VS83) and rapid cooling to avoid ice formation [71]. VS83 contains a mixture of formamide, DMSO and propylene glycol, which has been already applied to heart valves [117]. It has been proven that the procedure maintains the ECM, but it has a cytotoxic effect on valvular cells [116, 81]. Both methods require storage ad ultra-low temperatures and frozen transport. Another procedure that can be used is freeze-drying. This can be done by using a programmable freeze-dryer and sucrose as lyoprotectant. It allows storage and transport at room temperature by using specific protectants that form a glassy state [60,61]. In the study of Wang et al., the use of this method has been investigated and the findings were that the valves treated with the protectant showed a well-preserved histoarchitecture after rehydration [108]. While freeze-drying without a lyoprotectant has been found to result in structural and mechanical alteration of the tissue matrix [62, 63, 66, 108]. In the study of Sabra Zouhair et al. [119] the effects of these preservation methods have been evaluated in terms of biochemical composition, histoarchitecture, protein stability, cytocompatibility and biochemical integrity leading to interesting results. The investigation was performed using histology, biochemistry, biochemical testing, spectroscopic and thermal analysis. All preservation methods conserved the ECM structure and its components, fibres waviness and bundle organization of collagen were maintained with no signs of deterioration, denaturation or loss of GAGs and elastin. Proteins thermal stability was not affected, and no changes were evident in the protein secondary structure. Also, the cytocompatibility of the preserved matrices has been verified with contact cytotoxicity testing enlightening that all treated scaffolds induced excellent cell migration in vivo when implanted into large animals [88,120]. However, biomechanical testing highlighted a loss of extensibility in cryopreserved samples in comparison to vitrified or freeze-dried scaffolds, which both showed similar biomechanical behaviour. Low-strain rate uniaxial loading to failure has been used for the experiment. It has been shown that cryopreserved groups had a higher elastic phase modulus when compared to control, freeze-dried, and vitrified samples, suggesting a decrease in compliance of the cryopreserved samples. The differences, however, were statistically significant when observations were made on collagen phase modulus behaviour. An important

increase in the collagen phase modulus has been also observed in the freeze-dried group but the difference with respect to cryopreserved groups was still significant. Brockbank et al. [117] highlighted that the mechanical properties variations are most likely caused by the formation of ice crystals which affected the crimping and/or the collagen fibres mobility leading to an increase in stiffness (decrease of compliance) [90]. Alterations and deterioration in collagen and elastin have been reported in Schenke-Layland et al. [76,95] studies based on multiphoton imaging regarding cryopreserved porcine conduits. While in vivo and in vitro experiments with ice-free cryopreserved porcine aortic conduits showed a reduced inflammatory response, better ECM preservation and superior with respect to conventional cryopreservation [115,81,104]. However, Gerson et al. showed suitable preservation of the ECM structure of human aortic and pulmonary heart valves and a non-significant reduction in collagen and elastin after cryopreservation [105]. These contrasting results regarding the cryopreservation method have been hypnotized to be due to DMSO residuals, known being an optical clearing agent, thus affecting the interaction of interfiber collagen and subsequently influencing the visualization [75,106]. However, additional studies would be necessary to predict the scaffold durability and functionality and to assess if the decreased extensibility of the cryopreserved matrix might have negative effects upon function, repopulation, remodelling and regeneration. In conclusion, several groups investigated the effect of the cryopreservation method on decellularized pericardium and valvular scaffolds in vitro [86, 121] and in vivo [87,88] reporting contrasting and inconclusive results. Novel preservation techniques, such as vitrification and freeze-drying have been evaluated to assess their effects on the functional and biological integrity of the tissue showing promising results. Both methods offer significant advantages over the standard cryopreservation thanks to the simplified storage and transport procedure and by eliminating the use of liquid nitrogen. However, vitrification has relatively complicated processing compared to the freeze-drying procedure and involves the use of agents that may be potentially toxic. On the contrary, the freeze-drying method is performed without toxic protective agents, and it doesn't require storage at ultra-low temperatures, thus enabling cheaper and easier storage and transport. Given these advantages, freeze-drying methods are preferable for the preservation of the decellularized pericardium for direct use in clinical applications [119].

6.6 Supercritical Carbon Dioxide

Different types of decellularization procedures are used to ensure reduction of immunogenic properties without changing the biomechanics, such that Elastic Modulus, ultimate tensile strength (UTS), fracture toughness and suture retention

strength. However, in general, the acidic, detergent, or enzymatic treatments employed for cellular removal have an impact on the mechanical and biological properties (122). The major drawback of this kind of scaffolds is the long-term durability and for this reason, continuous research is ongoing to improve decellularization techniques. Recently, an alternative gentle decellularization method has been developed, known as supercritical carbon dioxide decellularization (scCO2) leading to promising results. This procedure requires CO2 conditioning above 31.1°C (304 K) and 73.4 bar (7.3 MPa) to obtain a supercritical phase such that CO2 can penetrate the tissue. In combination with acidic and oxidative reagents, the technique can sterilize the tissue at low temperature [123, 124] providing a great advantage over the common sterilizing methods such as autoclave, gamma irradiation or ethylene oxidation which have the major drawback of affecting the mechanical structure due to cross-linking formation [125,126]. Complete cell extraction was observed in the porcine aorta (127), and recently a mechanical characterization of the scCO2 treated bovine pericardium has been reported. The investigation proved that the treatment preserved the mechanical properties of the pericardium. The UTS of scCO2 treated pericardium has been observed to be like the native one while the suture retention strength was significantly higher than glutaraldehyde BP. Moreover, tissue rehydration was reached in 2 minutes, thus, it is not a limiting factor during the surgery procedure. However, there is a lack of information regarding the long-term stability and durability, thus, further investigations are mandatory but anyhow, since scCO2 is a solvent-free procedure and only CO2 and H2O can be released, no cytotoxic or genotoxic effects are expected. Nevertheless, long-term stability depends also on DNA and α-Gal epitope remotion, but nowadays there is no method able to guarantee complete removal of these elements without modifying the mechanical properties [128], therefore, further research is necessary to assess scCO2 effect on xeno-antigen elimination.

6.7 Examples of decellularization procedures

Several studies are investigating the synergistic effect of using combined decellularization agents to obtain an acellular scaffold without compromising mechanical structure, thus, evaluations regarding decellularization efficiency, tissue strength and collagen structure have been performed. Decellularization of bovine pericardium has been performed using a combination of 0.5% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate (SDC) and 1% TritonX-100 and it has been compared to the use of each detergent individually. The results obtained from the experimental assessment show that detergent mixtures performed better than detergents individually used leading to an effective decellularization with minimal effects on the

structure and no scaffold strength has been observed. On the contrary, when considering the agents independently, they showed detrimental effects and ECM damages. It has been shown that SDS significantly affects the matrix in all the parameters estimated because it causes thickening, it affects the collagen structure and tissue strength, moreover, it is not efficient in removing the nuclear components thus leading to an incomplete decellularization. Also, the SDS leads to similar results since it severely affects the collagen organization and ECM structure, and it is not effective as decellularization detergent. Finally, Triton X-100 did not alter most of the parameters evaluated but it showed the worst in cell removal. The combined detergent protocol, instead, performed the most effective with minimal influence on the tissue thickness, a little impact on the total collagen and tissue structure and effective decellularization with no alteration of the tissue strength. The synergistic effect can be explained by the fact that synergy in mixed surfactants increases with the degree of charge difference between the surfactants (129). SDC is classified as an anionic detergent, due to its polar properties, but it acts like a non-ionic detergent. SDC may aid in the net charge difference in combi nation with SDS, which can explain the synergistic effect of the combination of SDS, SDC and TritonX-100 in the decellularizing protocol.

Low concentration SDS (0.1% SDS) has also been investigated in combination with cross-linking EDC-NHS for the fixation of the tissue [130]. The effectiveness of the decellularization procedure has been assessed by performing the analysis reported in Table 2.

Method	**Results**
DAPI and H&E staining	Microscopic examination of DAPI samples confirmed that most cellular and nuclear material were removed, and the ECM components were maintained
Qualitative microstructure analysis of SEM	No significant difference in the structure and distribution of collagen and elastin fibres of the decellularized sample vs control (native BP)
DNA estimation	The estimated level was 3 ng/mg at day 35. Degree of matrix purification from nuclear material was 99.8% compared to native samples
Biomechanical properties	No alteration of matrix natural properties was observed, although, an increase in maximal tensile strength value was detected (almost 2 times greater than control) by longitudinal tissue testing.

Cytotoxicity	Samples cultured in a suspension of human fibroblasts for 2 months. Cell monolayer was observed on the surface of the BP sample
Biocompatibility in vivo	The scaffold was implanted subcutaneously into the dorsal skin of rat model and explanted after 3 months. Histological data confirmed successful biointegration of the implant, growing immature connective tissue was observed and also increased vascularization of the tissue.

SDS is an ionic detergent which breaks the hydrogen bonds of the collagen fibres promoting tissue swelling. [131, 132]. Since SDS has the tendency to strongly interact with ECM proteins, its total removal is not simple [133]. Implantation studies in rat models showed that the residual SDS in the scaffold could result in an incomplete repopulation with host cells leading to lower durability of the implant and inhibiting tissue growth. Peri-implant necrosis was found around scaffolds treated using 1% SDS [134]. Tran Ha Le Bao et al. showed that low- concentration 0.1% SDS solution compared to higher concentration 0.3% or 0.5% SDS is more indicated for treating pericardium thanks to the better preservation of the biological properties [135]. These finding has also been confirmed by [130] in which microstructure analysis showed no alteration of collagen structure using SDS at low concentration (0.1%) performing at the same time an efficient decellularization of the BP.

Nowadays, early results have been reported regarding the potential cytotoxicity of the crosslinking of the tissue with glutaraldehyde, thus, alternatives have been considered. EDC/NHS is a glutaraldehyde-free cross-linking agent which seems to be promising. Experimental findings highlighted that after EDC treatment collagen fibrils were more densely packed and had a more uniform structure compared to native pericardium [136]. Besides the biocompatibility and the absence of cytotoxic effects of the EDC-treated scaffold, histological data also confirmed an increase in cell concentration on the implanted matrix with successful integration in rats' models. Based on these results, it can be stated that the protocol is an optical decellularization method for bovine pericardium and its potential for clinical application is promising.

In Ning Li et al. study a decellularization method resulting from the combination and modification of other procedures has been studied. The bovine pericardia have been decellularized by [137]:

- Sodium dodecyl sulphate (SDS)
- SDS+ sodium deoxycholate (SD)

- Triton X-100 (TX)
- TX+SD (TS)
- freeze–thaw cycles + SDS + SD (FSS)
- freeze–thaw cycles + TX+ SD (FTS)

and native pericardia have been used as controls. In Table are reported the investigations performed to assess the efficiency of the decellularization method and the mechanical properties preservation of the matrix.

Methods	**Results**
H&E	A reduction of visible cells in all decellularized BP was observed with respect to the native tissue. However intact cells could be observed in the TX-PB, DNA components were evident in the SDS, SS and TS-BP. While no obvious visible DNA components could be observed either in the FSS or in the FTS bovine pericardia.
Immunohistochemical analysis	Assessment of α-Gal and MHC molecules shown a significant reduction of antigen components. However, several α-Gal-positive cells were found in TX-BD suggesting that the protocol performed the worse in removing the antigens
Qualitative and quantitative biochemistry to measure content of collagen, elastin, GAG.	All the methods caused reduction of elastin and GAG content, in particular elastic fibres in SDS, SS, FSS BP were significantly reduced, fragmented, and disorganized. GAGs, instead, were mainly preserved in TX, TS, FTS-BP. TX BP has the best preservation of the components, followed by TS and FTS BP. Collagen content had significantly decreased after SDS, SS and FSS content while in TX, TS, and FTS it was comparable to the native one. No significant

	change upon the components was identified using freeze-thraw cycles.
Biomechanical assay (tensile test)	Significant differences in ultimate tensile strength (UTS) were observed between native BP and SDS, SS or FSS BP. Instead, no significant difference was found between native BP and TX, TS, FTS BP. No significant change was observed by adding the freeze–thaw cycles procedure. Although the differences did not reach statistical significance, SDS, SS or FSS- BP also had a lower elastic modulus than native BP.
Cytotoxicity	Contact cytotoxicity assay was performed using HUVECs-infected adenovirus-carrying enhanced green fluorescent protein.
Biocompatibility	L929 murine fibroblasts and human umbilical vein endothelial cells (HUVECs) were used for in vitro biocompatibility assays. No significant difference in HUVECs viability was observed between control, TX, TS, FTS-BP, while SDS, SS, FSS-BP inhibited cellular growth. Fluorescence microscope observation after 12-h of culture has shown cells adhesion on TX, TS, FTS BP surface suggesting that these treated BP had no cytotoxic effects on HUVECs and promoted cell migration.
	In vivo biocompatibility was investigated using subcutaneous implantation in rat models to assess the host immune response. A moderate-to-serious chronic inflammatory response was detected in the native, SDS, SS, TX, and TS implants. Calcification was identified in the native, SDS, SS, TX, and TS implants, while FSS and FTS-BP showed no signs of calcification. Specific immunohistochemical staining of CD68, CD4 and CD8 was performed to detect the macrophages, T-helper T- cytotoxic. The number of macrophages, T-helper, T- cytotoxic in the FTS implants was significantly lower compared to native implants.

Given the results, FSS and FTS protocols were those performing better. They allowed efficient DNA remotion and significant reduction of antigen components, although

difference have been found between TX and SDS-based protocols. **SDS** is an ionic detergent that heavily interacts with the ECM proteins, making the total removal challenging, moreover, it tends to disrupt the native tissue structure [138,139,140]. Residual SDS causes an inefficient cellular repopulation of the scaffold inhibiting cell adhesion and tissue growth thus decreasing the implant durability.

TX is a mild non-ionic detergent which disrupts lipid-lipid and lipid-protein interactions. It allows a better ECM preservation compared to SDS, but a worse decellularization effect since both DNA components and α-gal have been found in histological analysis. However, TX combined with SD in low concentration could significantly improve decellularization efficiency. SD is an ionic-detergent effective in removing cellular and nuclear components but tends to cause greater disruption of the matrix when compared to SDS, thus, it is generally used in combination with zwitterionic or non-ionic detergents to find a balance of the effects. Moreover, the use of the physical method leads to cell lysis thanks to the ice-crystal formation in the intracellular space during rapid freezing and thawing [141]. Findings reported that this step increases the effectiveness of the decellularization result and moreover, also the use of deionized water to wash samples has been proven to facilitate the removal of cellular debris. FTS (1% TX, 0.5% SD, freeze–thaw cycles) treated pericardia showed optimal preservation of collagen, elastin and GAGs content, no cytotoxicity effects on HUVECs and good tolerance after implantation in rat models with minimal infiltration of inflammatory cells and no histological evidence of necrosis and calcification in the peri-implant region. Moreover, the physical method had no influence on the ECM components and biomechanical properties, which is consistent with previous studies [142, 143]. Thus, in conclusion, experimental findings established an efficient decellularization method with optimal ECM preservation and biocompatibility.

7. Recellularization

After the procedure of decellularization, the next step of TEHV is to repopulate the scaffold with patient's cells to generate a tissue able to grow and renew itself. The choice of the cell's typology needs to consider the capacity of proliferation, renewal, and the ability of re-building the heterogeneous composition of cells necessary for the formation of a functional tissue. For the recellularization of the scaffold two main approaches can be used:

- *Traditional approach*, that is, in vitro seeding. The scaffold is seeded with cells together with growth factors and cytokines to promote cell attachment and proliferation. The construct is then inserted into a bioreactor system which provides favorable conditions for the formation of the tissue, its organization and function. The bioreactor exposes the tissue to mechanical conditioning, through a cyclic change in the flow and pressure that mimics physiological conditions. In particular, shear stress, to which cells are subjected in vivo, represent an important stimulus even during cell culture. The optimal conditioning protocol depends on numerous parameters such as the response of the cellular phenotype to mechanical stimuli, the way in which these are transferred to the cells, the scaffold used, its size and the type of mechanical stimulation. This conditioning is followed by the implantation of the engineered construct in vivo, where the tissue should grow and integrate with the surrounding tissues [144].
- *Tissue guided regeneration* involves the in vivo implantation of an unseeded scaffold, which contains all the biological information necessary to attract circulating endogenous progenitor cells (endothelial or mesenchymal) or those of the surrounding tissue. These cells should then be able to invade the scaffold and differentiate into native cell phenotypes completely repopulating it. In this case it is the host itself that acts as bioreactor, with endogenous biomechanical and cytokine signals. This approach simplifies the manufacturing process of TEHV. To attract the cells, the scaffold needs to be covered with signal molecules in order to encourage their adhesion and differentiation. To repopulate the matrix of in vitro engineered heart valves, different cell sources and cell types are needed. Parenchymal cells, that is the type of cells responsible for the specific function of the organ, are strictly necessary. In addition, non-parenchymal cells such as fibroblasts and endothelial cells increase the functional phenotype of parenchymal cells and contribute to the organization

of tissue cell architecture [145]. Endothelial cells are necessary to create an antithrombotic barrier for the decellularized matrix [146] and for protecting parenchymal cells from shear stress caused by blood flow [147]. Fibroblasts are also important since they have a role in reshaping the ECM. In TEHV they have been used in combination with endothelial and mesenchymal cells [146].

Numerous studies have been carried out to directly compare different methods of decellularization for obtaining scaffolds and there has been an effort to identify protocols that reduce the risks, optimize the procedure and the result, and that could be made standardizable. Despite the numerous research, currently there is no optimal protocol for obtaining a decellularized matrix and it can be said that each method modifies all or part of the properties of the ECM and therefore must be chosen according to the intended application. [148] In general, the pericardium fixed in glutaraldehyde of animal origin is the elective material for the manufacture of biological valves. However, the pericardial tissue used for this purpose undergoes severe calcification due to chronic inflammation resulting from an immunological incompatibility of the pericardial tissue of animal origin resulting from the failure to remove xenoantigens of animal origin. In the research for new procedures to maximize biological compatibility, the research group of the Centro Cardiologico Monzino has developed a procedure for the decellularization of the pericardium as an alternative to fixation with aldehydes based on the use of ionic and non-ionic detergents in sequence. The protocol consists in a sequential incubation of the pericardial tissue into a hypotonic buffer (10 mM Tris-HCl; pH 8.0), a detergent hypotonic solution containing 0.1% (w/v) sodium dodecyl sulfate (SDS), and a nucleic acid removal solution containing 50 U mL21 deoxyribonuclease-I and 1 U mL21 ribonuclease-A [149]. This allows to optimize the decellularization procedure but also allows not to compromise the collagen of the matrix. The protocol is permissive for the sowing and culture of cells that should be able to colonize and rebuild a tissue like a native valve. Subsequently, the recellularization of the pericardium was also performed through a perfusion bioreactor and in a subsequent study the protein content of the tissue was studied. After following the decellularization protocol, tests were performed to verify the goodness of the procedure. First, the efficiency of removal of cells from the pericardium was verified with histological analysis and observation with fluorescence microscope after DAPI staining, confirming the absence of cells after treatment. In addition, the material has been tested to investigate the presence of 1,3-α-galactose, which is one of the main xenoantigens involved in valve rejection, with immunofluorescence analysis and has been demonstrated the absence of the antigen after the treatment. In Figure 7.1 on the left there is the result of Masson's trichrome staining, a test that allows to qualitatively observe the integrity of the post-treatment collagen bundles and a decrease in thickness, also confirmed by quantitative analysis

as shown in the graph on the right side. Uniaxial tensile loading (UTL) tests were then performed to verify the mechanical tensile behavior and a specific experimental set-up has been developed to assess distensibility and permeability of the tissue. From the results obtained it is concluded that the mechanical properties of the pericardium are maintained with an increase in the permeability of the decellularized tissue, as a consequence of the massive removal of cells. This fact, however, can be exploited to deepen the tissue for sowing with the bioreactor.

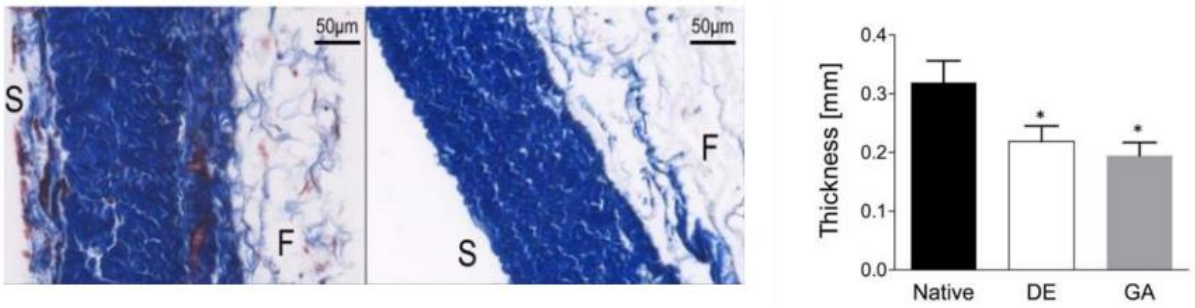

Figure 7.1: Masson's thrichrome staining. On the left side: tissue before decellularization; on the right side: tissue after decellularization. In the figure on the right quantitative analysis is reported.

Once these results were obtained, it was decided to recellularize the tissue using a U-Cup bioreactor. This allows sowing in a 3D scaffold thanks to the continuous perfusion of the cell suspension into the pores of the scaffold. The direction of oscillation of the medium is genuinely controlled by the movement of the syringe pump which allows an appropriate mass transport. For the sowing it has been chosen to use the valve interstitial cells to create favorable conditions for the maturation of the tissue thus choosing cells that have a valvular physiological role. Cell adhesion on both surfaces of the pericardium was then studied after 14 days of culture, and it was observed that the cells preferred to adhere to the sierosa [149]. In a subsequent study, permeability tests were carried out observing that the decellularized pericardium provides insufficient permeability to perfusion with cells via bioreactor. So, in order to increase permeability, a modification was introduced in the decellularization protocol, which is the incubation with Triton X-100, a non-ionic detergent that specifically breaks the lipid-lipid and lipidprotein interactions without denaturating the extracellular matrix. The increase in permeability causes the decrease in the pressure gradient ΔP during the culture with the bioreactor thus allowing to obtain a high sowing efficiency with full penetration of the cells inside the tissue, followed by a maturation phase [150]. In a third study, following the recellularization of the pericardium, the maturation of the living material was studied with attention to protein deposition and cell phenotype.

To do this, a 21-day cell culture was conducted, and a quantitative mass spectrometry analysis was performed to study the protein content and it has been observed that the recellularized pericardium compared to the native one showed a different number of specific proteins, and this is an indication of the fact that a de-novo protein synthesis took place by the valvular cells with deposition of new ECM. Analyzing the proteomic data in more detail, it has been observed that despite the lack of some components of the ECM such as aggrecan, decorin and osteoglycine, the repopulation and maturation of the tissue had occurred, even if the lack of these proteins suggested that the process was not yet concluded [151]. The work sets a new standard in tissue engineering compared to the existing literature, in fact previous investigations have shown insufficient cell growth throughout the depth of the tissue due to limited cell penetration. The method is therefore efficient and potentially scalable at an industrial level to achieve substantial growth of competent valve cells in the pericardium of animal origin and with the potential to use it in the production of living tissue for valve replacement therapies.

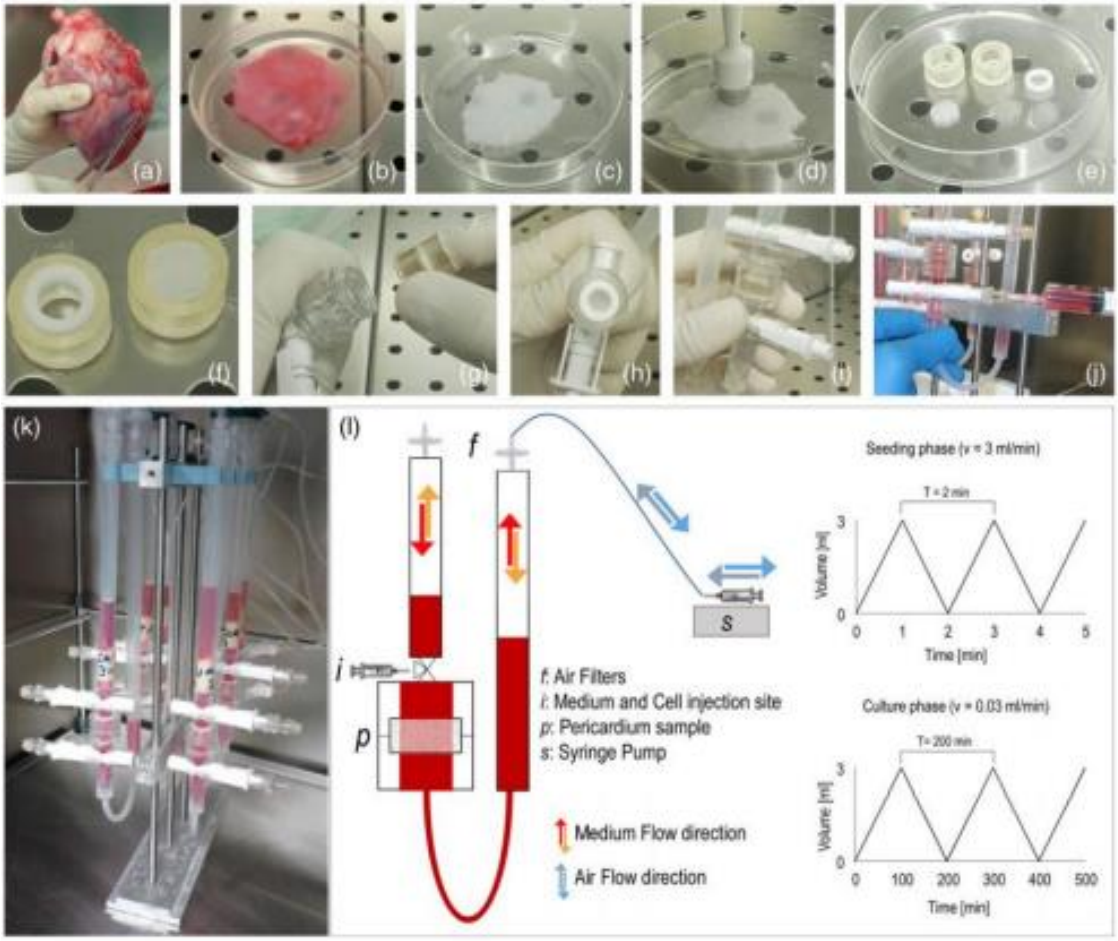

Figure 7.1: Steps of the procedure

8. Conclusions

Heart valve disease has a prevalence of 2% in the world's population and is continuously increasing considering the increase in average age. The main pathologies of interest are stenosis and insufficiency in the aortic or mitral valve of the left heart because they are subject to higher pressures. Current surgical therapy uses mechanical or animal-derived biologic valve replacements, but these frequently lead patients to reintervention or complications. In general, animal-derived glutaraldehyde-fixed pericardium is the elective material for the manufacture of biological valves. However, the tissue undergoes severe calcification due to chronic inflammation resulting from immunologic incompatibility due to failure to remove animal-derived xeno-antigens. In the research for new procedures to maximize biological compatibility, researchers around the world have focused their attention on solutions proposed by tissue engineering. The idea is then to recreate in vitro the valve leaflets starting from the patient's cells and then proceed with the replacement of the pathological valve. Also, in this case bovine pericardium appears to be the elective material for the construction of the valve leaflets thanks to its great flexibility, which allows an excellent manipulation of the tissue, suture retention, excellent hemodynamics thanks to a complete and symmetrical opening of the leaflets, viscoelastic behaviour which ensures operation for millions of cycles of opening and closing during the cardiac cycle without fatigue. Excellent results have been demonstrated following reconstruction of aortic valve leaflets with bovine pericardium. Several studies have also confirmed excellent clinical results in terms of postoperative hemodynamic, good ventricular function, and minimal structural deterioration of the valve over a 23-year follow-up. Particular attention must be paid, however, to the heterogeneity of the pericardium from a histological and biochemical point of view, which therefore requires a mechanical characterization of its properties in order to select portions characterized by uniformity in thickness and orientation of collagen fibres so as to have regular mechanical properties within the selected part. Before its use the material must be deprived of the cellular part to avoid having hyperacute rejection due to the presence of xenoantigens in particular the epitope α-Gal, which is present in most mammalian tissues except in humans and some monkeys. So, the idea that arises is to implement a process of decellularization of a donated tissue, in order to eliminate the cellular components responsible for the immunological reaction, but without damaging the biological and mechanical properties or altering the composition of the native ECM, whose components are generally preserved between species and therefore do not lead to immune reaction. The advantage is therefore to obtain a substrate that is recognized

by the cells, promoting their adhesion and vitality. Several studies have in fact shown that one of the main factors determining the longevity of heart valves is precisely the ECM, composed of a mix of structural and functional molecules whose tasks are to provide mechanical support and regulate various cellular activities both in the development of the tissue and in the repair and regeneration of the same. Some problems related to this procedure are:

- Limited availability of donated tissue
- Low reproducibility due to interspecific differences
- The decellularization process is not standardizable, is expensive and time consuming to perform.

Generally, a distinction is made between physical, chemical, and enzymatic methods. In most cases, these methods are used in combination sequentially to achieve a better effect. However, the effectiveness of the protocol depends on the tissue in question, the combination of the detergents themselves and the duration of exposure. The risk of using these substances is that the matrix may be damaged resulting in changes in mechanical and structural characteristics, Or residues may become trapped in the matrix causing a post-implant inflammatory response due to the release of toxic substances not removed. The synergistic effect is a mechanism still not fully known, but it is hypothesized to be related to the difference in charge that is created between the supernatants, this would also explain the balance effect that an agent has on the action of others. In the literature there are several methods aimed at solving the clinical problem, however, to date, there is still no method that can be defined as a gold standard because of the difficult reproducibility of the experimental set-up and all the factors that contribute to the definition of the effectiveness of the process of decellularization. In addition, not all combinations of detergents have a synergistic effect leading to an optimal result, the underlying mechanism is not yet fully known and even less the factors that influence it. The efficiency of the decellularization process strongly depends on the type of solutions/methods used, the concentration of the solutions used, the type of tissue and the exposure time of the tissue. Therefore, significant progress has been made in the optimization of decellularization protocols, and in the study of the synergistic effect of the combination of different detergents leading to excellent first results; on the other hand, however, the decellularization procedure is still far from being a standardized procedure, longer follow-ups are needed to determine the durability and viability of the tissue and further studies on the potential calcification of the implant are necessary. However, in recent years research has moved quickly in this direction offering increasingly advanced and sophisticated solutions, so it is plausible to think that in a short time the problems mentioned above will be solved.

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