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Tesi di Dottorato

From the anatomical study to the application of different bioengineering techniques for the creation of new vital heart valve substitutes

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‘Volli, e volli sempre, e fortissimamente volli’

Vittorio Alfieri

To my Mentors

Contents

Summary

This doctoral thesis focuses on the approaches currently in use to develop new vital valve devices: heart valve tissue engineering and heart valve tissue guided regeneration. Indeed, it investigates some aspects able to modify the preservation of the implanted construct, i.e. the immunogenic properties of the extracellular matrix and the biological entity of stem cell populations in heart valve leaflets.

The porcine semilunar valves, as the bovine pericardial-derived ones, find large clinical usage for the substitution of dysfunctional valves. The rational of their employ is the high morphological and functional analogies with the native human ones and hence they can provide a valid extracellular matrix (ECM) for bioengineered prosthesis. Once eliminated the xenogeneic cell component through a decellularizing treatment, it is possible to benefit from the extracellular fiber mesh as a template for repopulation with human stem cells in order to develop autologous-like replacements. So far the influence on cellular attachment exerted by the anisotropic ECM distribution in *fibrosa* and *ventricularis* has never been investigated. Porcine pulmonary leaflets were decellularized with a Triton X110/Sodium Cholate-based protocol. The absence of alpha-Gal epitopes, highly immunogenic for the human species, has been demonstrated through a double fluorescence technique using an isolectin and a specific monoclonal antibody. After treatment with fetal bovine serum and fibronectin to increase adhesion, human bone marrow mesenchymal stem cells have been seeded either onto the *ventricularis* or *fibrosa* and statically maintained in culture for 30 days. Not only *ventricularis* is able to consent a higher attachment, but also increased spreading and early cell differentiation in the cusp stroma. The interaction between the same cells and decellularized human leaflets has been further evaluated by *ventricularis* seeding. The homologous combination favors proliferation, with decrease of apoptotic events and enhanced cell maturation level, so that it is possible to appreciate the expression of typical mature smooth muscle markers in *ventricularis*. ECM performs consequently an essential role for cell integration by furnishing specific signals for the acquisition of the correct valvular phenotype.

A further hypothesis for the achievement of new vital substitutes is likely represented by tissue guided regeneration operated *in vivo* by ECM in respect to the recipient's cells. Decellularized aortic roots were used to mimic a reconstruction of the RVOT in minipigs and performances were followed echocardiographically for 12-14 months, revealing a progressive function improvement. A continuous engraftment is observed in the explanted specimens, even if the layers less exposed to blood circulation are still devoid of cells. The observations on primary cultures of the vessel and leaflet tissues lead to hypothesize the contribution of two main phenotypes to repopulation: pulmonary artery smooth muscle cells and mobilized mesenchymal stem cells.

Particularly important is the preservation of the bioconstruct once the function has been rescued, but at the same time it is essential to understand the key cell effectors participating to valve dysfunction in order

to prevent the phenomenon. Scarcely studied, possible stem cell populations in the valves could be involved in the homeostatic tissue remodeling or in adverse pathophysiological events. Aortic roots (n=27) with related mitral leaflets (n=27) have been classified in 5 groups depending on allograft donor's age (10-60 years). In addition, aortic and mitral pathological leaflets (n=10) were harvested during valve replacements. Cusp cryosections have been analysed through classical histology, Mallory's trichrome, Von Kossa, Oil Red O and immunohistochemistry for differentiated, inflammatory, calcifying, different lineage-derived stem cell markers. With aging the typical observed feature is the accumulation of lipids, both as small droplets in the *subventricularis* layer or as cholesterol crystals in the *fibrosa*. Calcifications appear rare. Stem cell epitopes are highly expressed in the leaflet to reveal a specific spatial distribution. Primary cultures obtained from these specimens showed a phenotype and a differentiation potentiality, suggestive of mesenchymal stem progenitors without apparent osteogenic induction. Further studies are needed to better understand the contribution of these cells in heart valve calcification.

In conclusion, both proposed modalities for the achievement of new valve substitutes appear valid instruments for the aim, because relying on fully decellularized alpha-Gal negative matrices permissive of cell-repopulation. Future efforts and application of new biomimetic strategies would surely implement the encouraging results here demonstrated. New insights on stem cell valvular biology have been proposed here for the first time with important confirmations on the continuous cell remodeling interesting the cusp tissue.

Riassunto

Tale tesi di dottorato si focalizza sugli approcci correntemente in uso per lo sviluppo di nuovi sostituti valvolari con caratteristiche di vitalità: l'ingegneria tissutale valvolare e la rigenerazione valvolare tessuto-guidata. Inoltre, mette in luce alcuni aspetti in grado di influenzare la preservazione dell'impianto, come le proprietà immunogeniche della matrice extracellulare e l'entità biologica delle popolazioni staminali nei lembi valvolari cardiaci.

Le valvole semilunari porcine, come quelle di pericardio bovino, trovano largo impiego per la sostituzione di valvole non più funzionanti. Il razionale per l'impiego di esse è l'alta analogia morfologica e funzionale con le valvole umane, che le rende fonte ottimale di matrice extracellulare (ECM) per protesi bioingegnerizzate. Una volta rimossa la componente cellulare xenogenica attraverso un trattamento decellularizzante, è possibile beneficiare della trama di fibre extracellulari come supporto tridimensionale per il ripopolamento con cellule staminali umane in modo da sviluppare dei sostituti simil-autologhi. Finora, non è mai stata indagata l'influenza esercitata dalla distribuzione anisotropica delle fibre dell'ECM in *ventricularis* e *fibrosa* sull'adesione cellulare. Lembi polmonari porcini sono stati sottoposti a decellularizzazione mediante un trattamento a base di TritonX100/ Sodio Colato. L'assenza dell'epitopo alpha-Gal, altamente immunogeno per l'uomo, è stata dimostrata attraverso una doppia tecnica di fluorescenza con un'isolectina e uno specifico anticorpo monoclonale. Dopo condizionamento con siero bovino fetale e fibronectina per aumentare l'attaccamento, le cellule staminali mesenchimali del midollo osseo sono state seminate sia su *ventricularis* o *fibrosa* e mantenute staticamente *in vitro* per 30 giorni. Non solo la *ventricularis* favorisce una maggiore adesione, ma anche un'aumentata diffusione all'interno dello stroma e un precoce differenziamento cellulare nei citotipi valvolari. Successivamente, è stata valutata l'interazione fra cellule umane e matrice decellularizzata umana, effettuando ancora una volta la semina su *ventricularis*. La combinazione omologa permette di ottenere una maggiore proliferazione , una diminuzione degli eventi apoptotici e un maggiore grado di maturazione del costrutto, tanto da apprezzare l'espressione di proteine tipiche del muscolo liscio proprio nella *tonaca* di pertinenza, la *vetricularis*. L'ECM effettua quindi un ruolo essenziale per l'integrazione cellulare fornendo segnali specifici per l'acquisizione del fenotipo valvolare corretto.

Un'ulteriore ipotesi per l'ottenimento di nuovi sostituti vitali può essere rappresentata dalla rigenerazione tessuto-guidata, favorita *in viv*o dall'ECM in grado di esercitare un ruolo attrattivo e di guida per le cellule dell'ospite. Radici aortiche decellularizzate sono state impiantate in minipig per mimare la ricostruzione del tratto di efflusso del ventricolo destro e la funzionalità valvolare è stata monitorata ecocardiograficamente per 12-14 mesi, rivelando un progressivo miglioramento della funzione. Un ripopolamento continuo può essere osservato attraverso le analisi *ex vivo* negli espianti, anche se le regioni meno esposte al flusso sanguigno sono ancora prive di cellule. Le osservazioni sulle colture primarie dei

tessuti parietale vascolare e del lembo portano a ipotizzare il contributo di due maggiori fenotipi al ripopolamento: cellule muscolari lisce dell'arteria polmonare e cellule staminali mesenchimali mobilizzate. Particolarmente importante non è solo la creazione, ma anche il mantenimento del biocostrutto: una volta che si è ripristinata la funzione, perciò, è essenziale comprendere i reali effettori cellulari coinvolti nella disfunzione valvolare per prevenire il fenomeno già nelle prime fasi. Scarsamente studiate, eventuali popolazioni cellulari staminali a livello valvolare potrebbero partecipare al rimodellamento tissutale omeostatico, ma anche a eventi patologici avversi. Homograft valvolari aortici (n=27) con annesso lembo anteriore della mitrale (n=27) sono stati classificati in 5 gruppi a seconda della fascia d'età di appartenenza del donatore (10-60 aa). In aggiunta, sono stati esaminati anche lembi aortici e mitrali (n=10), rimossi in sede di sostituzione valvolare. Criosezioni di tessuto cuspidale sono state sottoposte all'analisi istologica classica, istochimica (von Kossa, Oil Red O) e immunoistochimica per marker di cellule differenziate, citotipi calcificanti precoci, flogosi e cellule staminali di vari lineages. All'aumentare dell'età, il tipico aspetto osservato é l'accumulo di lipidi, sottoforma di piccole gocce nella tonaca sottoventricolare o depositi di colesterolo nella *fibrosa*. Il riscontro di calcificazioni è raro. Gli epitopi delle cellule staminali sono espressi nel lembo valvolare a rivelare una precisa distribuzione spaziale. Le colture primarie ottenute da questi campioni mostrano un fenotipo e proprietà di transdifferenziazione, caratteristiche di un possibile progenitore mesenchimale, senza tuttavia apparente induzione all'osteogenesi. Ulteriori studi sono necessari per meglio comprendere il coinvolgimento di tali cellule nella patologia valvolare.

Concludendo, entrambe le modalità considerate per la realizzazione di un nuovo sostituto vitale sembrano validi strumenti a raggiungere lo scopo, basandosi su matrici decellularizzate prive di alpha-gal e prontamente colonizzabili. Esperimenti futuri e l'applicazione di .nuove strategie biomimetiche potranno sicuramente migliorare i risultati incoraggianti finora ottenuti. Sono state inoltre proposte per la prima volta nuove informazioni sulla biologia cellulare staminale della valvola cardiaca con importanti conferme sul processo di continuo rimodellamento cellulare a carico del tessuto cuspidale.

Chapter 1

Introduction

Historical notes and new insights on the comprehension of
valve biology and pathology for
the development of new vital substitutes

1.1 A historical *excursus* in the studies of the heart valve apparatus

> 'There is a pair of them [two vessels] at the entrance of which there have been constructed three membranes for each, rounded at the extremity at least, to the extent of a half circle, and when they come together it is marvelous how they close the outlets, the ends of the veins (aorta and pulmonary artery).' [1]

This is one of the first anatomical descriptions of the semilunar valves belonging to the outflow apparatus of the heart. Within *De Corde* of the Hippocratic Corpus, this quotation found temporal attributions from the medical School of Locres in Sicily to the fourth century Cnidian School or even to the magnificent period established in Alexandria by the Macedonian King Alexander the Great [2-4]. In this long embracing period and even before, still fascinating, anatomy notions could be only achieved by the study of animal organs and attributed to the human ones also by inferences from the outer observation of the human body. Natural philosophy contributed to complete the 'image' of each organ by teleologically establishing its ontological function. Until the Alexandrian period (third century B.C.), the soul and the reason, *psyche* and *gnome*, were retained to be seated in the heart. As powerfully describes in the *Historia Animalium* one of the maximum followers of the cardiocentric theory, Aristotle (384-322 B.C.), who probably did not recognize the cardiac valves, the heart is 'central, mobile and hot, and well supplied with structures which served to communicate between it and the rest of the body' [5]. The assertion of the heart as a central organ for the living beings therefore well describes the interest for the study of its conformation and justifies the speculations about its nature. The discovery of heart valves, both semilunar and atrioventricular, is although prior to *De Corde* and more accredited to Erasistratus (310-250 B.C.), as supported by Galen [6]. From the careful distinction of the arteries and veins prepared by Herophilus (335-280 B.C.), founder and colleague of the Medical school where he taught, the anatomo-physiologist Erasistratus continued the study of the circulatory system describing other blood vessels, as the azygous vein or the superior and inferior *venae cavae* through the first dissections allowed on the human body [7]. In particular, he described any leaflet of the cardiac valves as a *glochin*, the Greek term for the Latin *cuspis*, simply referring to a point of projection [8]. The more recent dating of *De Corde* than Erasistratus's work could be supported by the fact that previously any anatomical investigation could be achieved only in animals and the following quotation would temporally frame the treatise:

> 'And if someone... should remove the heart of a person who has died and should push aside one of these heart valves but bend the other upward, then neither water nor air that is pushed against (the valves) would pass through the valves into the heart, especially not through those on the left side of the heart.' [9]

Again a powerful report of an important property of the heart valve, the unidirectionality of blood flow and hence the continence, that is reflected *in vivo* in the capability to maintain the separation of the ventricular chamber in respect to the artery compartment during the blood filling phase. The specification attributed to the left part of the heart still experiences the cardiocentric vision of the innate intellectual competence emanating from it. The statement of the irreversible direction of blood flow was once more tightly bound to Erasistratus's definition of the heart as a pump with four valves, two ensuring the inflow and two the outflow, of the two-chambered organ. Fascinated by the idea of *techné,* as a useful tool to figure the complex and perfect architecture of the bodies' *physis*, Erasistratus was probably inspired by the mechanism of the water pump proposed by his contemporary Ctesibius to illustrate the functionality of the heart, taking into account both blood and air (*pneuma*). The first, coming from the *vena cava*, enters the right ventricle by a valve and reaches the pulmonary vessels through another one, while the pulmonary *pneuma* is sucked in the left ventricle and propelled to the aorta passing through other intake and outflow valves [10]. The two flows, although distinct inside the organ, share a common pneumatic force which rises from the *horror vacui* proved by *physis* and in a more mechanical prospective which is at the basis of the progression of the pulse as a wave [11]. During the first century B.C., the scientist Galen (129-190 B.C.) accepted Erasistratus's cardiac valve anatomy and consolidated his experiences by reporting them in *De Hippocratis et Platonis placitis* [12], a treatise with wide diffusion not only in the western medical society, but also in the Islamic world.

> 'The more strongly the thorax, in its exertion of a compressing force, tends to drive the blood (out of the heart), the more tightly do these membranes (the sigmoid valves) close the opening. Invested in a circular manner from within outward, extending throughout the entire conference of the interior of the vessel, these membranous valves are, each one of them, so accurately patterned and so perfectly fitted that when they are put upon the stretch by the column of blood, they constitute a single large membrane which closes (watertight) the orifice. Pushed back by the return of the blood, they fall back against the inner surface of the vein, and permit an easy passage of the blood through the amply dilated orifice (which they an instant before closed so perfectly).' [12].
>
> On this basis, the Iranian physician Rhazes (Ray, 864-930) contributed to a further portrayal of the heart valve apparatus in the book *Tebb-e Mansouri*:
> 'the right ventricle has two orifices, one of which is the orifice through which the blood coming from the liver via a great vein enters the right ventricle and its orifices has three membranes which close backwards, but open to allow the blood to enter the heart. The second orifice is at entrance of a vein, which travels from this ventricle to the lungs. This

vein has no pulsation but its wall is thick and for this reason, the anatomists have named it the arterial vein.' ' the left ventricle has two orifices one of which is at the exit of the large artery called the aorta. This valve has three membranes, which close from the outside inwards such that it allows the blood and the vital spirit (*pneuma*) to exit the heart freely. The second valve is at the orifice of the artery which comes from the lung through which the air reaches the heart from the lungs. This valve has two membranes which close from inside outwards, so that it opens to allow the air into the heart.' [13]

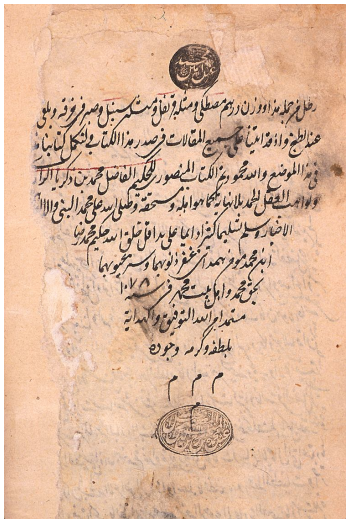


Fig. 1 *Tebb-e Mansouri* by Rhazes [13]

Ali ibn Abbas al-Majusi, who lived in the same period, reported a similar vision of the heart and blood vessels in the *Liber Regius*, the Latin version of Kitab Tebb-e al-Maleki (a treatise page is below reported [14]):

'The heart has two cavities, the right and left ventricles which are separated by a thick septum. This septum has a passage which some has called it 'the third ventricle' which is totally untrue.' 'The right ventricle has two orifices from one of which the blood from the liver is transferred to it by the '*vena cava*'. This orifice has a valve with three small membranes which fall together once the blood has entered the right ventricle and thus preventing the blood from returning into the *vena cava*. From the second valve, a vein exits whose structure is like an artery and thus is known as the 'arterial vein'...

Fig. 2 A page of the *Liber Regius* by al-Majusi [14]

From the left ventricle two arteries exit, the smaller of which has a soft wall and is therefore called the 'venous artery'. This artery conveys a large portion of the blood and the vital spirit (*pneuma*) to the lungs for its nourishment. In the lungs, the arterial vein divides into many branches and obtains air from the lungs and conveys the air to the

> heart from the opposite side. The second large artery is called aorta which divides into two branches, one of which travels upwards and the other downwards.' [14]

Ali Husain Gilani (A.D. 948), a predecessor of the more famous Avicenna, asserted the presence of eleven cusps in the heart and especially considered the aortic valve for its position at the outflow of the left ventricle and its resemblance to the Greek letter Sigma when open or to a triangle when closed (in contrast to the actual echocardiographic triangular vision during closure and Y form during opening of the valve) [15]. However, these works are dramatically influenced by Aristotle, Galen and his forerunners' ideas of the heart: even if Ali Ibn Abbas al-Majusi refused Aristotle's theory of the 'triventricular' heart, he did not introduce as his compatriots any substantial modification to the anatomy proposed earlier. Although some medical interpreters sustain that Aristotle's anatomical description of the heart was not correctly understood and try to compel dissertations about the mistranslation of the Greek term 'ventricles' in the modern connotation, rather than with the more correct 'cavities' (hence speculating the existence of at least the left atrium in a 'triventricular' heart) [16, 17], it is widely accepted that the left and especially the right atria started to be considered as true cardiac chambers, and not as venous dilatations, only with the Renaissance scientists Leonardo da Vinci (1452-1519) and Andreas Vesalius (1514-1564) [16]. The practice of human dissection has been for Leonardo particularly essential: the careful examination of the corpses and the innate ability to graphically reproduce any observed feature represented not only a noteworthy manual exercise, but also introduced new insights on the cardiac organ and its valves - even if in some passages he was surely inclined to the Galenical anatomy. [11]. Driven by a teleological need of investigating human anatomy, at the end of his life he has been able to analyze 30 bodies: one belonged to a centenarian whose peculiarities Leonardo described:

> 'And this old man, a few hours before his death, told me that he has passed 100 years, and that he was conscious of no failure of body, except feebleness. And thus sitting upon a bed in the hospital of Santa Maria Nuova at Florence, without any untoward movement or sign, he passed away from this life. And I made an anatomy to see the cause of a death so sweet, which I found to proceed from debility through lack of blood and deficiency of the artery, which nourishes the heart and the lower members. I found this artery very desiccated, shrunken and withered.' [17]

but in the same *Codex* another passage refers to this dissection:

> 'the other anatomy I made was of a child of 2 years in which I found everything to be the opposite to that of the old man.' (*Windsor 19027v*). [17]

Not only Leonardo emphasized the pathological aspects of the old man's arteries, but he also attributed them the cause of his death, both diagnosing the advanced atherosclerotic signs and

inferring their absence in a younger subject. The application of the deductive reasoning made Leonardo hypothesizing the effect of aging on blood vessels:

> 'The tunics of the vessels behave in man as in oranges, in which the peel thickens and the pulp diminishes the older they become. And if you say that it is the thickened blood which does not flow through the vessels, this is not true, for the blood does not thicken in the vessels because it continually dies and is renewed.'(*Windsor 19028v*) [17]

The comparison of different aged men as well as of different species (animal and human) reported in his notebook (*Quaderni I, folio 3*) let Leonardo to better understand further characteristics of the cardiac structures, as the intraventricular moderator band (*catena*) and the ventricular *trabeculae*. In the same chronological period (1508) in which Leonardo studied the movement of the water and the liquid vortex (*Leicester Codex*), he turned his attention also to the investigation of the heart valve apparatus. It is therefore not surprising his approach as engineer and in some way as technologist to the examination of the heart valves. The marvelous drawings of the cardiac valves, actually part of the *Windsor Codex*, are to be considered valid representations even nowadays. Particularly interested by the mechanical forces in action during the cardiac function, his greatest account was for the left side and hence for the mitral and aortic valves, but he did not ignore the right ones. The left atrioventricular valve, named mitral in the Renaissance probably later by Andreas Vesalius (1543) for its similarity to the *mitre* of a bishop [17], was studied by Leonardo mainly in the bovine heart, fact explained by the quite enormous dimensions of the sketched connections. He reported the closure conformation of the valve as it would be formed by 4 cusps with the juxtaposed margins shaping an H letter and made the typical indentation of the posterior leaflet appreciable. He also paid attention to the drawing of the *chordae tendinae*, attached at the ventricular aspect of the mitral cusps and in particular to the basal or strut cord, which modern imaging techniques revealed necessary for the correct heart functioning during the cardiac cycle.

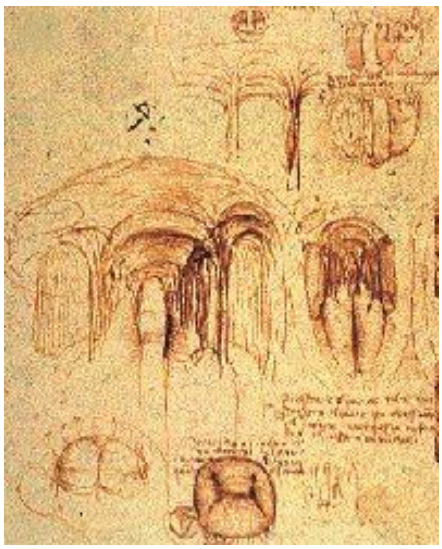


Fig. 3 Sketches of the papillary muscle and *chordae tendinae* by Leonardo da Vinci in *Windsor 19080r*.

Leonardo was convinced that the leaflets, he termed *uscioli*, were composed of two layers in the atrioventricular valves: the atrial aspect was called *pannicolus carnosus* in the name of the smooth surface and the fleshy thickness, while *chordae tendinae* of the papillary muscle formed the *pannicolus nervosus* or the armor of the ventricular side with the function of preventing possible flapping. The closure of the atrioventricular cusps is provoked on Leonardo's hemodynamics by the force generated by the exit of the blood from the 'lower ventricles' (proper ventricles) to the 'upper'

chambers, the auricles; a reflux of blood in the inverse sense facilitated their re-opening. By evaluating the chain of events interesting the atrioventricular valves during the cardiac cycle, Leonardo ascribed to the systolic relaxation of the papillary muscles together with the ventricular contraction the cause of their closure, while the opposites were able to open the orifices during diastole (p.218). In particular, Leonardo's pictures about the tricuspid valve of the right part revealed the importance of three papillary muscles, the septal or 'on the intermediate wall', the inferior or 'in the angle' and the anterior or 'in the middle of the covering of the right ventricle', in the mechanism of closure that he also emphasized in this passage:

> 'Cut out these three muscles with their cords and valves and then join them in the manner in which they exist when the right ventricle closes itself, and then you will see the true shape of the valves [and] what they do with their cords when they shut themselves.' [17]

Teleological explanations of this anatomical arrangement of the *chordae tendinae* are supplied by Leonardo:

> 'Nature has made the *chordae* [to attach] on the reverse side of the fleshy membrane of the three cusps with which the gateway of the right ventricle is shut. She has not made them on the posterior aspect because these cusps experience more effort when they draw the blood in than when they squeeze it out.' [17]

And he also specified that:

> 'The cusps of the right ventricle are not entirely double because it would follow, as has been said in this treatise of mine, that at the place [continuous margins of the valves] where it is not necessary, it would be doubled to four [layers] and where it is necessary, it would be twofold only: and in this case Nature would be wanting in her law, as was said at the beginning of this treatise.'

He also examined other qualitative appearances of the atrioventricular leaflets, as the lower thickness and higher abundance of *chordae tendinae* in the right side by inferring that

> 'This thing was ordained by Nature so that when the right ventricle begins to close, the escape of blood would not cease abruptly since some of the blood had to be given to the lung and none of it would be given if the valves prevented its escape'. [17]

The panel *Windsor 19080r* also evidences the relationships among the heart valves: Leonardo pointed out that the connection in between the aortic and mitral valves has a bony consistency, *os cordis*, an anatomical feature once again derived from the ox observation. Leonardo thought that the pulmonary and aortic valves belonged respectively to the pulmonary artery, named at that time *arteria venale*, and to the aorta, rather than to consider them true cardiac components. Their

topographical position inside the cardiovascular system could be defined by Leonardo as the boundary between the heart and the arteries originating from it, but surely they had to be regarded as the first part of these latest ones. A mechanical deduction was subject to his belief: not the ventricular mass, but only the arteries showed the elastic capability of recoiling, a necessary condition for the opening and closure of the semilunar valves, otherwise

> 'if these valves had been constructed within [the ventricular substance] at the base of the heart, which is very strong and resistant, it would necessary follow that the revolution of the blood would turn back from this resistance and beat against the weak valves which in a short time would be staved and destroyed.' [17]

The functioning of the valve of the aortic artery was impressively attractive for Leonardo: for its derivation within the aorta, the valve is always represented with its two coronary sinuses or Valsalva sinuses. At the level of these latest aspects, the base of the cusp is identified as part of the aorta and by its transversal cut as a triangle inserted in its circumference.

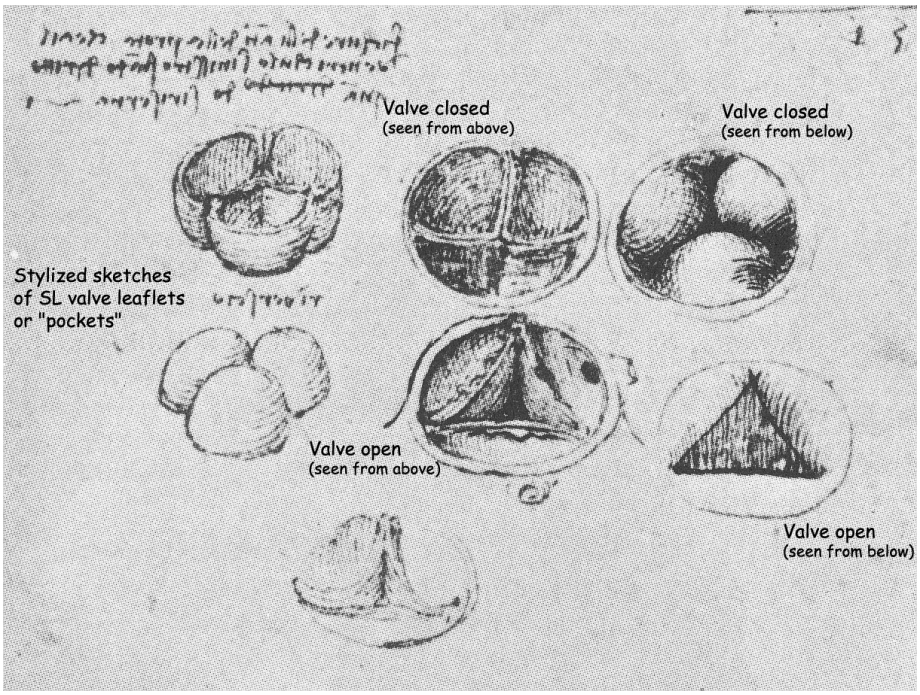


Fig. 4 Aortic valve closure and opening from different views, as designed by Leonardo. Modified from [17]

Reaching the free margin of the *usciolo*, the thickness decreases and the projection assumes a quite triangular morphology. A short statement is made about the pulmonary valve, which is described by Leonardo as a duplicate of the aortic valve in the right ventricle. Returning to the aortic valve, coronary sinuses, triangular base and leaflets are not considered as simple anatomical elements, but assumed for him a key role during the rheological events against the valvular district. Leonardo already had the occasion to fully appreciate the dynamic phenomena occurring to the water passing through a level pipe and to illustrate the flow turbulence as caused by the 'impetus of water under other water', in other words by the sum of different forces generated by the water in respect to an obstacle.

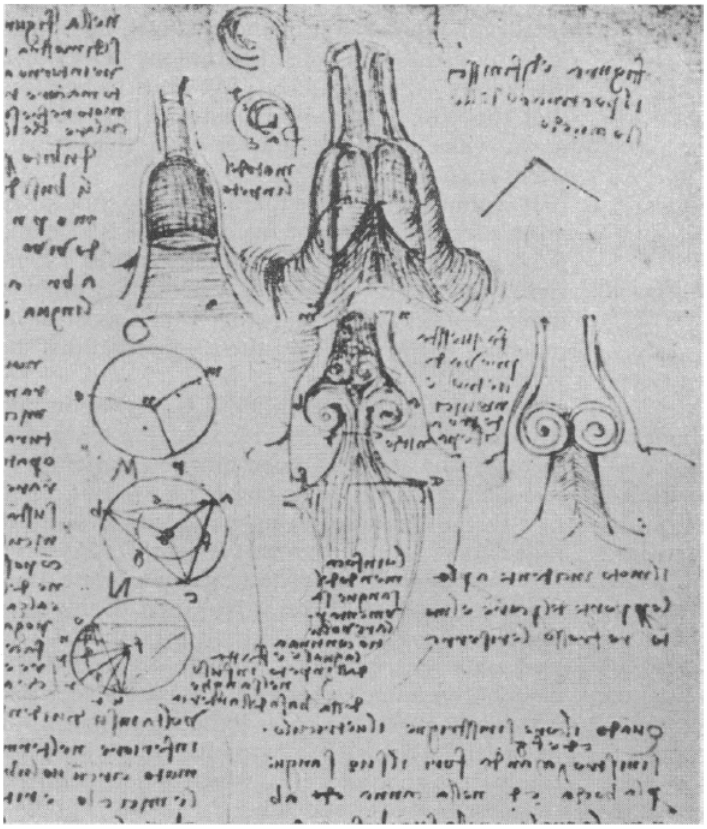


Fig 5 *Leonardo's model of the aorta and its valves, divided into three parts to show its mode of construction; the design is represented diagrammatically below in three circular figures. The closure of the aortic valve cusps by the return eddies of blood is shown in the central figure. Beside this are the words 'fa questa prova di vetro e movici dentro a cise panicholo'. (Make this glass-trial and move in it the pannicles). (QA IV 11v)*

Fig. 5 Aortic valve blood rheology as studied by Leonardo. From *The Royal Collection 2005, Her Majesty Queen Elizabeth II*

He found easy to apply his previous observations to the blood dynamics and for this purpose, he intended the aortic valve as subdivided in three main portions, the *emicicle*s, each composed by a leaflet and its corresponding sinus of Valsalva. The concavity of the *emicicle* was responsible on Leonardo's opinion for the closure of the valves because

> 'it reflects the percussion of the blood with a large and speedy momentum towards the center of the triangle [aortic aperture] where it presses against the apex of the cusp.' [17]

He believed the friction provoked by the sinuses's vortices could generate at first 'fever' and hence, seen that the aortic orifice 'emits blood as often as the heart beats', the 'innate heat'. His deductive activity made him speculating about another contention but it is somehow intriguing how he tried to formulate a sort of thermodynamic theory of the cardiac function. As a general rule of his descriptive approach, he not only applied deduction but he also tried to reproduce the dynamic events he observed following the philosophy of *regressus demonstrativus*. The application of this method to the aortic rheology can be esteemed in this passage translated from the *Windsor 19082r*:

> 'How the blood which turns back when the heart reopens is not that which closes the valves of the heart. This would be impossible because if the blood beats against the valves of the heart while they are corrugated, wrinkled and folded, the blood which presses from above, would weigh and press down the front of the membrane upon its origin, as is shown at the valve "r", "o" [in the figure], the folds of which, being weighted

> down from above, would close in solid contact, whereas Nature intended it to be stretched in height and width.'

that analyses the typical phenomenon of tripped opening of the valve during the infusion of antegrade aortic cardioplegia during cardiac surgery interventions [18].

A similar approach was at the basis of the iconographic investigation of Andreas Vesalius, recognized as the initiator of the dramatic changing perception of medicine from the Renaissance on. For the first time, an anatomist critically rose against the scientific method applied so far, giving life to a profound medical revolution: a true investigator had to examine a corpse not so much as a mean to repeat the predecessors' lessons, but to make his own hypothesis, verify them even by confuting the work completed before. He therefore triggered the beginning of the empirical methodology era. Vesalius is surely a strong promoter of this phenomenon, but as described before, even Leonardo tried to understand the function of the body parts rather than only to depict them. It was more and more clear that pure speculative reasoning was not able to explain the observed reality and so surprisingly philosophers and scientists begun to apply the motto '*entia non multiplicare praeter necessitatem*' supported by William of Ockam [19] especially for the heart ,to which many body and soul faculties had been attributed so far. Differently from Leonardo, Vesalius leaned on an engraver to visually translate his observations: that this could be even the famous Titian –as some tradition wished to consider true- or one of his skilled pupils, the fact remains that the drawings of his treatise *De Humani Corporis Fabrica* [20] are a superb representation to complete the notes he wrote. Vesalius dedicated to the heart an entire book, the seventh, within which the twelfth and the thirteenth chapters were respectively entitled *On the Vessels of the Heart and their Orifices* and *On the Eleven small Membranes of the four orifices of the heart*. As first, he discredited Galen's theory about the presence of invisible pores in the interventricular septum, a fundamental also in Leonardo's anatomy. In two editions of his treatise, the doubts about the existence of these holes have been gradually increasing (1543): in addition to say that he was really unable to see any perforation, he also claimed as God could create such a complexity in the cardiac organ. This statement opened a new scenario in heart physiology, setting the foundation for the later discoveries of pulmonary and systemic circulations respectively by Michael Servetus (1553)/ Realdo Colombo (1559) and William Harvey (1603). At the light of the new scientific philosophy he imposed, a particular concern is aroused by his confutation of the heart bony skeleton in the *Book 1, Chapter XX*, he dedicated to the bones and cartilages:

> 'Not wishing to give the impression in this book of having passed over any bone in silence, we have decided that mention should be made at this point of the osseous substance of the heart. Galen recorded that an extraordinary large bone was excised from an elephant's heart by one of his friends, and says that a bone exists in the hearts

> of larger animals and a cartilage in a smaller. In all my researches, so far I have not found a pure bone in the human or any other heart; at the point where Galen says this bone occurs I find a cartilaginous substance which in my opinion is merely the roots of the great artery and arterial vein as they come forth from the heart. These roots, that form the basis of the membranes which prevent the material in these vessels from flowing back into the heart, are so much harder than the substance of the rest of these vessels that they might well seem to same extent to partake of the nature of cartilage. Now, in oxen cartilage hardens… The roots of these vessels are mutually coterminous like two circles coming together and coalescing at an appoint, the circle on the right being the root of the arterial vein and that on the left (which is larger) that of the great artery.' [20]

Indeed a further passage is centered on the 'mistakes of anatomists' and 'dishonesty of physicians and druggists', to reconfirm his disappointing against Galen's followers. Another example can be found in the *Book VI, Chapter XII* where Vesalius discussed about the already mentioned controversy of Aristotle's tri-chambered heart:

> 'Furthermore, in the left ventricle, at the highest part of the septum, arising structure behind the right membrane of the orifice of the pulmonary artery deceived Aristotle into thinking that there was a separated, hidden, elevated chamber constituting the third ventricle, which he believed to be the origin of the great artery. For this he is much less to be blamed than for writing that the number of the ventricles of the heart varies with the size of the animals.' [20]

In Vesalius's vision, the heart is therefore composed of two ventricles and the upper parts assume the aspect of a dilatation of the *vena cava*. This latest one is described very carefully by Vesalius as much as the other main cardiac vessels, the 'artery-like vein', the 'veinlike artery' and the 'great artery', putting on special evidence the orifices belonging to them. In net disagreement with Galen's anatomy, the *vena cava* became the first vessel of the heart: its origin was demonstrated by the observation of the adult body and confirmed through dissection of newborns and even fetuses:

> 'From the right side of the base of the right ventricle, therefore, the hollow vein takes origin at the lowest part of the base by means of a very broad, open orifice: its origin is much ampler than the capacity of the vein as the whole, and a sort of protuberant circle can be seen where the substance of the heart ends.' [20]

The protuberant circle, the first part of the vein, was illustrated to have a smaller diameter than the vessel, but a greater consistence from which three membranous prolongations arise, each ending with a 'blunt apex'. Vesalius proceeded to describe also the *chordae tendinae* attached to these leaflets by specifying their connection with the rest of the right ventricle ('close to its apex') and

composition ('fleshless where they are joined to the membranes' and 'supported by fleshy substance' at the opposite insertion), and interestingly made use of a similitude with the Turkish sharp weapon, in vogue at that time, to explain the 'triangular spike' of the closed orifice. He continued his analysis of the cardiac vessels with the 'artery-like vein', even this belonging to the right side of the heart and whose denomination was once more elucidated:

> 'is called a vein because it conveys blood to the lungs and so performs the function of a vein, and artery-like because its body is that of an artery'. [20]

The orifice appeared smaller in relationship with the mentioned right ventricular valve, the tricuspid one (as Vesalius identified) and it is localized in the highest part of the ventricle nearby to the septum. His depiction of the orifice continued on the page 592 [20] and again he used comparison to evidence its characteristics: these leaflets are quite different from those of the tricuspid valve, they are dissimilar in 'position or shape or strength' and do not protrude into the cavity of the right ventricle. Vesalius seemed in his visualization of this right ventriculo-artery valve to agree with Leonardo's subdivision in three semicircles proposed for the aortic valve. He in fact underlined that in contrast with the atrioventricular valve, each leaflet appeared to originate from its own semicircle to resemble 'half moons' and a lateral curvature prevented it to form a perfect 'half circle', hence the Greek description ' as a sigmoid (*sigmoeideis*) from the shape of their letter sigma.' For the 'vein-like artery', Vesalius proposed the similar descriptive approach used in the case of the 'artery-like vein': as for the latest, he began his explanation of the name referring to the venous nature of the vessel and its role of air conveying. Then, he proceeded with another parameter of comparison, the *vena cava*, with which it shared the 'origin from the softer part of the ventricle' -in this case the left one- and the morphology of the origin. The orifice is circular, membranous and like the *vena cava*'s one, its concavity protrudes into the ventricle chamber. The leaflets are described as resembling the ones belonging to the tricuspid valve, but in this circumstance, they are in number of two and can recall a particular shape:

> 'the experts in dissection state that only two membranes control its orifice; one might not inappropriately liken them to a bishop's miter (*mitre*) in that the part that goes around the head corresponds to the membranous circle and the anterior and posterior apexes of the miter to the processes.' [20]

For this similitude, the left atrioventricular valve had been named as 'mitral of Vesalius' or simply 'mitral'. However he pointed out that the mitral leaflets vary also in dimensions and consistence respect to the tricuspid valve cusps because they are 'larger and tougher'. After having well topographically framed the mitral projections as regards the ventricle wall, he went forward by characterizing the *chordae tendinae* of the left part, quite similar to the ones of the right side.

Using Vesalius's words, 'our account must now turn to the fourth vessel, which is the great artery' [20]. Once more again the Author advanced in his explanation by comparison with the cardiac parts he already illustrated, but also with skeptical attitude toward his forerunners' deductions about the heart bone or the triventricular heart. As first, he discussed about the origin of the two left vessels and their quite mutual positioning in the heart:

> 'So the beginning of these two hard, thick vessels, namely the artery-like vein and the great artery, correspond so closely that the left side of the artery-like vein is actually attached as its origin to the right side of the great artery, and both vessels have a common beginning or root here at the top of the septum between the ventricle of the hearts.' [20]

About the valve of the 'great artery', little is said but the close analogy with the one of the artery-like vein. The three cusps, which were 'created by Nature', differ from those of the artery-like vein for their largeness and potency and at the same time even the vessel shares the same properties of them.

About twenty years later (1569), the anatomist Andrew Caesalpini was able, perhaps even before Harvey and still influenced by Aristotle's theory, to focus new aspects of blood circulation and in particular he attributed to the semilunar valves the important role we actually estimate:

> 'Now, while awake, the movement of the innate heat is from within outward, and during sleep it is the reverse; hence it follows, that while awake a great quantity of the vital spirit and blood are carried to the arteries, which transmit them to the nerves; and during the sleep the same heat returns to the heart, not by the arteries but by the *vena cava*, which alone communicates with this organ.' [21]

The successor of Vesalius in the medical teaching in Padua was Realdus Columbus (1510-1599), whose contribution to the description of the pulmonary circulation in his book *De Re Anatomica*, although to him attributed, combined the vision of Vesalius and Servetus, another anatomist condemned for heresy for his religious ideas published in *Christianismi restitutio*. Another Vesalius's contemporary was Giulio Cesare Aranzi (1530-1589), which had the possibility to observe the heart of a fetus, by comparing it to the adult organ in his treatise *On the foetus*. In particular, he described new cardiac structures as the closure of the *ductus arteriosus* and *foramen ovale* at birth, in addition to the nodules of the semilunar valves, which were dedicated to him. The right atrial remnant of the valve of the inferior *vena cava* was discovered in the same years by Eustachio (1520-1574).

Biomechanical studies upon the heart, almost interrupted after Leonardo, were resumed by William Harvey, who considered the organ as a mechanical unit composed of a pump- the same heart-, the valves and the vasculature [22]. In his book *De motu cordis*, Harvey contrasted Galen's theory by

evidencing for instance the unfeasibility of the mitral valve to allow the passage of '*vapours*', but not of blood [23].

It is with Anthony van Leeuwenhoek (1632-1723) that the microscopical visualization became available by enabling the observation of red bloods in the capillaries, but extending the use of the method also to the investigation of the other cardiovascular structures. Other important anatomists during the seventeenth until the nineteenth centuries had the occasion to illustrate pathological aspects encountered during autopsies. One of these, Giovanni Battista Morgagni (1682-1771), also demonstrated the presence of a nodule in the pulmonary valve leaflets, as Aranzi did for the aortic counterparts. Important contributions to the understanding of the normal heart valve biology have been settled later in 1931 by the work of Gross and Kugel [24]. However, it is the extensive evaluations of Mano Thubrikar and colleagues to definitely establish correct biological and mechanical properties of the valves of the cardiac organ [25-28].

1.2 Macroscopic evaluation of the heart valves

Despite the great historical contributions of Scientists as Leonardo or Vesalius, the valves of the cardiovascular system have not always had the interest they deserve. Not surprisingly little is still sufficiently known about their characteristics and rarely it is ascribed them a vital role, precisely because their poor connective tissue components could indicate a structure devoid of complexity and specialization.

The heart valve apparatus is composed as previously seen by 4 different valves which guarantee blood unidirectionality from the atria to the efflux arteries: two, the mitral and tricuspid valves, establishing the connection between atria and ventricular, two, the pulmonary and aortic semilunar valves, in between the ventricular chambers and the arteries.

When the cardiac anterior structures are removed, the triangular anteromedial leaflet of the mitral valve becomes visible with an extension from the posteromedial septum to the anterolateral ventricular wall in the left part of the heart. Defined as aortic leaflet, it is much closed to the noncoronary and left coronary cusps of the aortic valve, establishing the so-called aortomitral fibrous continuity [29]. In contrast for morphology, position and motility, the other leaflet, the posterolateral one, interests at least two-thirds of the mitral orifice with its elongation but with lesser height. They are connected each other by commissural tissue and to the two papillary muscles by *chordae tendinae* of first level if inserted to the free borders, of second and third ones if attached to the ventricular surface by originating respectively from the same papillary muscles or from the ventricular wall. In spite of their equal importance in the closure mechanism, it is the anteromedial cusp that defines with its surfaces the boundary between the inflow and outflow tracts in the left ventricular cavity. When blood enters the atria, the inflow structure, composed of the two leaflets,

their *chordae tendinae* and the mitral *annulus*, ensures its direction into the cavity and then at systole, the ventricular septum and free wall along with the inferior surface of the anteromedial leaflet act as a propeller unit of the blood towards its expulsion from the heart.

At the right side, the tricuspid valve is characterized by three leaflets, which similarly to the mitral ones are of different sizes, but seriously interconnected and less thick. The area comprised by the *infundibulum* and the inferolateral ventricular wall is the expansion of the anterior leaflet, also called anterosuperior, whereas the septal leaflet, smaller than the first one, finds connections in the membranous and muscular portions of the ventricular septum. The slighter cusp is however the posterior one which is attached to the tricuspid *annulus* along the inferior and posterior segments of its borders. Even for the tricuspid leaflets, the anchorage is allowed through *chordae tendinae* by papillary muscles that with a special arrangement in single unit or in groups connect to the wall. Apart from the cusp number and size, many other differences distinguish the mitral valve from the tricuspid one, as the *annulus* circumference (8-10 cm vs. 10-13 cm), the ring morphology (elliptic vs. oval), the tridimensional position in the heart (they are not on the same plane and the mitral orifice is lower) and the connections with the other valves (differently from the mitral valve, the tricuspid one does not establish a proximity with the pulmonary valve due to the presence of the right ventricular *infundibulum*). However, they can be considered a complex with the atrium, the *chordae tendinae*, the papillary muscles and the ventricular wall for their performance as a mechanical unit during the cardiac cycle [30, 31].

Different aspects are even evidenced in the examination of the semilunar valves, the aortic and the pulmonary ones. The main dissimilarities derive from the evaluation of the thickness, for which the pulmonary cusp appear thinner, and from the valve location, with the pulmonary valve higher, anterior and to the left of the aortic one.

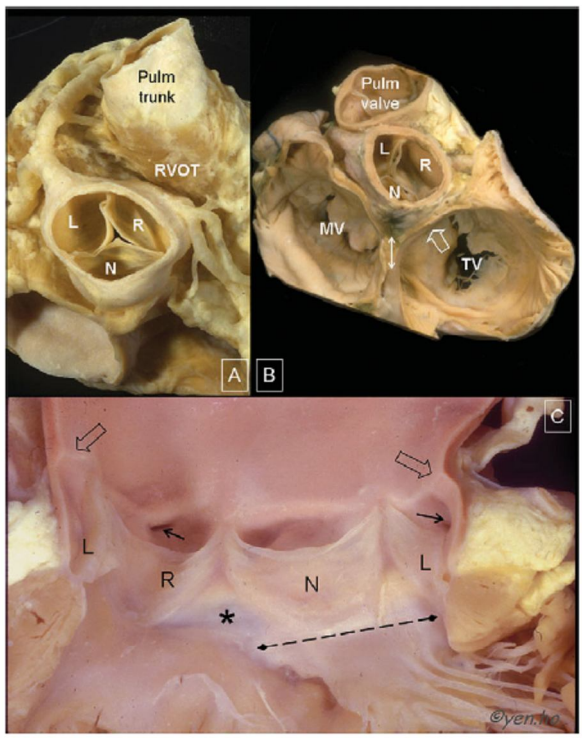


Figure 1 (*A*) The muscular sleeve of the right ventricular outlet (RVOT) has been pulled forward to show the left (L) and right (R) aortic sinuses that give origin to the main coronary arteries. The non-coronary (N) aortic sinus is furthest from the pulmonary trunk. (*B*) This overview shows the central location of the aortic root and the relationship of the non-coronary aortic sinus to the plane of the atrial septum (double-headed arrow). The open arrow indicates the area of the aortic mound. MV, mitral valve; TV, tricuspid valve. (*C*) The aortic root has been opened longitudinally to display the level of the sinutubular junction (open arrows), orifices of the coronary arteries (small arrows), and the area of fibrous continuity (broken line) between aortic and mitral valves. The asterisk marks the pale-coloured area that is the membranous septum.

Fig. 6 Anatomical identification of the heart valves and particular of the aortic valve by Siew Yen Ho.
From [32]

Conversely, they are both at the higher point of the corresponding ventricle assuming an outflow function and displaying a similar conformation. For both, it does not exist a real fibrous ring as for the atrioventricular valves: for instance, the aortic *annulus* has a 45% muscular composition (ventricular septum), while the remaining percentage is represented by fibrous tissue, with no gradual anatomical shifts between the aortic wall and the cardiac muscle. It is therefore preferable calling it aortic root rather than *annulus*. Especially for the aortic valve, this structure is not circular, but composed by three consequential paraboloids, each representing a cusp attachment.

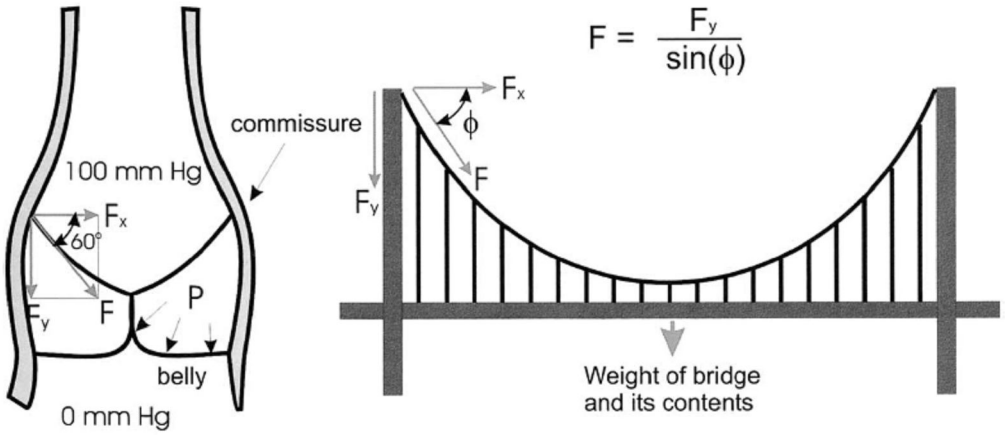


Fig. 3. Representation of the analogy between the diastolic loads transferred from the leaflets to the aortic wall, and the vertical loads carried by a suspension bridge that are transferred to the supporting towers. The angle of the leaflet fibers and the suspension bridge cable affects the force (F) induced in each, as per the equation above.

Fig. 7 Representation of the paraboloids in aortic valves as schematized by Ivan Vesely. From [33]

In continuity with these elements, are the wall dilatations outward from the lumen, which are termed sinuses of Valsalva. These pouches display a thinner wall in respect to the distal aorta. As the free portion of the projection is reached, it is noticeable a slight decrease in thickness, but at the same time the presence of a nodular formation just at the centre of it, which is identified as *nodulus Arantii* or nodule of Morgagni in the pulmonary valve. In the right semilunar valve, the leaflets are termed anterior, right and left pulmonary cusps.

The nodule of Arantius is not a sake valve element, but it displays a fine function during valve closure by concurring to the coaptation of the slightly overlapping cusps, hence as a support to avoid blood regurgitation. Despite this duplex control mechanism during closure and the presence of valve commissures- which ensure at least for a short distance from the base a strict contact between nearby cusps-, it might happen that fenestrations exist in these areas even in normal condition.

The three leaflets of the aortic valve do not share equal size in most cases [34] and are named noncoronary, left and right coronary cusps, in respect to the connections with the left and right coronary arteries, whose *ostia* are detectable at the upper third of the corresponding sinuses of Valsalva. For its central position in the cardiac organ, the aortic valve is in relationship to many heart structures. The right coronary leaflet is near the anterior wall of the right atrium, being overlaid by

the *infundibulum* of the right ventricle; conversely, the left cusp establishes a connection with the left side of the heart and together with the mitral valve composes the intervalvular *fibrosa*. The noncoronary leaflet arises in intermediate position between the two atria. Another considerable aortic valve constituent is the sinotubular junction: as the same term suggests, it represents the connection between the tubular and sinusal valve portions and establishes the starting edge of the ascending aorta. Its diameter is smaller than that of the aortic root, but superior to the height of the aortic sinuses. Not only the leaflets open, but even the rest of the valve dynamically participates in the cardiac cycle. In particular, the radius length at commissural level modifies in linear function with the aortic pressure variation [29]: the teleological reasons of this radius variation reside in the necessity to avoid bending during leaflet opening. In contrast, the root displays a higher resistance to modify its circumference in relationship to the different pressure loads because of its extracellular matrix composition [29]. In addition, in order to guarantee a laminar blood outflow, the truncated-conical geometric figure identified in diastolic closed position moves in systole to expand as a cylinder, where the *annulus* and valve radii tend to be similar and the leaflet base enlarges inwards up to 20% [35]. However, as well evidenced by Leonardo, a passive reflow generates at the telesystole with the starting valve closure and contributes to stabilize the newly acquired position of the leaflets and to avoid blood stasis.

1.3 Heart valve embryology

In the development of the cardiac valves, the endocardial cushions appear to be the primordial of the valvular leaflets and the membranous septa: they derive from expansions of extracellular matrix regionalized between cardiomyocyte sheets and endocardial cells of the cardiac tube. A subset of endothelial cells acquire specific capabilities of delamination and invasion of the cardiac jelly, by assuming a typical mesenchymal phenotype, with high proliferative activity and able to remodel the cushions in definitive cusps. The work of Markwald and Colleagues about Endothelial-Mesenchymal Transition (EMT) first evidenced the involvement of soluble factors in the extracellular matrix and nevertheless a close myocardium-endothelium relationship, likely to emphasize the unique responsive property of the endocardial cells to cardiomyocyte mechanical and paracrine stimuli [36]. At the end of the developmental process, at least four are the cellular contributions which constitute the different valvular phenotypes: the myocardium, whose appearance is early in the development of the atrioventricular valves; the endocardium, which encounters an endothelial-mesenchymal transdifferentiation; the epicardium for the atrioventricular valves; and the neural crest cells, which migrate from the brachial arches to the distal outflow tract, participating to the aorto-pulmonary septation of the semilunar valves (Fig. 8) [37-39].

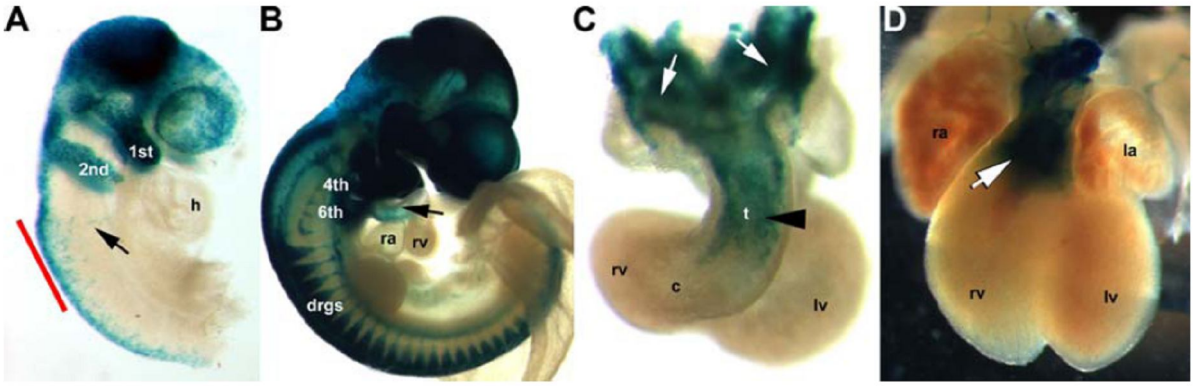


FIGURE 1. Specificity and efficiency of NC marking using the *Wnt1-Cre*[11] and *R26R* reporter Cre/loxP system. (A) Whole-mount staining of an E8.0 embryo. Labeling is seen in the dorsal neural tube, first and second pharyngeal arches (numbered), and migratory CNC (arrow). Location of CNC progenitors is indicated by red line. The OFT of the heart (h) is unlabeled, but is in obvious proximity to the pharyngeal arches. (B) Staining in an E11.0 embryo. Extensive labeling is seen in the head, dorsal neural tube, NC-derived dorsal root ganglia (drgs), all the pharyngeal arches (fourth and sixth numbered), and in CNC colonizing the OFT of the heart (arrow). (C) Isolated E11.0 heart. *LcZ* labeling can be seen in the aortic arch arteries (white arrows) and the truncus (t) of the OFT cushions (large arrowhead), but not in the conus (c). (D) E14 mature septated heart. Note robust *lacZ* reporter staining is present in the condensed mesenchyme of the OFT conus (arrow) and the anterior divided truncus, but is absent from the ventricles and atria. Abbreviations: rv, right ventricle; lv, left ventricle; ra, right atria; la, left atria.

Fig. 8 Neural crest cell specification as studied by Snider P and Colleagues. From [38]

Different molecular pathways are involved in the heart valve development and the high complexity of regulation can be appreciated in the following chart:

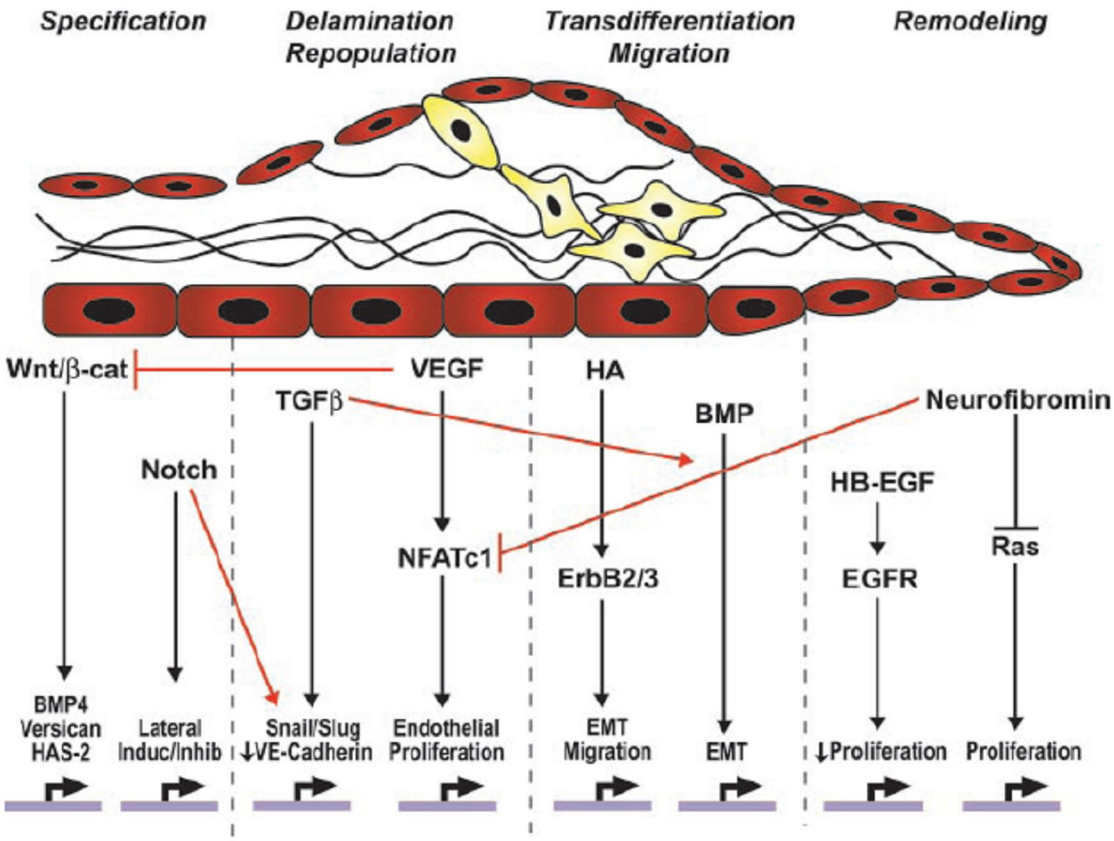


Fig. 9 Valvulogenesis signaling complexity as proposed by Armstrong EJ and Bischoff J. From [40]

The pleiotropic factor VEGF is considered a specific mediator in the endothelial-mesenchymal transition, favoring endothelial cell proliferation. Downstream of VEGF, the transcriptional factor

NFATc1 has intranuclear expression, limited to the endocardium not activated in the EMT process during the developmental window of the cushion, while it is expressed by some valve endothelial cells in the post-natal life, leading to hypothesize their participation in the repopulation of the adult endothelium. Even RANKL (receptor activator of nuclear factor κB ligand) exercises a control role on NFATc1 when the endocardial cushion is more mature by inhibiting the cell proliferation induced by VEGF [41]. Upstream of NFATc1, two elements, connexins and DSCR1 (Down Syndrome Critical Region 1) play their function by signaling pathways associated to calcium cellular gradients. In the cardiomyocytes, DSCR1 seems to be overexpressed during the increase of the cytosolic calcium and acts as modulator of the calcineurin dephosphorilating activity on NFATc1, by preventing its translocation to the nucleus.

Connexin 45-composed gap junctions might allow the calcium extracellular diffusion in the endocardial cushions after the VEGF-mediated activation of the calcium transients in a subpopulation of endothelial cells [40].

The transcriptional factor Notch1 rules the osteogenic differentiation as well as the valve development: its signaling in the endocardium might induce an increase of TGF-β2 expression in the myocardium, which is able to trigger EMT in endocardial endothelial cells through the cytosolic activation of Snail and Smad [42]. Activators of the TGF-β transduction pathway are even the bone morphogenetic proteins (BMPs), in particular BMP2, which is able to cause a powerful induction of Smad6 in the cusp endothelium [43, 44]. Another pathway induced by VEGF is the one involving Wnt1/β-catenin. During EMT the down-regulation of CD31/PECAM in the endothelial cells is followed by the hyper-regulation of smooth muscle actin: β-catenin can be considered a link in between the mesenchymal activation and the populating process of the cardiac jelly with mesenchymal cells.

Beside these transduction pathways, it is equally important the integration of the signals of the extracellular matrix. ErbB proteins are involved in the cell proliferation of the cardiac cushions and their signaling is mediated by the hyaluronic acid, a highly hydrophilic glycosaminoglycan which is able to extend the extracellular space and control the ligand availability [40]. For both development and maintenance of the mesenchymal cells in the valve leaflet, Sox9 is necessary because it favors cell proliferation and ECM correct alignment [45]. Recently, it has been recognized to an epicardium cell-secreted protein, periostin, a particular importance during the atrioventricular valve development for the promotion of the differentiation of the epicardial stem cells [46, 47].

New high-speed fluorescence microscopic technologies have allowed verifying a large interplay of different pathways in the development of the cusps and in particular, they enable a better understanding of the first events of valvulogenesis, arguing in favor of an invagination rather than a formation of endocardial cushions [48].

1.4 Biology of the cardiac valves

The leaflets of the heart valves, both atrioventricular and semilunar, display a similar composition in terms of microstructural characteristics, but these ones are arranged in different distribution and spatial relationships with the other cardiac structures.

The extracellular matrix is definitely the major component of the postnatal adult cardiac valve and it performs an essential role in the maintenance of valve function not only at the macroscopical level but also at the microscopic one. The fibers that compose the scaffold of the normal leaflet are present in dissimilar amount and allocation, so that it is possible to identify the typical stratification at the histological examination.

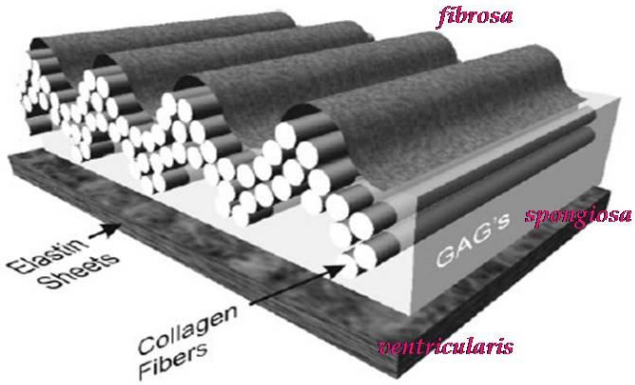


Figure 4. Schematic diagram of the multilayered configuration of an aortic valve cusp, showing the location of the Collagen fibers in the fibrosa, the elastin sheets in the ventricularis, and the GAG-rich matrix of the watery spongiosa.

Fig. 10 Heart valve matrix characteristics in a 3-D model proposed by Vesely I. Modified from [33]

Considering the semilunar aortic valve -but in a similar way to the pulmonary outflow, the spatial orientation of the extracellular matrix fibers lets recognize three layers, the *ventricularis*, the *spongiosa* and the *fibrosa*, each characterized by a precise cell population (Fig. 10). The *ventricularis* derives its name from the fact it faces the ventricular chamber. Elastin sheets, radially disposed, are the main constituents of the *tunica* and allow expansion and contraction during the cardiac cycle thank to the property of elastic recoiling. Smooth muscle cells and myofibroblasts reside among the elastin fibers contributing to the elastic properties of the layer [49]. The layer surface is smooth and glossy, features that prevent blood turbulence at the valve closure in diastole. Similar aspects are not shared by the *fibrosa*, at the opposite side of the leaflet. This layer appear at a macroscopic analysis opaque and corrugated, due to the thick collagen I bundles crossing it in the surface and more deeply in all its thickness, surely higher than the *ventricularis* one. The presence of circumferentially oriented collagen permits the cusp to sustain resistance to the pressure forces created at the diastole. Cells populating the *tunica* seem to be less specialized and simple fibroblasts: conversely, their capabilities of matrix synthesis and remodeling have strictly to be considered regarding the extracellular matrix maintenance. Particularly in the last years, new insights are emerging about further specialization and sensibility to intercellular and extracellular

interactions [50-52]. Endothelial cells cover the entire free surfaces of the cusp, but display a different morphology and role in respect to the layer of pertinence. The ventricular endothelium has a more flattened appearance than the aortic one, but it displays also a higher resistance to external injuries (Fig. 11) [53-56]. A healthy valve endothelial lining carries out an important protection against thrombotic events [57].

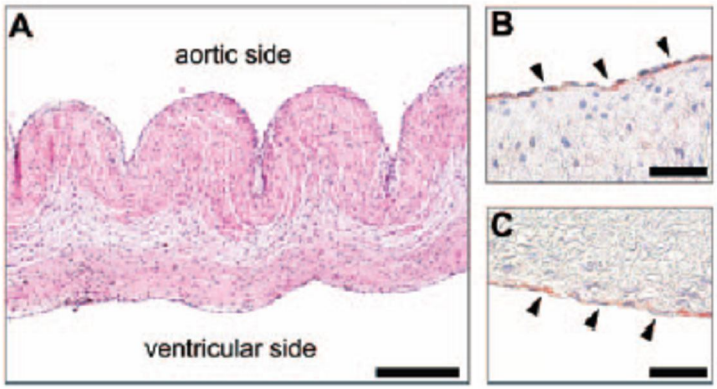


Figure 1. A, Histological section of a normal porcine aortic valve stained with hematoxylin and eosin. B and C, Immunostaining for vWF demonstrated intact endothelium on the aortic (B) and ventricular (C) surfaces of the valves (arrowheads). Bars=200 μm in A and 40 μm in B and C.

Fig. 11 Endothelial cells at the fibrosa and ventricularis layers studied by Simmons CA et al. From [53]

The intermediate layer is represented by the *spongiosa*, the Latin version for sponge, where a dense and loose connective tissue guarantees to appease the shocks provoked by the enormous pressure variations during the entire cardiac cycle. The main contribution to the *spongiosa* composition is given by highly hydrophilic molecules as the proteoglycans and glycosaminoglycans, protein complexes formed by the assembly of hyaluronan, cheratin and chondroitin sulphates [58, 59]. The distribution of these fibers and cells taken throughout the cusp observation varies in function of the already cited thickness decrease from the attachment base to the distal part of the projection, thus evaluating a progressive *spongiosa* thinning. Moreover, the atrioventricular valve possesses a further layer, named *atrialis*, based on elastin and with tendency to disappear at the very distal part [60]. As further specification, other ECM fibers are present in the leaflet: collagen III is distributed widely in the scaffold contributing to the 24% of the total collagenous fibers [61], while laminin is a normal constituent of the basal lamina of the endothelial cells; fibronectin and vitronectin are more associated to the inflow aspect, whereas byglican is ubiquitous [62]. Elastin content is concentrated in form of sheets in *ventricularis,* but sparse fibers can also be identified in the rest of the leaflet. At the base of the leaflet it is frequent to observe cardiac muscle cells and smooth muscle bundles especially for porcine and bovine heart valves and also the human atrioventricular ones. In the animal cusps, some arterioles and venules can be distinguished in this abrupt zone between the fibrous and the muscular connective tissue, but in humans the presence of vascularization should

remind to a pathologic condition. An innervation mesh has been described in all valve leaflets with active sympathetic, parasympathetic and peptidergic neurotransmitters [63-65].

Considering the entire microstructure, the leaflet is hence an anisotropic tissue, whose components, differently stratified, display a unique property in respect to their precise localization. In particular, this matrix and cell organization appears quite conserved across different mammalian species [66].

It could seem quite impossible that such a fine tissue could bear so high forces, also considering that opening/closure happens for 40 million times in a year course. For the same reason, the active processes regarding its maintenance but also pathobiology have been often underestimated. Conversely, the leaflet is a very dynamic metabolic cardiac component, where not only the synthetical activity is particularly high in order to preserve and produce new matrix elements, but also the control pathways regulating them are precisely defined. For instance, transcriptome profiles of valve endothelial cells reveal the involvement of different functions for the leaflet homeostasis, from the biosynthesis to the cell motility or to the gluconeogenesis [53] and more information on the other cells populating the cusp could be achieved in the near future through the use of this technique. However, a quite abundant literature focuses on the valve interstitial cell population, as generally are described all the non-endothelial cells residing the leaflet stroma. Immunocytochemical and flow cytometric analysis have been applied to better understand VIC characteristics [60, 63, 67, 68]. VICs are a very heterogeneous population and as already mentioned, it is highly probable that not only smooth or cardiac muscle elements inhabit with endothelial cells and fibroblasts (activated or not to a contractile phenotype), but there might be many other differentiated cells still unknown. As elegantly resumed by Taylor and Colleagues [67], the main aspects of VICs are to be found in this diagram:

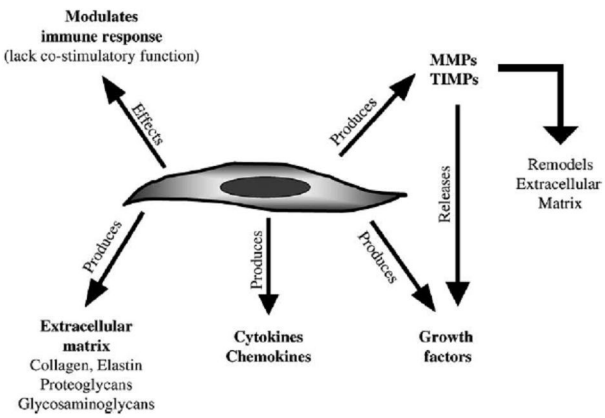


Fig. 1. Cellular functions of valve interstitial cells. Valve interstitial cells synthesise and secrete cytokines, chemokines, growth factors, extracellular matrix components, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), which modulate the matrix and also release growth factors sequestered within the matrix. In addition, these cells may have an immunomodulatory role.

Fig. 12 Valvular interstitial cell (VIC) and their functions as described by Taylor PM et al. From [67]

Interstitial cells of the valve cusp carry out synthetical activity, but also secretion of metalloproteinases which help in the ECM turnover [69]. Growth factors and chemokines are released by VICs, which interestingly evidence also a capability of immunomodulation. This property reveals itself primary to maintain controlled any possible inflammatory pathway induced by the

continuous mechanical stress sustained by the leaflet [67]. At the basis of the immunomodulation, it is probable the synthesis of some molecules as interleukin 10 (IL-10) [68]. Moreover they are also sensitive to the nervous stimulation [63-65, 70]. On their cytoplasmic membranes, VICs display different types of integrins, both α and β at various expression grades, important for integration in the whole scaffold and for cell-cell communications [50, 71]. Primary cultures of VICs can be obtained either by explant or digestion with specific enzymes for ECM degradation. Various morphological phenotypes have been reported: from the similarity to the aortic medial population for the hill and valley confluence pattern to the typical ridge formations. A limited dilution technique after predigested explants has allowed separating at least 60 clones, where the four main phenotypes were characterized by cells with cobblestone morphology with two different cell sizes, hill and valley appearance or multilayer growth. Among these clones, even the doubling index and the capability to cover the entire plastic support during culture were dissimilar [52]. For an ultrastructural consideration, VICs generally show an elongated shape with prominent adhesions and gap junctions. Basal lamina is often incomplete, while they tend to firmly anchor to the ECM. In the cells actively participating to the ECM biosynthesis, both the rough endoplasmic reticulum and the Golgi's apparatus appear prominent. Generally, thin and intermediate filaments can be appreciated in the cytoplasm in variable amounts [60].

In a normally avascular human cusp, an active transport has to be fundamental. Previously, the relatively few reports on the topic assumed that the process happened only on a normal direction to the endothelium, as a molecular diffusion through a one-dimension unstratified thickness [72]. Recently, studies on rat valves and theoretical models proposed with the aim to generally describe the leaflet of whatever species put on evidence the existence of a valvular subendothelial intima-like space, similarly to the one of the aorta, where the macromolecules diffuse but also convey by entering not only in a normal, but also parallel direction to the endothelium thanks to the leaks here present [73, 74].

The remodeling process interesting the valve cusp is not rare: conversely it has a character of continuity from the postnatal to the adult life and involves both ECM fibers and cells. At the birth, when the blood circulation changes, the aortic and pulmonary valves seem to be interested by two different response events. In the left district the sudden variation of pressure induces the aortic cells to maintain a contractile phenotype, seen the high positivity for smooth muscle actin (sm-actin), while in the pulmonary cusps the content of sm-actin positive cells is reduced. As the adult life is reached, VICs appear to be quite quiescent and inhabit an ECM with fully stabilized collagen [66, 75]. Effectively, the different pressure regimens encountered by the aortic and pulmonary VICs might be a significant source of heterogeneity for the two residing populations especially for what concerns the potential of tissue remodeling [76, 77].

Another aspect to enlighten is the quite improbable definition of the valve cell population as purely resident. This continuous remodeling, the dynamic activity of the valve and its direct contact to the blood circulation have to suggest that the leaflet does correspond to a circumscribed cardiac region, but at the same time it represents an attractive microenvironment for outer cells, as evidenced after transplantation procedures in animal models and in human cardiopathic patients [78, 79].
All these features make VICs absolutely different from any other body fibroblasts or interstitial cells.

1.5 Cardiac Valvulopathies: some notes on congenital and acquired pathobiology

Among cardiovascular malformations, the defects interesting the valvular apparatus have a quite high annual incidence, 1-2 subjects per 1000 newborns in the United States, corresponding to the 20-30% of all cases [80]. Any dysfunction or insufficiency of an ECM protein during valvulogenesis risks to compromise the normal development of the heart valve: if some gene mutations able to produce these modifications are known, the real mechanisms provoking the defects are still misunderstood.

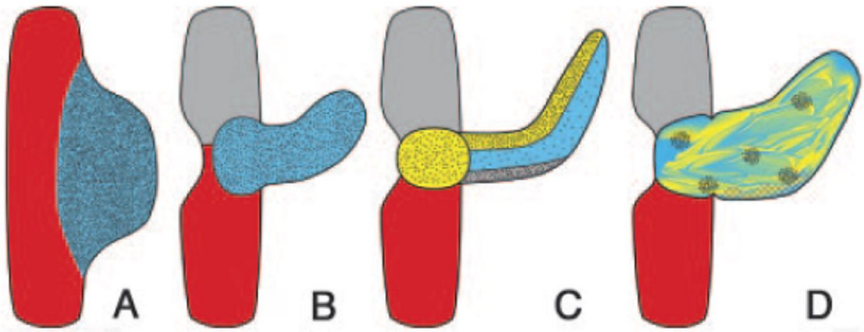


Figure 7. Model of semilunar valve development and disease. During early embryonic valve development, endocardial cushion formation (A), and cushion elongation (B) occur, and during late embryonic valve development, cusp remodeling (C) results in stratified layers of ECM and differential distribution of VICs. Cell density decreases dramatically during valvulogenesis. ECM organization and VIC compartmentalization begin during remodeling, and the highly organized mature valve structure is realized in postnatal life. Pediatric aortic valve disease (D) is characterized by ECM disorganization and VIC disarray. Red corresponds with myocardium, blue with proteoglycans, yellow with collagens, and gray with elastin.

Fig. 13 A proposed model of valve disease development by Hinton RB et al. From [66]

Bicuspid aortic valve (BAV) represents the most diffuse congenital valvular pathology with 1-2% of the entire world population affected [81]. In these aortic pathological valves, the correct dynamics is altered above all during opening while the leaflets are submitted to stretch during systole, provoking in both cases progressive modifications of the same ones as thickening, fibrosis and calcifications. These degenerations occur much earlier than for normal tricuspid valves (BAV (49±7 years) versus

tricuspid aortic valves (68±12 years) [82] and can be complicated by stenosis (substantial flow obstruction due to a root size reduction), insufficiency (valve incontinence) or are more easily sites for infective endocarditis [83]. BAVs were already recognized and drawn by Leonardo in his pictures, but it is only with the last century that the gravity of the pathology was recognized together with the associated aortopathy [84]. The left and right leaflets appear generally fused together and sometimes it is possible to observe a ridge in correspondence to the fusion zone, indicated as *rafe*.

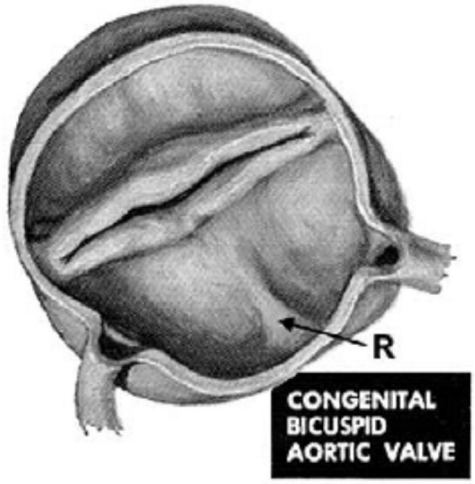
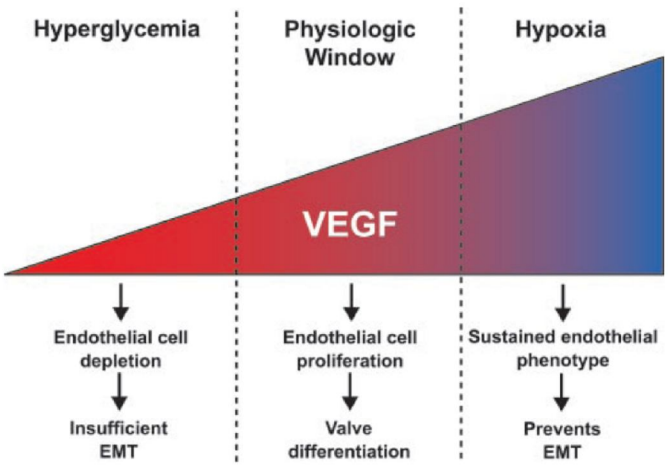


Fig. 14 Congenital bicuspid aortic valve with rafe (R).
From Netter FH. Collection of Medical Illustrations [85]

The genetic cause responsible for this two-leaflet valve is still not known, but different hypothesis have been proposed. In particular the observations obtained by inbreed Syrian Hamsters, frequently affected from this pathology, have evidenced that the leaflet fusion is an important step in the development of this phenotype [86].

Some Researchers find a linkage correlation between Notch1 mutation and the genesis of BAV [87, 88], but for Others it is a mutation of the fibrillin 1 gene to provoke the presence of only two leaflets instead of the three normally expected, but also of the mitral prolapse [89, 90].

Even due to modifications of the endothelial nitric oxide synthase pathway, BAV can establish. Another fascinating theory is associated to abnormalities of the neural crest cells, in fact other cardiac structures derived from them appear to be interested by dysfunction [91, 92].



As a general statement regarding congenital valvulopathies, Some indicate a correlation between cardiac valve anomalies and hypoxic or hyperglycemic fetal settings.

Fig. 15 Impaired valvulogenesis.
From Armstrong EJ and Bischoff J [40]

A proof of this is the increased tendency to develop cardiac malformations in newborns from diabetic mothers [93].

In the adult, stenotic acquired conditions are typical manifestations affecting both aortic and mitral valves. Often they find etiological explanations in rheumatic fevers, but more recently at least in the industrialized countries where antibiotic therapies are readily available, they can be provoked by valve degeneration with aging. Aortic stenosis can also be associated to hypertrophic obstructive cardiomyopathies and in some cases can be isolated in respect to a mitral valve involvement [94]. Aortic and mitral insufficiencies display as common feature the inability of the valve to maintain continence with consequent blood regurgitation. Causes of these disorders can be searched in the primary disease of the leaflets, often of rheumatic nature, but in some cases are linked to a dilatation of the *annulus*, progressively achieved in the presence of a genetic mutation (Marfan or Ehlers-Danlos syndromes, myxomatous proliferation of the aortic valve). Besides these, the mitral prolapse, commonly indicated as floppy valve, is a condition for which the leaflets do not coapt, provoked by lots of different genetic, infective and traumatic events.

Often to unite so many dissimilar clinical manifestations is the injury response activated by the interested valve tissue. For instance, the aortic myxomatous degeneration and the mitral prolapse are characterized by a progressive leaflet thickening, with a complete loss of the ECM normal stratification. In particular, an abundant production of glycosaminoglycans along with an uncontrolled collagen metabolism is responsible for the increased *spongiosa* thickness [95, 96].

A true common aspect of particular adverse weight in the maintenance of valve function is the appearance of calcifications along with the already cited fibrotic degenerations: the onset of this feature, characterized by a mineral deposition, is relatively late in the adulthood and share many similarities with the atherosclerotic process interesting the aorta and other arteries. It is associated with the accumulation of incorrect reparations during valve remodeling [97]. Hence, it has to be a control pathway in the homeostatic regulation of the valve tissue, which tends to lose efficacy with aging. Natural effectors of this control are mostly non collagenous matrix proteins, among which osteopontin and osteocalcin. Osteopontin has a controversial role: it is described as inhibitory element in calcification, even if its expression has been documented in the sites of valvular mineralization. This effect can be ascribed to the phosphorilation state of the protein that, free from the link with the phosphate group, displays a high affinity towards hydroxyapatite [98]. Osteocalcin is a protein containing carboxyglutamic acid, even observed in the calcific valvular lesions and whose expression levels increase when the osteoblastic phenotype prevails [99]. Other events verified in the calcific impairing are the increased levels of bone alkaline phosphatase and of specific metalloproteinases.

The *fibrosa* is the preferential site of calcification and it is not surprising to note that both the adult endothelium and valvulogenetic pathways against the layer denote a conceivable behavior towards calcific stimuli [53, 100]. In effect, the typical ECM proteins, i.e. osteocalcin, osteonectin, periostin and collagens, which are expressed in the mineralization-prone tissues, as the bone, are normally

found in the *fibrosa* layer [52, 66]. Even at the genetic level, many pathways are shared between valve calcification and osteogenesis (Fig. 16 in the following page), as the Wnt, TGFβ, Notch and BMP signaling [101, 102].

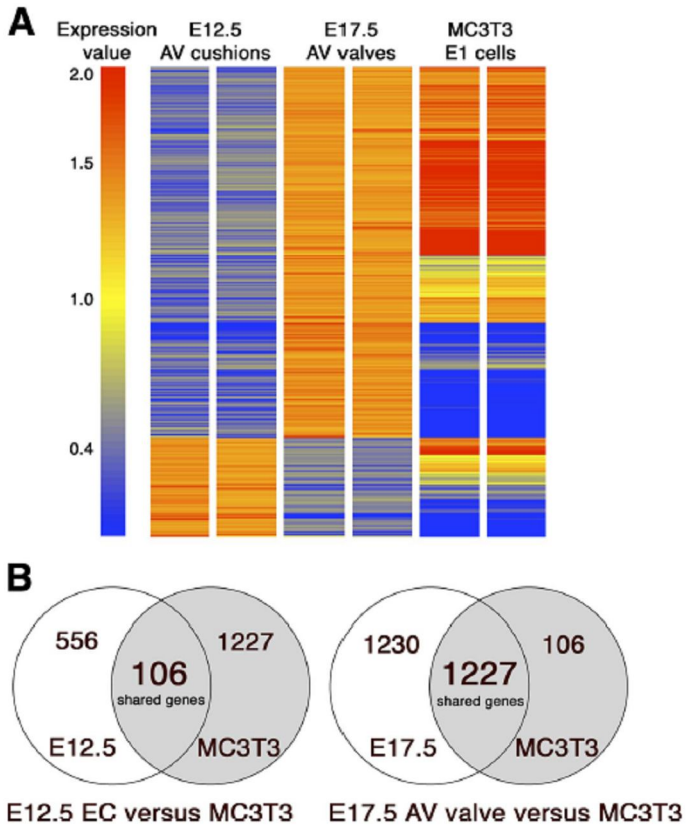


Fig.16 Affymetrix gene expression profiling analysis of murine embryonic day E12.5 AV endocardial cushions (EC), E17.5 AV valves, and preosteoblast MC3T3-E1 cells. Unchanged expression is identified by red color, decreased one by blue, while red means an increased appearance. From Chakraborty S et al. [103]

Ex vivo analysis on calcific heart valves revealed an impairing in the expression of RANKL and osteoprotegerin, both participating in the turnover of the bone. Higher levels of RANKL are able to promote cell proliferation, but also the acquisition by aortic valve myofibroblasts of a calcific phenotype for the expression of osteocalcin, alkaline phosphatase and metalloproteinases [104]. Targeting VICs with salmeterol enables the abolishment of any previously activated osteogenesis pathway by acting against the β2 sympatho-adrenergic stimulation, seen that the used drug is a selective β2 adrenergic receptor agonist [105].

1.6 Valve replacement devices

The injury responses tend to accumulate in the valvular tissues often without evident clinical signs until the valvulopathy reaches a complete deterioration with secondary suffering of the close cardiac structures, as the same myocardium. At the end-stage, valvular substitution is absolutely necessary to rescue valve function and substitutive prostheses represent the key instruments to this aim.

The first attempt of designing a valvular device has been made by Leonardo, who depicted the appearance of a prosthetic aortic valve to be reproduced in glass material. A reference to the

construction of a glass model of the heart valve is found on another sheet of the *Windsor collection* (1513) [18].

Fig.17 Glass model design of an aortic valve prototype by Leonardo da Vinci in *19082r*
The Royal Collection 2005
Her majesty Queen Elisabeth II

The first real manufacture of valvular substitutes goes back to the '50es of the previous century, when the application in heterotopic position of an aortic mechanical valve by Hufnagel and Colleagues triggered the beginning of the surgical therapeutical era of valvulopathies [106]. It was however the contributions of Harken, Starr and Edwards to demonstrate the feasibility of orthotopic valve replacement with these early devices [107].

Along the developmental history of mechanical prosthesis, many generations of valves have been proposed with the aim to progressively ameliorate the hemodynamic performances once in the patient. The first generation of such mechanical devices comprised valves whose occluding mechanism was represented by a caged silicon ball [107]. The constituent material demonstrated *in vivo* an impressive affinity towards circulating lipids and provoked in few years the necessity of re-substitution, but another adverse characteristics of the device was associated to the maintenance of high transvalvular gradients. This drawback lead to develop a second prosthesis generation, in which a tilting plate disc -of plastic Delrin and then of pyrolytic carbon- was introduced instead of the ball

[108]. Eight years later, a third mechanical model has been proposed: it was a device able to better mimic the valve structure with its bileaflet system, totally in pyrolytic carbon, hence contributing to a more correct dynamic performance than the previously proposed ones [109].

Despite the high durability of these mechanical devices, the major adverse effects associated with them are still represented by the onset of thromboembolic events if the cardiopathic patient is not opportunely treated with a long-life anticoagulation regimen, which in turn exposes him to an increased hemorrhagic risk.

For that reason, above all for subjects with coagulation defects or pediatric patients with scarce compliance, this kind of prosthesis does not find any indication. To overcome these inconvenient and to consent the treatment of a larger range of patients, a new category of substitutes has been proposed: the bioprostheses. In this term it is possible to include various devices, each of biological nature, such as the animal-derived valves, the allografts and the autografts.

Heterologous valves were the first to find application already in the 1970es to deal with the usage limitations of the mechanical devices [110]. Composed of animal tissue, they can be either total valves obtained from the pig or bovine pericardium opportunely valve-moulded. Prior to implantation, these natural materials have to undergo a chemical fixation with glutaraldehyde with the aim to guarantee higher stability at the macromolecular level and hence better performances, and indeed to create an immunological barrier between the xenogeneic tissue and the receiver's blood. They can be mounted on stent to ease the surgical implant, even if its presence can provoke rigidity and subsequently compromise the dynamic behavior [110]. In addition to the quite absolute resemblance with the native heart valves, these substitutes offer a further value: if the anticoagulation therapy has a necessity character during the first months of implant, later it is not more required [111]. However, even in this case a remarkable problem is able to decrease their durability during time: the structural deterioration. This phenomenon is very similar to the degeneration encountered by native valves at least for what concerns its pathological evolution.

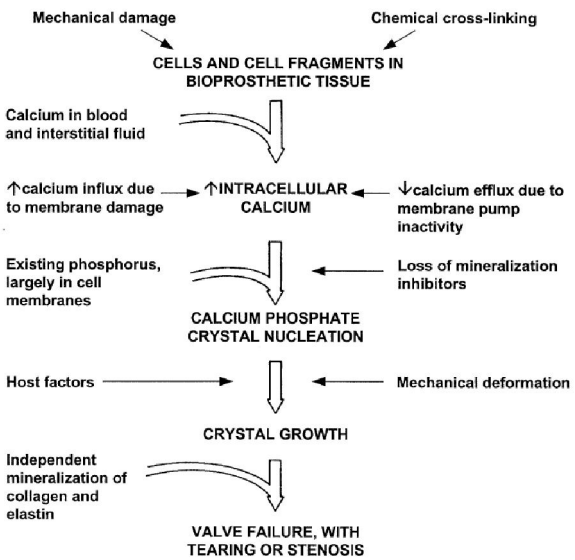


Fig. 18 Evolution of the bioprosthesis structural deterioration as proposed by Schoen FJ and Levy R. From [112]

Mineral deposition occurs in fact in the bioprosthesis too, but the underlying mechanism is different and strictly related to the presence of phospholipids (phosphatidil serine and phosphaditil choline) and phosphate backbone of degraded nucleic acids in the extracellular *milieu* of a no more vital

tissue [113, 114]. These extracellular phosphate groups are perfect nucleation sources of mineral deposition. The progression and entity of calcification, as previously discussed, is dependent on many factors and surely the lack of a cell-favored homeostatic pathway, provoked by glutaraldehyde cytotoxicity, can be useful to accelerate the phenomenon of diffuse mineralization on the ECM fibers.

In the course of the cardiosurgical replacement practice, other biological devices have been considered as the human-derived allografts and autografts. In particular, the first ones, commonly called homografts, have attracted considerable attention, both to avoid xenogeneic tissues and disadvantages of fixation, but also for the feasibility to create a tissue bank, from which to draw according to the patient's needs. Once harvested from the donors, these valves are previously sterilized in antibiotic solution and then cooled in opportune conditions of culture medium (use of dimethyl sulfoxide as cryoprotectant) and temperature (-1 °C/min until reaching -196°C) [115]. The same allografts are used in the revolutionary technique of entire outflow tract reconstruction proposed by Ross and Colleagues, where the pulmonary native valve is used to replace the LVOT and a pulmonary cryopreserved allograft is used to replace the RVOT [116]. This approach demonstrated strong implications in the treatment of congenital heart defects and still nowadays shows positive outcomes [117].

Although the higher homology degree of these substitutes in respect to mechanical and heterologous valves, failure occurs in a time almost comparable to the second ones. Events able to induce allograft deterioration are still unclear, but manipulation during harvest and mismatch conditions, related to cellular or mechanical factors, have been formulated as hypothetical causes [118, 119].

1.7 New heart valve substitute designing approaches

The non durability of the previous described commercial devices has shift the attention of surgeons and engineers towards a new therapeutical concept: heart valve tissue engineering. The first general definition of this approach has been advanced by Langer and Vacanti, as the *in vitro* creation of a viable tissue by combining separate elements, i.e. cells plus an ECM, opportunely conditioned to attain the correct mature function [120]. This universal paradigm has to be applied also to the reconstruction of the valve tissue. The rational idea is to obtain a valve with adequate mechanical function, good hemodynamic performance, vital capability, growth and remodeling permissiveness and lack of inflammatory/ immunological reactions. Such demands have required the synergistic application of different scientific disciplines, from the cell biology to the engineering and surgery. Quite numerous have been the attempts to create the perfect valve substitute, but it is possible to

recognize among them two main modalities with some exceptions (Table 1 in the following page) [121].

Scaffold	Source	Examples
synthetic	biocompatible and biodegradable polymers	polyglycolic acid (PGA) polylactic acid (PLA) polyhydroxyalkanoates (P3HB) PGA and PLA (PGLA) PGA and P4HB
biological	xenogenic or allogenic	decellularized porcine pulmonary heart valves pulmonary heart valves on allogenic acellular matrix conduits
gels	fibrin	heart valves based on fibrin-myofibroblast cell suspension
hybrid	decellularised heart valves coated with synthetic polymer	porcine aortic heart valves dip coated with biodegradable poly(hydroxybutyrate)

Table 1 Matrix typologies for heart valve tissue engineering. Modified from Schmidt D and Hoerstrup SP [121]

The choice of the ECM scaffold is not only a distinction parameter among different approaches, but more a prerequisite for the successful realization of tissue engineered heart valves (TEHV). ECM is able to establish the necessary 3D configuration and guides cell attachment and structural development of the new tissue. Synthetic materials represent often the preferential option in terms of biocompatibility. For their correct shaping, organ printing is fundamental prior to any cell seeding [122, 123]. One of the major positive aspects of the use of biomaterials resides in the possibility of their controlled degradation by colonizing cells, while the last ones operate a new matrix synthesis. Thus, after an initial guiding effect, the non-self material is eliminated in favor of a complete autologous tissue. To this aim different biopolymers with specific chemo-physical properties are used: *inter alia*, 90% porosity is recommended to enable cell adhesion and spreading in the selected biopolymeric mesh [124]. Shinoka et al. firstly reported in 1995 the application of biomaterials in TEHV. The constructs were composed of aliphatic polyesters, as polyglactin, polyglycolic acid (PGA) and polylactic acid (PLA) [125-127], whose scarce pliability did not allow a perfect shape modeling [128]. Conversely, polyhydroxyalcanoates, as polyhydroxyoctanoate (PHA) mixed or not with poly-4-hydroxybutyrate (PH4B), have demonstrated better capabilities in this sense, so that their use in vascular constructs is at the preclinical animal model. The chemical composition of these last polymers is characterized by a polyester group, but combined with bacterial-derived hydroxyacids [129, 130].

Despite the evident capability of these biomatrices to encounter cell remodeling, a proper architectural tissue reconstitution operated by engrafting cells might require a long time chemical and mechanical stimulation. For this reason, another research stream inside the heart valve tissue engineering approach prefers the usage of animal-derived decellularized scaffolds. Decellularization

allows removing xenogeneic cells, maintaining all the fiber composition and distribution of the natural ECM. In addition, the absence of xenogeneic cells might even avoid the treatment with glutaraldehyde, by exposing the matrix to the metalloproteinase action for its continuous remodeling.

Cell-free matrices can be achieved with different methods: trypsin-based enzymatic and detergent decellularization procedures are only two examples of the proposed treatments. An interesting paper by Grauss and Colleagues compared various protocols actually applied to decellularize heart valves by verifying that trypsin and Triton X-100, an anionic detergent, could be able to provoke a loss of matrix integrity [131]. Not only can the enzymatic treatment with trypsin induce elastin defragmentation, but also the use of sodium dodecyl sulphate [132]. Conversely, the use of sodium cholate- and deoxycholate-based methods allows the achievement of fully nude matrices, able to be cell-recolonized *in vitro* and further implanted in clinical patients [49, 132-133].

Although no viral infections have been recorded yet, the risk of transmission of porcine retroviruses has not to be underestimated in order to avoid new trans-species viral combinations.

Other devitalizing methods have been advanced from the observation of the pathological events inducing valve deterioration. Treatments based on ethanol or surfactants display as rational the elimination of the cell membrane phospholipids merely [134]. An incomplete removal of donor's cells is able to trigger immunological responses in the recipient -the major drawback from the employ of xenogeneic tissues-, therefore the application of devitalisation must be followed by fixation prior to implantation.

Not necessarily the natural-derived scaffolds have to be of pericardial or valvular tissue, in effect new matrices based on chitosan, a polysaccharide found in Crustaceans and mixed with synthetic biopolymers, can successfully consent the accomplishment of a hybrid TEHV. Electrospinning techniques have been demonstrated to be valid methods to obtain such composite scaffolds [135, 136].

The second key component of a TEHV is the cell: it is this element that provides vitality to the tissue and hence its remodeling and maturation towards a functional organ.

Different cell types have been combined to the previously described scaffolds: some examples of cell sources employed to create both vascular and valvular bioconstructs are provided in Table 2 (page 34) [121]. For a similar selection principle operated by those researchers preferring the more committed animal-derived matrix, endothelial cells, fibroblasts, myofibroblasts and/or smooth muscle cells isolated from vascular or valvular conduits have been extensively utilized to seed nude matrices, by obtaining both endothelial coverage with antithrombotic activity and tissue repopulation [49, 137]. As demonstrated by Schmidt et al, the obtained cell constructs display similar features to the native tissues both in term of cellularity, growth properties, mechanical behavior, without any influencing from aging [121].The same group verified the feasibility of the

application of less differentiated cells in the creation of functional TEHVs. Marrow stromal cells, umbilical cord myofibroblasts and progenitor cells, *chorionic villi*-derived cells and amniotic fluid progenitors share not only the potential to transdifferentiate in the valve phenotypes after appropriate stimulation, but are associated to numerous positive aspects, which make them particularly attractive for bioengineering applications [138, 142].

Human cell source
dermal fibroblast (covered with bovine endothelium)
foreskin fibroblast (covered with human endothelial)
marrow stromal cells
aortic myofibroblasts
aortic myofibroblasts and venous cells
venous myofibroblast
umbilical cord myofibroblast from vein
umbilical cord myofibroblast (covered with human endothelial cells derived from umbilical cord blood endothelial progenitor cell)
chorionic villi-derived cells (covered with human endothelial cells derived from umbilical cord blood endothelial progenitor cell)

Table 2 Human cells used in the creation of bioengineered constructs as reviewed by Schmidt D and Hoerstrup SP. Modified from [121]

While neonatal progenitor cells can be cryopreserved at birth in view of a future use, bone marrow derived cells, and especially the mesenchymal compartment, can be easily harvested from the same cardiopathic patient for a fully autologous TEHV or even employed for the creation of autologous-like constructs with no risk of cell rejection thanks to their immunomodulatory properties [143].

Scaffolds and cells are not the only necessary conditions for a successful TEHV, but conditioning is indispensable to achieve a perfect maturation of the construct prior to implantation. This last step in the manufacture of the new vital valve can be guaranteed by the employ of bioreactors able to submit it to a physiological pulsatile flow [144, 145] or chemical stimuli [146].

TEHV is not the only approach investigated to obtain new vital valve devices: tissue guided regeneration is proposed as an alternative method for the direct *in vivo* tissue reconstruction, by exploiting ECM instructive capabilities.

1.8 Aim and outline of this doctoral thesis

This thesis evaluates different methods for the creation of new vital and functional heart valve substitutes based on decellularized matrices. Some biological aspects able to influence newly formulated biodevices, as well as native valves are also assessed. The just developed Chapter 1 has focused on the biological and pathological notions about the valvular apparatus, which were achieved by the ancient anatomists and implemented by the modern researchers, evidencing the necessary development of new therapeutical approaches for valve replacement. The second Chapter discusses about the immunogenic properties of the extracellular matrix, both of xenogeneic and allogeneic nature, and in particular, analyses the expression of the xenoantigen alpha-gal in native and decellularized animal heart valve leaflets, suitable to obtain a bioengineered construct. Chapter 3 describes an *in vitro* approach of heart valve tissue engineering using porcine and human decellularized leaflets in combination with human mesenchymal stem cells of bone marrow origin. Effects of the differential leaflet ECM fiber distribution on cell engraftment are examined. In Chapter 4, it is proposed an alternative regenerative approach to the classical tissue engineering paradigm. Allogeneic decellularized aortic roots have been implanted in orthotopic-like position to reconstruct the right ventricular outflow tract and evaluated up to 14 months *in vivo*. Chapter 5 investigates the presence of stem cell populations in the atrioventricular and semilunar leaflets derived from donors' allografts or excised during valve replacement surgery. Each Chapter is organized in a general introduction on the topic, materials and methods, results and cited bibliography. Finally, Chapter 6 discusses the different proposed studies and their biological and clinical implications in the creation and maintenance of new bioengineered heart valves.

1.9 Chapter references

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Chapter 2

Immunogenicity of xenogeneic cells and extracellular matrices

Alpha-Gal expression in heart valve cusps of different mammalian species and effect of decellularization: an *ex-vivo* study

2.1 Introduction

Allotransplantation has been widely consolidated as valid therapy to rescue a failed vital function, but the shortage of human cells, tissues and organs dramatically increases the waiting lists for replacement: in 2006 Eurotransplant referred almost 16000 patients were attending to receive a substitute, while in the United States these ones reach the number of 90000 [1]. The numerical entity of these registers is expected to sensitively grow during the near future. Surely, the employ of unlimited supplied animal-derived organs could allow an immediate intervention to recover the lost function and in combination with tissue engineering methodologies, might favor the achievement of human-like organs through strictly controlled manufacture procedures. So far, animal tissues fixed in glutaraldehyde have been the reserve to exploit at the time of clinical need, but in accordance to maintain vitality, this can be no longer considered a viable way above all for those organs that, devoid of a proper cell physiology, cannot perform a correct function. The use of non-human sources could not be unaccompanied by a major raising issue for a broad clinical application, i.e. the immunological barriers. For a donor-receiver mismatched allocombination, the major medical concern is the inability of accommodation and therefore the onset of chronic rejection, whose main manifestation in the case of heart transplant is graft vascular disease. This allotransplantation drawback is characterized by an unchanged gravity and entity in respect to 40 years ago, when this research line started to be investigated as possible route of treatment of end-stage pathologies [2]. A peculiar atherosclerotic process interests the heart transplant with few calcifications, but increased cellularity and extracellular matrix at the entire intimal level with a concentric distribution [3]. A similar event could occur in the xenotransplantation approach where an even more severe expression could be attended. Furthermore, in addition to a chronic response, hyperacute antibody mediated rejection represents a dramatic hurdle to early term xenograft survival, when a trans-species setting is to be considered. By developing in a time period from minutes to hours in the pig-to-primate combination, hyperacute rejection, commonly defined as HAR, is a typical humoral immune response in vascularized organs with deposition of xenoreactive natural antibodies and complement activation [4]. Pig-to-primate xenotransplantation has properly enabled to discover the progression of the delayed immunological answer to the cardiac graft (DXR): besides a strong humoral activity, an acute cellular infiltrate and the activation of the endothelium seriously compromise the function of the newly transplanted organ [5]. However, less known -and in the last years very debated- is the real immunological trigger able to cause the complete loss of the xenograft during time. Probably the prompts of this phenomenon are not to be found in a unique opponent, but in more factors which alone or in cooperation provoke it. One of the most powerful antigens is definitely Galα1-3Galβ1-4GlcNAc, commonly identified as α-Gal. This oligosaccharide is a component of the glycoproteins and glycolipids, displayed on the surface of vascular endothelial

cells in all mammals except apes, Old World monkeys and humans, unable to metabolize it for the evolutionary gene silencing of the related enzyme α1-3-Galactosyltransferase [6, 7]. Similarly to ABO antibodies, alpha gal, which reaches an expression concentration of at least 10^7 epitopes per pig cell, is recognized by human cells in a highly specific pattern soon after birth. In fact, microorganisms colonizing or transiting through the intestinal flora express it on their surface and due also to the dietary use of animal-derived nourishment, 1% of serum circulating IgGs are specifically directed against this epitope with a quite pure protective role against parasite and viral attacks [8, 9]. Already at the end of '80s in the previous century, a restricted but well developed body of evidence considered alpha-gal as a possible suspected immunological concern, but it was only ten years later that a full demonstration of its causative role in HAR was made available by studying the interaction pig-to-primate [10]. Other important observations were obtained in this research about the amount of natural anti-pig antibodies in humans and their specific subclasses. Even the 85% of humoral anti-pig players are specific for alpha-gal and belong to the IgG and IgM classes: more recurrently, the latest ones, even without the first, tend to deposit on the graft endothelium [10-12]. Anti-Gal antibodies have been shown to contribute both to HAR and lately DXR, if a tolerance regimen has been introduced [13]. Therefore the issue is unquestionably alarming for new therapeutical approaches of biomedicine using xenogeneic biological materials.

In order to face this problem, different strategies have been developed just from the observation of the glyco-composition differences between pig and human cells. In the endothelium of both species, N-acetyllactosamine and sialic acid are expressed, but the human one –as previously mentioned- lacks alpha-gal and is characterized by the presence of ABH-Galβ1-4GlcNAcβ1-R (ABO system) and a supplementary sugar, NeuGca2-3Galβ1-4GlcNAcβ1-R (N-glycolylilneuraminic acid). Seen that the depletion of anti-Gal IgMs resulted in a complete elimination of complement-mediated cytotoxicity by human serum, but it had no effect on the IgG counterpart, various methods to deplete the whole range of anti-alpha gal antibodies have been tested. When the first xenotransplantation experiments started in the 1960s, still unaware of the existence of alpha-gal and its antibodies, a decrease in xenoreaction could be achieved by perfusing the recipient's blood in a donor-specific organ, such as the liver which has a high immunoadsorbent capability. At the same extent, other depletion procedures appeared to ameliorate the survival of the xenogeneic tissue: plasma exchange and plasma perfusion through column systems based on specific protein interaction or on immunoaffinity could extend the implant time, but rarely for more than one month since accommodation was not established. The development of such mechanisms is still under study and certainly they show many complexities, being accompanied at different points of the filtration process by difficulties able to limit the success of the attended outcomes. A more direct method, which avoids the use of external devices, is the intravenous approach established on similar affinity principles. Infusion of idiotypic antibodies directed against idiotypes on anti-gal antibodies,

immunoglobulin (IVIG) or even oligosaccharides can behave as silencers, by blocking –at least quite completely- any possible immunological response against alpha-gal and in the case of IVIG, it has been postulated an indirect beneficial role through the acceleration of IgG physiological catabolism and inhibition of macrophage function [12].

A further procedure inducing an accommodation state can be identified in the suppression of anti-alpha gal effectors production. The full depletion of B lymphocytes and plasma cells is the only possibility to obtain this result and methods proposed for this purpose are irradiation, pharmacological therapy, anti-B cells specific antibodies (mAbs) and immunotoxins. The irradiation of the entire body with the clinically used dose of 300 cGy does not result in a lethal condition, but provokes a transient B-cell ablation, whose positive effects are therefore time-limited. The administration of mAbs underlies a more selective way of action than the previously mentioned one and benefits from the experiences completed in the haematooncological field. Anti-CD20 mAbs, for instance, have been used to treat baboons for 4 weeks: at the end of this course, a complete deficiency in B cells could be maintained both in bone marrow and peripheral blood for at least 3 months. A combination of immunotoxins, such as ricin A and sapporin, to these mAbs can facilitate better ablation results [12].

In the transplantation setting, a wide line of investigation has been directed to discover chemical agents able to perform an immunosuppressive effect. Even in xenografting, this kind of therapy seems to have an important role of support to other strategies in order to induce accommodation. Cyclophosphamide is one of the most common pharmacological agents with this task: in heart transplant, the effect of function prolongation is positive without doubt, but many risks are associated with a permanent use of this drug, as leucopenia [14]. Alkylating agents, as melphalan, or DNA polymerase blockers, as zidovudine, or even enzymatic inhibitors, as methotrexate, have been analogously experimented with different results. The first are particularly interesting because they are quite the lonely one to have plasma cells as target. Although reducing anti-alpha gal IgGs, the suppressive effects of zidovudine are not so important in a prospective of clinical use. Methotrexate, on the contrary, appears to be a valid agent if added to other B cell destroying therapies. In newborn baboons, mycophenolate mofetil (MMF), an inhibitor of the purine synthesis, has allowed, as therapeutical adjuvant, to prolong the survival of transplanted porcine hearts of at least the double time in respect to non treated animals [15]. These results in themselves could not seem particularly appealing if compared to the effects of other drugs, but MMF is able to offer a similar efficacy in the face of lack of side consequences and appear to be very promising for future uses on xenotransplanted patients. A special mention should be addressed to a therapy based on a quite new soluble glycoconjugate, GAS914, in combination with different drugs, among which MMF too. GAS594, used to maintain the graft, has demonstrated a constant capability to suppress the production of anti-gal IgGs and IgMs in cynomolgus monkeys transplanted with transgenic porcine

hearts [16]. All these methods search to abate the recipient's response to this carbohydrate, but generally a certain refractoriness to the therapies cited before does not allow a long permanence of the xenograft and hence provokes the inability to maintain the organ function [12].

Another possible way to avoid alpha-Gal-induced rejection is the modulation of the donor tissue, that is to bioengineer the graft in order to eliminate the source of antigenicity. Genetic engineering enables the silencing of the genes involved in the pathway of production of alpha-Gal, resulting in transgenic animals which do not expose the antigen on the surface of their cells. Mice and pigs expressing human α1-2-fucosyltransferase were produced with this rational, by obtaining a modification of the cell membrane alpha gal and therefore a decreased antibody reactivity [17]. The induction of the human decay-accelerating factor (hDAF) expression in animal donors could play an inhibitory role in the onset of HAR [18]. Nevertheless, a more direct approach is the knocking down of α1,3-galactosyltransferase. When transplanted in heterotopic position into immunosuppressed baboons, Gal KO porcine hearts were enabled to survive *in vivo* for at least 6 months with graft failure not linked to HAR, but to thrombotic microangiopathy [19].

A particular observation, frequently evidenced by all the researchers attempting to abolish anti-alpha-Gal response, is represented by a still acute humoral xenograft rejection (AHXR) elicitation [16]. Alpha-Gal, although recognized to be the most hazardous antigen in xenografting, is not the only one as demonstrated by Lam's study, where rejection of porcine hearts in immunosuppressed monkeys was due not to a titer increase of anti-Gal antibodies, but of anti-non-Gal ones. Similar results were confirmed in the work of Chen and colleagues in the renal transplantation field by directly neutralizing anti-Gal antibodies [20].

In all cases, it is commonly accepted that xenoantigens are cell-derived so the removal of the membranes, where these epitopes are displayed, should definitely solve this problem. Such a perspective could particularly fit the case of engineered arterial vessels or heart valves, which have undergone a prior decellularization procedure. When the first human pediatric implantations of decellularized porcine heart valves, produced in an industrialized and controlled manner by Cryolife (Kennesaw, GA), were performed in Wien (Austria), a severe inflammatory response provoked the failure of the graft [21]. If this dramatic event could be attributed to an incomplete cell removal, the same Authors pointed out that, despite full cell-devoid condition, residual alpha-Gal not correctly washed out from treated heart valves could be responsible of the previously observed HAR event and later suggested the use of the detergent IGEPAL CA-630 to extract it totally [22].

It is otherwise important to recognize that epitopes able to induce graft rejection are not only present at the cell level, but could be found in the matrix scaffold and such a phenomenon could be also at the basis of aging-correlated pathologies, autoimmune disorders and allotransplant degeneration. Already in the 1970es, there was evidence that if pure bovine elastin is injected subcutaneously or in the peritoneal cavity in rats, it is able to elicit an immunological response, for

which macrophage effectors try to eliminate it [23]. More recently a wide investigation about the immunogenicity of extracellular matrix has been undertaken by Allaire and Colleagues [24]. In this extensive work, both native and decellularized arterial grafts were tested in concordant and discordant trans-species combinations for a period from 15 minutes up to 30 days of implant. Excluding an alpha-gal-mediated rejection (all the mammals used in these studies express it), the failure of the fresh graft finds as putative cause the antigenic properties of elastin and develops less early in the concordant hamster-rat model, rather than in the discordant guinea pig-rat xenointeraction, where elastin resorption reaches higher values facilitating the onset of aneurysms at the end of the experimentation. By comparison with decellularized arterial iso- and allografts, their xenocounterpart tends to dilate *in vivo*, therefore an interspecies, rather than intra-species, extracellular matrix immunogenicity might be suspected. If a discordant xenografting of decellularized artery is to be considered, its survival *in vivo* is very limited because monocytes, macrophages, T lymphocytes and immunoglobulins are expected to attack the *intima*, infiltrate the *media* and provoke elastin lysis. According to the Authors, the presence of cells inside the graft can speed elastin resorption in the discordant xenografting and hence vessel dilatation, by accelerating the monocyte recruitment in the intimal and adventitial layers, without any effect on the enrollment of T lymphocytes.

2.1.1 Alpha-Gal expression in heart valve cusps of different species and effect of decellularization: an ex-vivo study

As pointed out previously, Alpha-Gal might quickly compromise the durability of transplanted organs from porcine donors to human recipients, by eliciting a hyperacute rejection. Even if not vascularized in normal condition, a heart valve is immersed in the blood circulation and hence directly exposed to circulating immunological effectors, which can immediately recognize any non-self epitope on their endothelial surface. Crosslinking with fixatives, as glutaraldehyde or carbodiimide, has allowed so far the tissue protection from a direct blood contact, but has not eliminated the chronic inflammatory response induced by the xenogeneic materials. Effectively, a hypothetical explanation of the early structural deterioration occurring in 10-15 implant years could be associated to the mechanical stress-induced microfenestrations in these tissues. In the need of an organ, able to physiologically remodel and grow with the patient, the use of chemical agents should be avoided because it drastically reduces the vitality of its cells and promotes pathological conditions. It is therefore essential that a newly formulated biocompatible valvular substitute should be devoid either of chemical crosslinking and cell membrane epitopes in accordance to the cardiovascular implant guidelines (ISO 5840).

2.2 Materials and methods

Hearts from different species (porcine and bovine) were obtained from local abattoirs and resected to obtain their semilunar valves. Pulmonary and aortic valve cusps were embedded in OCT (Tissue Tek®, Tokyo, Japan) and cryocooled in liquid nitrogen, as fresh samples. Some of the porcine specimens underwent TriCol decellularization [25; for a detailed description see Chapter 3] and then the same storing procedure described before. According to previous reports [22, 26], the isolectin B4 can be used as an alpha-gal detector due to its high affinity for the carbohydrate element. FITC-conjugated B4 isolectin (BSI-B4; Bandeiraea Griffonia simplicifolia, Sigma, St. Louis, MO) was initially used to visualize alpha-gal epitopes on 6-μm cryosections. A double staining with collagen I or elastin (both from Sigma) was applied to the slices to better evaluate the tissue distribution of the epitopes and nuclear material was marked by Hoechst 33258 (Sigma). Another technique is proposed to alpha-Gal recognition; based on immunoaffinity principles and it uses a monoclonal antibody directed against the xenoantigen. The different alpha-gal tissue recognition capabilities shown by the BSI-B4 isolectin and the specific monoclonal antibody M86, revealed with an anti-mouse AlexaFluor 594-conjugated goat Fab' (Molecular Probes, Carlsbad, CA), were compared in the same tissues by triple labelling with Hoechst.

2.3 Results

In native tissues, BSI-B4 revealed a moderate positivity both at the endothelial surface and in the inner stroma of the porcine and bovine leaflets, as visible in Figs. 1-4.

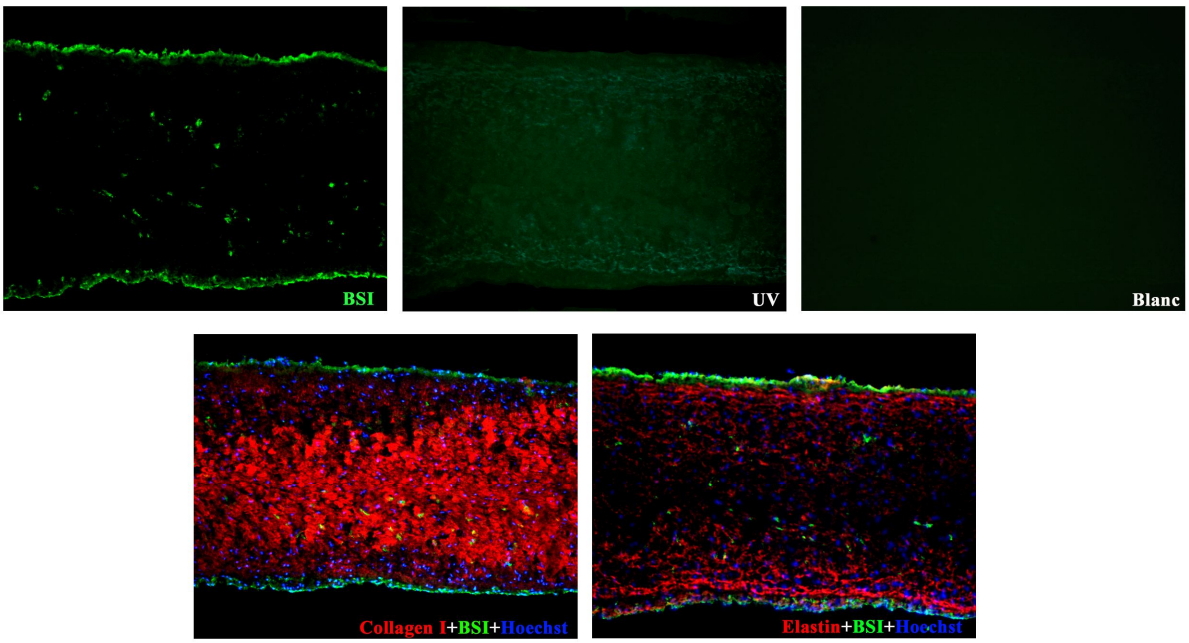
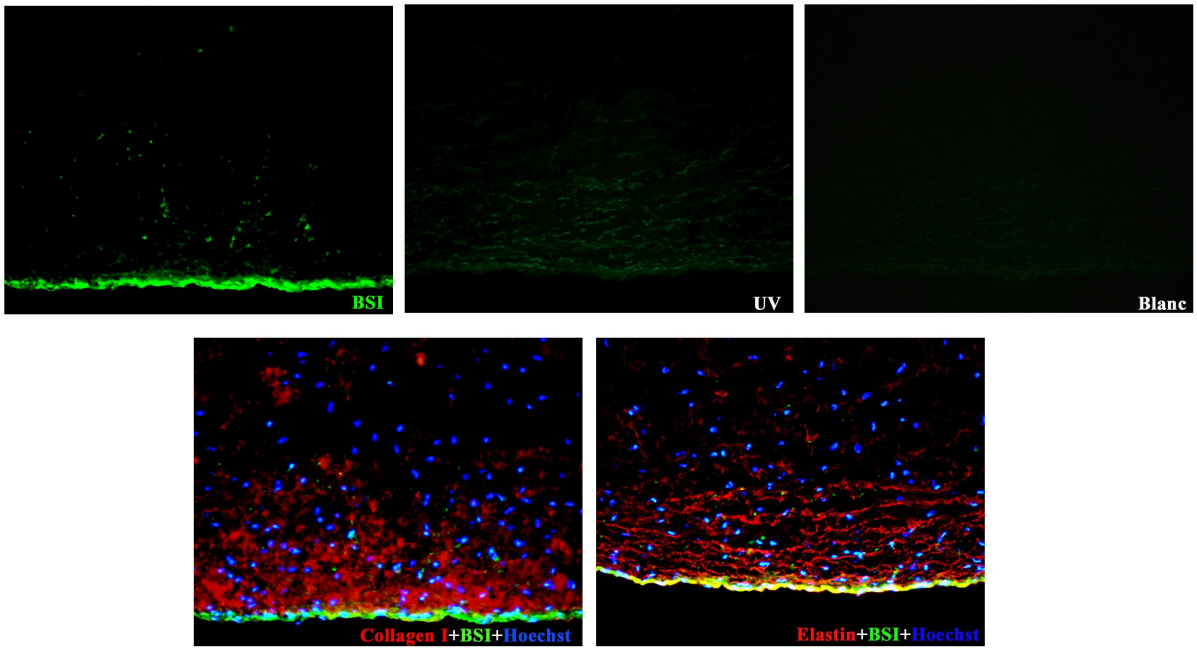
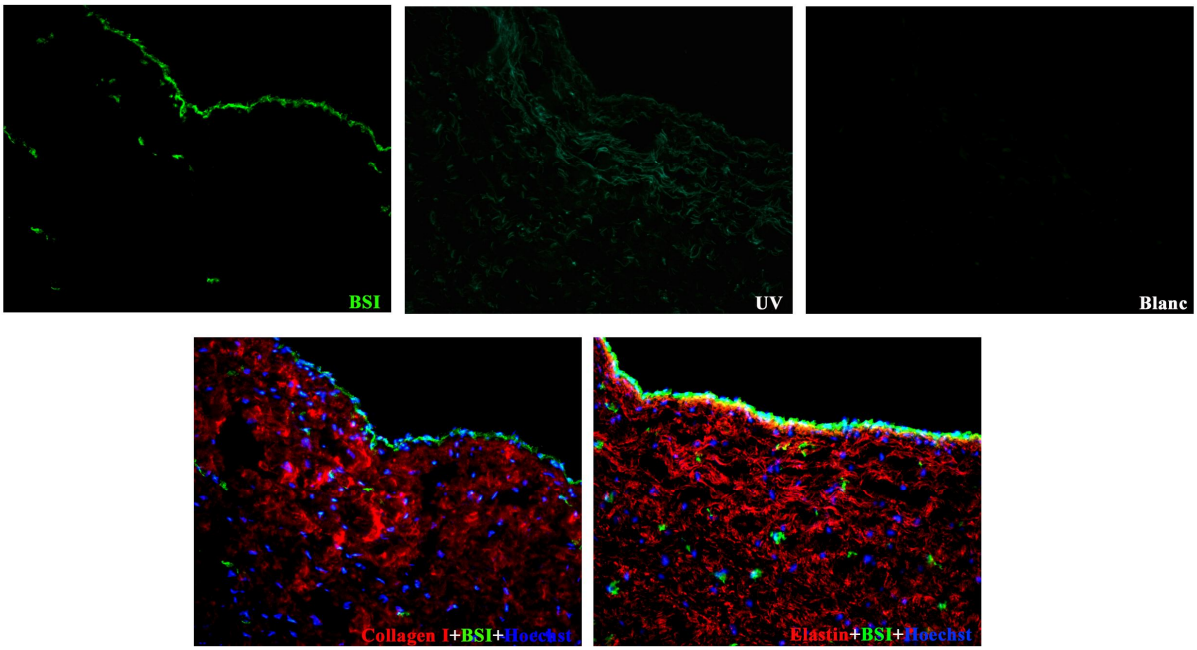


Fig. 1 Alpha-gal detection in fresh porcine pulmonary specimens. Note the positivity for the BSI-B4 isolectin both on the endothelial lining and among the inner stromal cells at 100x magnification. UV enlightens leaflet autofluorescence



Figs. 2 and 3 BSI-B4 positivity on porcine and bovine pulmonary cusps (respectively in the above and below middle parts of the page). Magnifications: 200x



In aortic coronary cusps, it was frequent to observe that the endothelium of the small arteriolar and capillary vessels at the base of the leaflet was attractive to isolectin (Fig. 4), hence revealing at least three alpha-gal sources in this tissue: outer endothelial lining, some valvular interstitial cells and in addition the endothelium of the leaflet vascularisation.

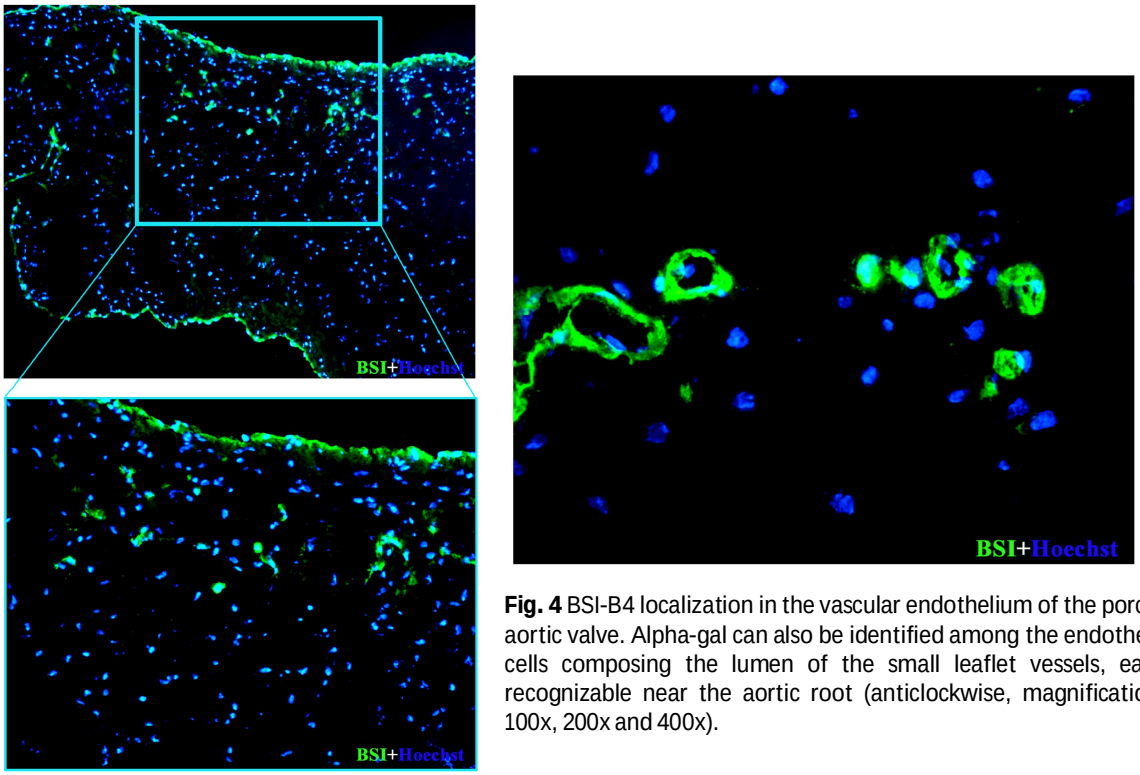


Fig. 4 BSI-B4 localization in the vascular endothelium of the porcine aortic valve. Alpha-gal can also be identified among the endothelial cells composing the lumen of the small leaflet vessels, easily recognizable near the aortic root (anticlockwise, magnifications: 100x, 200x and 400x).

A diffuse staining was observed when TriCol-decellularized specimens were incubated with the glycoprotein. No blue fluorescence (Hoechst) was appreciable but a wide co-localization of BSI-B4 with any matrix fibre, both collagen I and elastin, could be observed (see Fig. 5C and 5D).

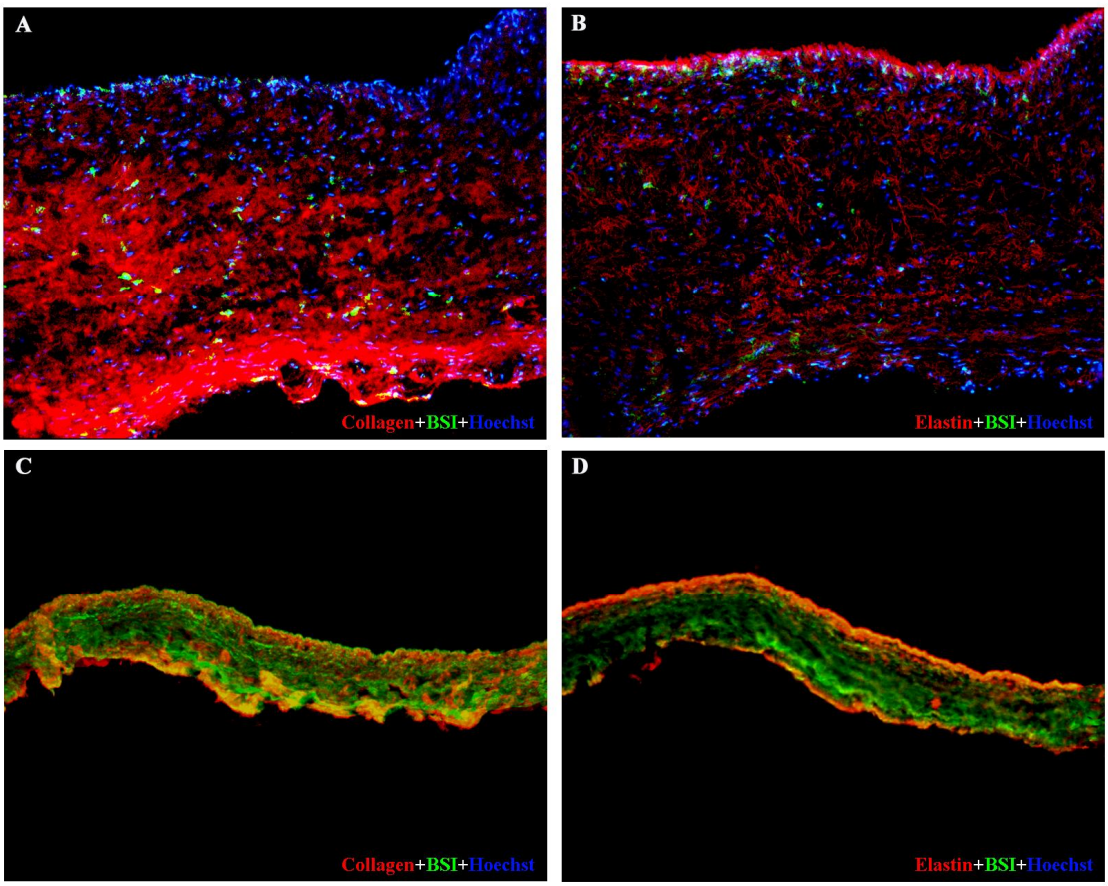


Fig. 5 BSI-B4 affinity pattern in fresh and TriCol decellularized porcine pulmonary leaflets. While in the fresh specimens BSI-B4 had a scattered distribution in the cusp stroma, in the decellularized ones (C and D), it showed a high overlapping positivity with collagen and elastin without any particular distinction among the two extracellular matrix fibres (Magnification: 100x). In the previous page

Such results could lead to hypothesise not only a small cellular expression of alpha-Gal in fresh cusps, but also an unrestricted positivity at the extracellular matrix level, made unmasked in the cell-devoid condition.

To confirm these data, the alpha-Gal specific antibody M86 was tested together with BSI-B4 in both native and decellularized samples, revealing a quite matched distribution pattern in the first ones (Fig. 6A). On the contrary, no binding of cell debris or matrix fibres to M86 could be detected in TriCol-treated leaflets (Fig. 6B), hence demonstrating an unspecific isolectin affinity in these tissues.

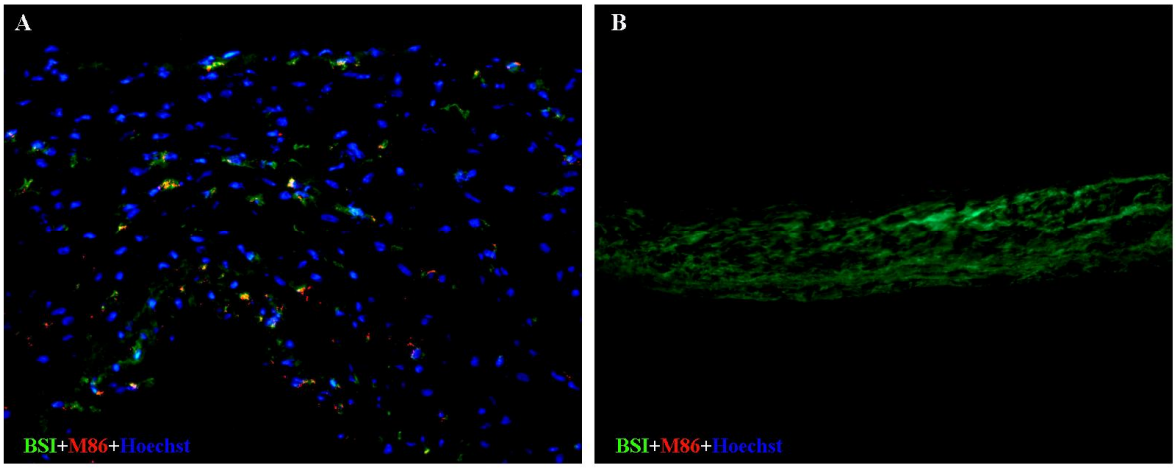


Fig.6 Alpha-gal contextual assessment in fresh and TriCol decellularized porcine pulmonary leaflets using BSI-B4 and M86 monoclonal antibody. Similar epitope detections were identified by M86 and BSI-B4 in fresh specimens (yellow merge in A); when used on TriCol cusps there was no M86 binding, as visible in B, due to the complete lack of red fluorescence, whereas BSI-B4 shows again an indistinct positivity for the entire leaflet. (Magnification: 100x).

No M86 positive signaling was also encountered when the detergent IGEPAL CA-630 (Sigma) was used as further step in the decellularization treatment, as suggested by Kasimir et al. [22] to completely eliminate any alpha-gal residues (data not shown).

However, when these leaflets were tested in an *in vivo* model of biocompatibility a strange phenomenon (Fig. 7) was observed in respect to the cusps treated with the basic procedure. When inserted in rat peritoneum for 30 days, despite a quite low immunological response –comparable to TriCol alone- an unrestricted neovascularisation was documented in the leaflet. Therefore, IGEPAL CA-630 might be provided as an-alpha-gal remover –at least as confirmed by the experiments performed by Kasimir and Colleagues [22]-, but induces extracellular matrix modifications able to establish the basis for further clinical pathological events, as the formation of vessels is considered in human heart valves.

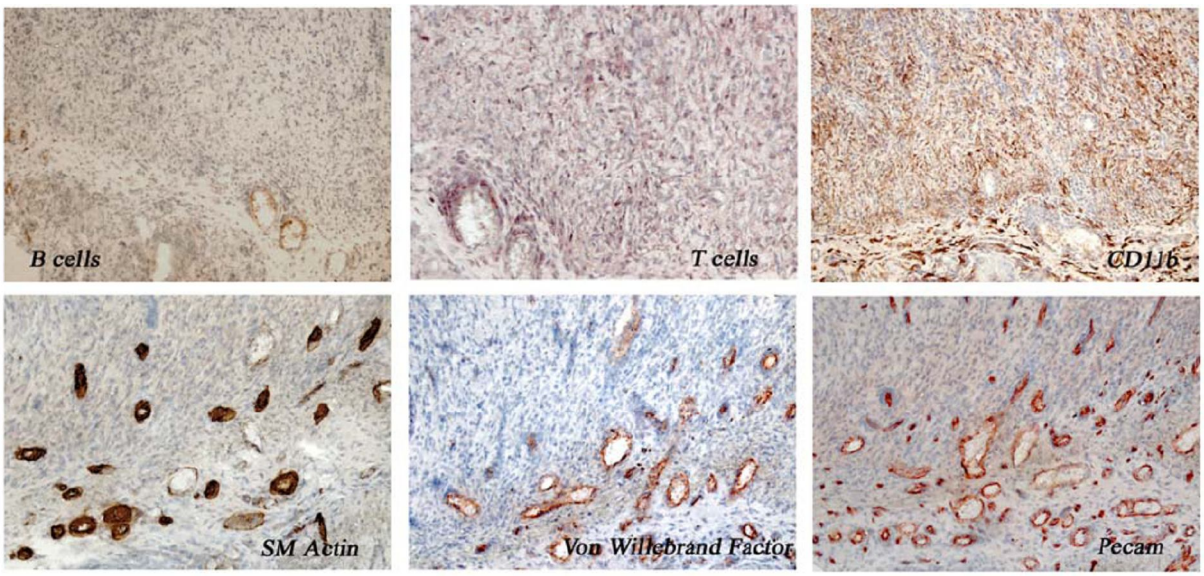


Fig. 7 Biocompatibility evaluation of TriCol/IGEPAL CA-630- treated porcine aortic cusps in a xenogeneic rat model. Porcine decellularized leaflets with the IGEPAL-implemented TriCol procedure were fully colonized by CD11b cells after 30 day-implant in the rat peritoneum cavity, but a wide neovasculogenic occurrence interested all the leaflet thickness ([27]; magnification: 100x). The precise mechanism able to trigger such an angiogenic program in these specimens is not clear, but surely it is indispensable to draw attention to the secondary effects provoked by the used reagents and detergents in the decellularization process.

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Chapter 3

In vitro Heart Valve Tissue Engineering

The influence of heart valve matrix anisotropy on the engraftment of human Mesenchymal Stem Cells

3.1 Introduction

Among heart diseases, end-stage valvulopathy still represents a condition for which valve replacement with mechanical or biological prosthesis is mandatory [1,2]. Xeno- and homograft biological supports are currently used in cardiac surgery and both have their pros and cons as regards durability and performance, particularly in pediatric patients with congenital heart defects (i.e. Fallot's tetralogy) [3]. The fact that current biological valve prosthesis do not enable the reconstitution of a viable valve structure has prompted new bioengineered applications based on decellularized valve scaffolds seeded with autologous cells from the recipient prior to implantation, or recolonized *in vivo* after surgery. Basically, this two-step procedure includes preparing xeno- or homogeneic valve matrices [4] with mechanical and functional properties suitable for cell engraftment. We recently set up a technique for decellularizing porcine heart valve leaflets using a non-ionic detergent, Triton X100, which enables scaffolds with an optimally preserved collagenous elastic network to be achieved [5,6]. It is particularly important to select cell type(s) suitable for appropriate valve cell colonization *in vitro* in static conditions or in a bioreactor. Ideally, the reconstituted valve should display an endocardial endothelium covering the engineered tissue and valve interstitial cells (VICs) distributed tissue-specifically, i.e. myofibroblasts and smooth muscle cells (SMCs) in the *ventricularis*, and fibroblasts in the *fibrosa* and *spongiosa* layers [7]. To achieve this result, researchers benefited from more mature cells, as myofibroblasts [8], endothelial cells (ECs), SMCs from the carotid artery media [9] or less differentiated cytotypes, i.e. endothelial progenitor cells (EPCs), or mesenchymal stem cells (MSCs) [10–12]. Among the various cells tested, MSCs obtained from bone marrow (BM-MSCs) offer some advantages over other stem or progenitor cells in terms of their prospects for employ in routine clinical practice, i.e. simple protocols for their isolation, storage and *in vitro* expansion [13], a surprising phenotypic resemblance to valve cells [14], a beneficial immunomodulatory capacity [15] and, in a more significant way, they can be phenotypically converted into ECs, fibroblasts/myofibroblasts, and SMCs [16–18]. So far, efforts to obtain valve substitutes with biomaterial moulds populated with extra-valve cells have failed to recreate the fiber arrangement of native matrix. This drawback can be overcome by using decellularized scaffolds, which act as ideal supports for cell attachment and differentiation because the native leaflet fiber integrity and alignment are preserved [5, 6, 19], as demonstrated by recolonizing valve scaffolds with VICs [4]. In this study, we first investigated cell adhesion, spreading and potential for differentiation of hBM-MSCs seeded statically onto the two anisotropic microstructures of *ventricularis* and *fibrosa* of porcine valve leaflets. After establishing which of the two valve layers was better able to allocate hBM-MSCs, we compared these cells in a hetero- vs. homotypic matrix setting, using decellularized porcine and human valve leaflets, respectively. The

results of this study are of importance to clinical applications of valve tissue engineering, and particularly to the assessment of whether a xenomatrix seeded with patient's cells can surrogate an autologously cell-colonized allomatrix as a valve substitute.

3.2 Materials and methods

3.2.1 Human bone marrow mesenchymal stem cells (hBM-MSCs)

Bone marrow obtained under local anesthesia from the iliac crest of healthy young voluntary donors (15–30 years old) was diluted with sterile Ficoll (1:2; Lymphoprep®). After centrifugation (1040 rpm, 5 min, RT), collected pellets were resuspended in a-MEM (Sigma, St. Louis, MO) plus 20% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 1% penicillin–streptomycin (Sigma) and 1% L-glutamine (200 mM; Sigma), and plated on non-tissue culture plates (NOTC; BD Falcon®, BD Biosciences, Franklin Lakes, NJ). Non-adherent cells and debris were discarded after seven days. Adherent cells were cultured until pre-confluence, then detached from plastic using a 0.05% trypsin–sodium EDTA solution (Sigma), and passaged up to the 4^{th} passage. Cell morphology was studied using a phase contrast Leica DM IRB microscope (Leica, Wetzlar, Germany) connected to a Canon Power Shot S40 camera.

3.2.2 Flow cytometry and cytocentrifugation

FACS analysis of hBM-MSCs was performed at the 3rd passage. Cells, detached with citrate buffer (Sigma), rinsed and re-suspended in PBS, pH 7.2 (Sigma) at a concentration of $5x10^5$ cells/100 ml, were directly stained with 10 µl of FITC fluorochrome-labeled anti-human HLA-ABC (Immunotech, Marseille, France), CD29 (Immunotech), CD44 (Immunotech), CD90 (Immunotech), CD146 (Immunotech), CD31 (Immunotech) and PE-conjugated anti-human antibodies to the progenitor markers CD45 (Immunotech), CD34 (Immunotech), CD117 (Immunotech), HLA-DR (Immunotech), CD73 (BD Biosciences), and CD105 (Beckman Coulter, Fullerton, CA). Cytometric analysis was done using a COULTER Epics XL-MCL cytometer (Beckman Coulter) and data were processed using EXPO™ 32 ADC software. Cytocentrifuged cell samples, acquired via a Shandon Cytospin 4 centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA), were fixed in 2% p-formaldehyde in PBS and incubated with primary antibodies to membrane and cytoplasmic antigens of differentiated valve cells as well as embryonic and mesenchymal stem cell antigens, for 30 min at 37 °C. The primary antibodies used in the immunofluorescence experiments were directed against the following epitopes: SSEA4 (Chemicon, Temecula, CA), OCT3/4 (Santa Cruz, Santa Cruz, CA), human CD117 (Dako, Dakopatts, Denmark), human CD34 (Dako), human CD133 (Abcam, Cambridge, UK),

human CD90 (Chemicon), CD271 (NGFr; BD Biosciences), human CD29 (Integrin b1; Chemicon), human CD105 (Cymbus, Chandlers Ford, UK), Stro1 (Developmental Studies Hybridoma Bank, Iowa City, Iowa), CD54 (ICAM1; Sigma), Flk1 (VEGF-R2; Santa Cruz), human nestin (Abcam), glial fibrillary acidic protein (GFAP, Chemicon), von Willebrand factor (vWf; Dako), SM a-actin (Sigma), SM22 (Abcam), vimentin (clone V9, Dako), platelet myosin heavy chains (MyHC-Apla1 and MyHC-Apla2) [20], SM-myosin heavy chain II (SM-MyHC) [20], smoothelin (Abcam), osteopontin (Abcam), osteocalcin (Abcam), osteonectin (clone AON, Hybridoma Bank), tenascin (Exalpha, Maynard, MA), pro-collagen I (clone M38, Hybridoma Bank), human aggrecan (not reactive to porcine aggrecan; Abcam), HLA-ABC (Chemicon) and MHC II (VMRD, Inc., Pullman, WA). The secondary antibodies were the Cy2 conjugated goat Fab' to mouse or rabbit IgGs (Chemicon). Cell nuclei were stained with Hoechst (Hoechst 33258; Sigma). Antigen distribution was studied using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and images were acquired using a Leica DC300F digital videocamera (Leica, Wetzlar, Germany). The cytocentrifuged cells positive to each antibody were counted manually by two independent examiners and the corresponding scores were expressed as percentages of total Hoechst-stained cells. Flow cytometry and cytocentrifugation analyses were performed in triplicate using distinct MSC preparations.

3.2.3 Endothelial, smooth muscle, adipogenic and osteogenic differentiation of hBM-MSCs

Cell lineage differentiation was assessed by stimulating passaged cells with induction media specific for ECs (Endothelial cell growth medium; PromoCell, Heidelberg, Germany) and SMCs (DMEM with 10% FBS, 5% horse serum [Invitrogen], and 50 mM hydrocortisone [Sigma]), which normally populate heart valve leaflets. Adipogenic and osteogenic conversion abilities were also investigated. Briefly, cells were plated at a density of 4 x10^3 cells/cm^2 on tissue culture dishes (Falcon, BD) and treated with: (1) adipogenic medium (low-glucose DMEM supplemented with 10% FBS, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 mM insulin, 200 mM indomethacin [Sigma]); and (2) osteogenic medium (low-glucose DMEM supplemented with 100 nM dexamethasone, 10 mM b-glycerol phosphate, 50 mM ascorbic acid 2 phosphate [Stem Cell Technologies Inc., Vancouver, BC]). Each stimulation lasted 21 days with medium changes 3 times a week. Coverslips with treated hBM-MSCs were fixed in 2% p-formaldehyde in PBS and tested in immunofluorescence with anti-vWf, anti-CD31, anti-SM a-actin, anti-SM22 and anti- SM-MyHC as primary antibodies; mouse or rabbit Cy2 conjugated-IgGs as secondary ones and Hoechst for nuclear staining. Adipogenic induction was determined by evaluating cells with fat vesicles, stained by Oil-Red-O staining (Sigma). Alkaline phosphatase (AP; Sigma) was used to visualize the effects of osteogenic stimulation. Nuclei were counterstained with Mayer's hematoxylin (Sigma). Antigen

distribution was evaluated using a Leica light microscope and images were acquired with a Leica DC300 digital videocamera (Leica).

3.2.4. Heart valve leaflets and decellularization procedures

Porcine pulmonary valve leaflets were used to study the effect of matrix fiber distribution on cell attachment. Porcine hearts (n= 2) were harvested under sterile conditions from a local abattoir and stored at +4 °C until further processing. In the presence of protease inhibitors, pulmonary heart valves were isolated in aseptic conditions and decellularized according to Spina et al. [6]. Briefly, the leaflets (n = 4) were treated with solutions containing Triton X100 and sodium cholate (TRICOL) in a nitrogen atmosphere. An endonuclease treatment (Benzonase, Merck, Darmstadt, Germany) was applied to remove any remaining nuclear fragments. All solutions used in the procedure were degassed and filtered in sterile conditions (0.22 mm filter; Millipore). Serologically-tested, intact cryopreserved human pulmonary heart valves (n =2) were obtained from the Tissue Bank of Treviso Hospital. Human leaflets (n =4) were decellularized using the same protocol as for porcine matrices. For each preparation, a sample was removed and snap-frozen in OCT (Tissue Tek®, Tokyo, Japan) to verify effective cell and nuclear debris removal. For this purpose, 6 µm cryosections underwent Hoechst staining to identify nuclear fragments, and hematoxylin and eosin staining (Bio-Optica, Milan, Italy) to additionally reveal cell debris. Alpha-gal xenoantigen expression was also assessed in porcine leaflets by double immunofluorescence using an FITC-conjugated B4 isolectin from Bandeiraea simplicifolia (BSI-B4; Griffonia simplicifolia, Sigma) and a specific monoclonal antibody (M86, Axxora, Nottingham, UK), revealed with an AlexaFluor 594-conjugated goat Fab' to mouse immunoglobulins (Molecular Probes, Carlsbad, CA). Native porcine leaflets were used as positive controls.

3.2.4 *In vitro* static cell seeding

Each decellularized leaflet was divided into two parts (pig pulmonary valve leaflets, pPVLs, n= 8; human pulmonary valve leaflets, hPVLs, n=8) and stored for 7 days in a 0.04% sodium azide solution (NaN3; Sigma) in PBS. After intensive washout with a sterile 5% sodium carbonate solution (Prolabo, Paris, France) to completely remove any residual NaN3, leaflets were subjected to a combined antibiotic–antimycotic treatment for four days. The cocktail contained lyncomycin (600 mg/ml; Lincocin®, Pharmacia Italia, Milano, Italy), vancomycin (250 mg/ml; Vanco, Bayer, Milan, Italy), cephazolin (1200 mg/ml; Cefazolina, Dorom, Milano, Italy), colymicin (500 mg/ml; Colimicina ®, UCB Pharma, Torino, Italy) and amphothericin B (5 mg/ml; Fungizone®, Bristol- Myers Squibb, UK), and was replaced every 24 h. Any residual antibiotic solution was removed by washing out in HEPES-modified DMEM (Sigma). PVLs were incubated with this medium for 24 h at 37 °C in a humidified atmosphere, then treated with FBS (12 h at 37 °C), followed by bovine fibronectin (5

mg/cm2; 24 h at 37 °C; Sigma). Porcine leaflets were used in the first set of experiments: equal parts of the same pPVL were placed in tissue culture 24-wells (TC; Falcon BD) and seeded respectively onto *fibrosa* (*fibrosa*-seeded, FS) or *ventricularis* (*ventricularis*-seeded, VS) with hBM-MSCs ($2.0x10^6/cm^2$), previously collected by trypsin–EDTA treatment, centrifuged and resuspended in HEPES-modified DMEM plus 10% FBS, 1% streptomycin–penicillin and 1% L-glutamine. After cell seeding onto these surfaces, *in vitro* engineered constructs were maintained for 30 days at 37 °C in humidified atmosphere, changing the medium every 3 days. Seeded leaflets were subsequently embedded in OCT, snap-frozen in liquid nitrogen and stored at -80 °C. In the second part of experiments, hBM-MSCs were seeded onto hPVLs applying the same procedure as for porcine valve scaffolds.

3.2.5 Tissue analysis and quantitative studies on bioengineered leaflets

Cryostatic sections (6–7 μm) were stained with hematoxylin and eosin, Masson's trichrome (to study ECM composition), and von Kossa (to evaluate any calcium deposits). Adhered hBM-MSCs were analyzed for the expression of any differentiation antigens pertinent to valve cell lineages and stem cell epitopes, using antibodies to: pro-collagen I, collagen I (Sigma), collagen III (Chemicon), elastin (Sigma), vimentin, SM α-actin, smoothelin, MyHC-Apla1, MyHC-Apla2, SM-MyHC, SM22, vWf, human CD31 (Dako), CD133, CD34, CD117, CD105, nestin, GFAP, CD90, Flk1, OCT3/4, SSEA4, osteopontin and osteocalcin. HRP-conjugated anti-mouse and anti-rabbit IgGs (Dako) were used as secondary antibodies. The substrate used to reveal the bound primary antibodies was 3-amino-9-ethylcarbazole (Sigma). Controls were obtained using non-immune IgGs instead of the primary antibodies. Nuclei were counterstained with Harris's hematoxylin (Sigma). Cell counting was performed using double immunofluorescence with FITC-conjugated, anti-SM α-actin (Sigma) and other primary antibodies (vWf and CD31 for ECs; MyHC-$Apla_1$ for fibroblasts; SM22 and SM-MyHC for SMCs; osteopontin, osteocalcin for pro-calcific cytotypes; pro-collagen I and aggrecan [applied to PFA-fixed, chondroitinase-treated sections] for neo-synthesized matrix elements; SSEA4 and OCT4 for stem cells). Primary antibody binding was revealed with AlexaFluor 594-conjugated goat Fab' to mouse and rabbit IgGs (Molecular Probes). Apoptosis and proliferation were analyzed in epifluorescence microscopy using ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon) and anti phospho- Histone H3 antibody (Ser 10; Upstate, Lake Placid, NY), respectively. Attachment, cell spreading and phenotypic expression of colonizing cells were examined in a blinded fashion by randomly choosing four microscopic fields in three sections for each tissue at 400x magnification (corresponding to an area of 0.0374 mm^2).

3.2.7 Transmission electronic microscopy on repopulated pulmonary valve leaflets

Specimens of about 1 mm^3 were fixed with 2.5% formaldehyde–2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, post-fixed with 2% OsO4 in the same buffer, dehydrated with graded

ethanols and embedded in Araldite/Epon. Thin sections were contrasted with uranyl acetate and lead citrate. Observations and photographic records were obtained with a Philips CM12 STEM electron microscope (Philips Export BV, Eindhoven, Holland).

3.2.8 Statistical analysis

Data are expressed as means +SD. Statistical differences between FS and VS, and between pig-VS and human-VS specimens, were determined using Student's paired or unpaired t-tests. Results were considered statistically significant if $p<0.01$.

3.3 Results

3.3.1 Immunophenotypic characterization and conversion potential of hBM-MSCs

hBM-MSCs, collected by density gradient centrifugation and selected for plastic adherence, displayed a typical spindle-shaped morphology *in vitro* (Fig. 1A, panel a) with a tendency to grow in multilayered clusters (Fig. 1A, panel b [inset a]). Cells were characterized by flow cytometry (Fig. 1B) and cytocentrifugation (Table 1 (for a review see at the end of the chapter)), revealing a ''classical'' pattern of ''mesenchymal stemness'', confirmed by a high expression for some characteristic markers, e.g. CD73, CD105, CD29, CD44, CD90. The hematopoietic antigens CD45, CD34 and CD117 were negative or expressed at very low levels by hBM-MSCs. Only a small fraction of the cell preparations was reactive for HLA-DR (one of the trigging elements of immune rejection in cell transplants), while HLA-ABC, which is essential to immune modulation, was shared by a large majority. Other stem cell markers, such as OCT3/4, SSEA4, Nestin, GFAP, NGFr, Flk1 and Stro1, analyzed by immunofluorescence on cytocentrifuged, were also positive. The presence of CD146 is compatible with an endothelial-pericyte commitment and in contrast with the absence of mature EC proteins, i.e. vWf and CD31. An early differentiating SMC profile could also be demonstrated given the weak positivity for the lineage-specificSM22 and SM α-actin, but the most committed smooth muscle proteins SM-MyHC and smoothelin were almost not found. A wide immunodetection of MyHC-Apla1, MyHC-Apla2 and vimentin endorses the description of a typical fibroblastic phenotype. Despite a relevant osteopontin display, the pattern for bone markers was substantially negative for the more mature osteogenic proteins, osteocalcin, osteonectin and tenascin.

Cell treatment with specific inducing media gave rise to different degrees of adipogenic, EC and SMC conversion: there was a roughly 90-fold increase in CD31 immunoexpression (77.56 + 4.78%; Fig.1C, panel a) and vWf enhanced about 6 times (97.61 +1.07%; Fig. 1C, panel b), compared with the untreated condition, while the smooth muscle markers SM α-actin, SM22 and SM-MyHC were augmented in proportions of 20.69+ 5.04, 63.20+ 2.71 and 8.67 +3.09% (i.e. 2.26, 4.10 and 2.75 times the standards, respectively). There was evidence of conversion to osteogenic cells (Fig. 1C, panel f) and fat vesicles were seen in almost all stimulated cells (Fig. 1C, panel g). Taken together, these data

identify the hBM-MSCs employed in this study as multipotent progenitor cells potentially suitable for repopulating decellularized matrices *in vitro*.

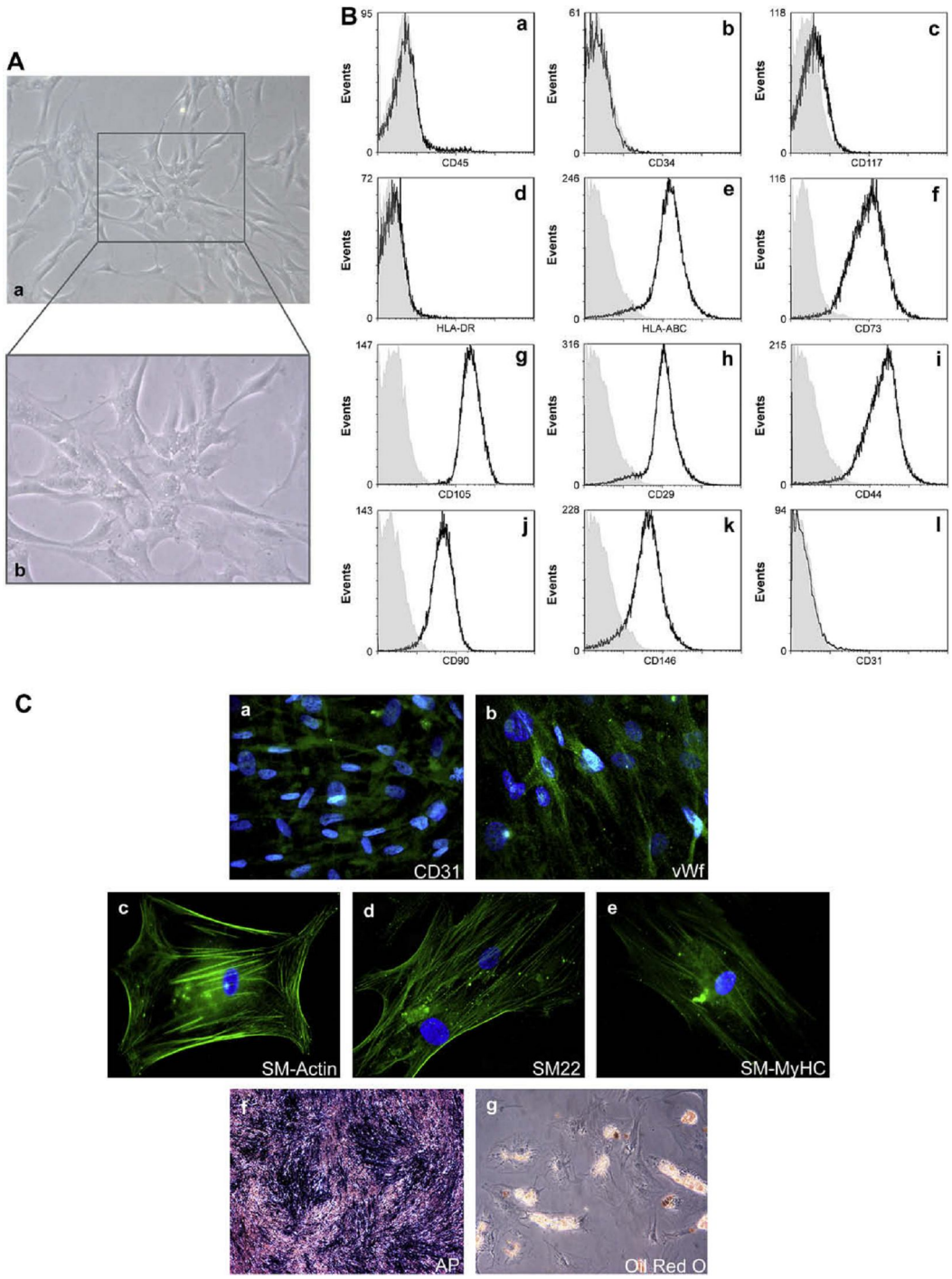


Fig. 1 Pre-seeding characterization of hBM-MSCs. Cells displayed a spindle-shaped morphology (A; panel a; magnification 100x) with multilayer growth (A; panel b; magnification 200x, typical MSC pattern in cytofluorimetric assay (B, panels a–l) and were able to differentiate into endothelial (C, panels a–b; 400x), smooth muscle (C, panels c–e; 400x), osteogenic (C, panel f; 100x) and adipogenic (C, panel g; 100x) cell lineages.

3.3.2 Decellularization and alpha-gal detection

Specimens of p- and hPVLs underwent a double control procedure involving hematoxylin/eosin and Hoechst stainings to reveal nuclear and cell debris. No nuclei or cell fragments were visible under light or fluorescence microscopy (Fig. 2, panels A, B, I for pPVLs and C, D for hPVLs). Carbohydrate

binding protein BSI-B4 and M86 antibody were both used for alpha-gal epitope visualization in porcine samples. Compared to the native condition, where these reagents co-stained Gala1–3Gal (α Gal) glycans (Fig. 2, panels E, F and G), isolectin glycoprotein linked aspecifically to the fibers of decellularized matrices (Fig. 2, panels H and J). Immunofluorescence with M86 (considered a more realistic assay for alpha-gal detection) gave negative results (Fig. 2G). In all samples, a decrease in leaflet thickness was qualitatively appreciable, possibly due to GAG loss of hexosamine and uronate in the *spongiosa* caused by detergent action, as reported in other publications by our group [6].

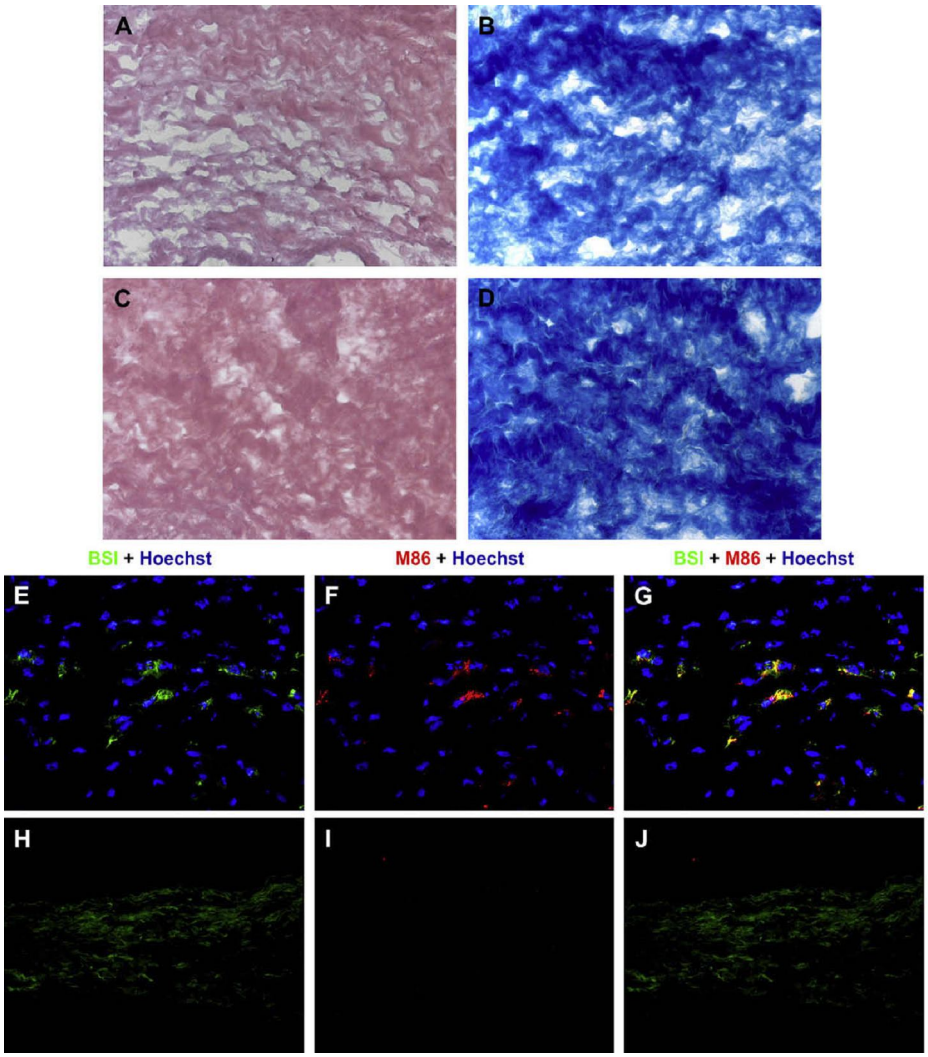


Fig. 2 Decellularization test and alpha-gal detection. Removal of cell debris, both cellular and nuclear, was studied in pig and human leaflet specimens using hematoxylin and eosin and Masson's trichrome (panels A–B for porcine samples and C–D for human ones; magnification 320x). In porcine tissues, alpha-gal epitopes were revealed by double immunofluorescence with BSI-B4 isolectin and M86 antibody; in native conditions, double-positive elements could be appreciated both in the endothelial lining and in some interstitial cells (E–G, magnification 400x); after decellularization, BSI-B4 revealed a specific binding to matrix fibers, while M86 revealed no reactivity (H–J, magnification 400x).

3.3.3 Histological and immunohistochemical evaluation of *in vitro* tissue-engineered leaflets

Fig. 3 shows the qualitative microscopic findings in porcine engineered leaflets after 30 days of static conditioning with hBM-MSCs.

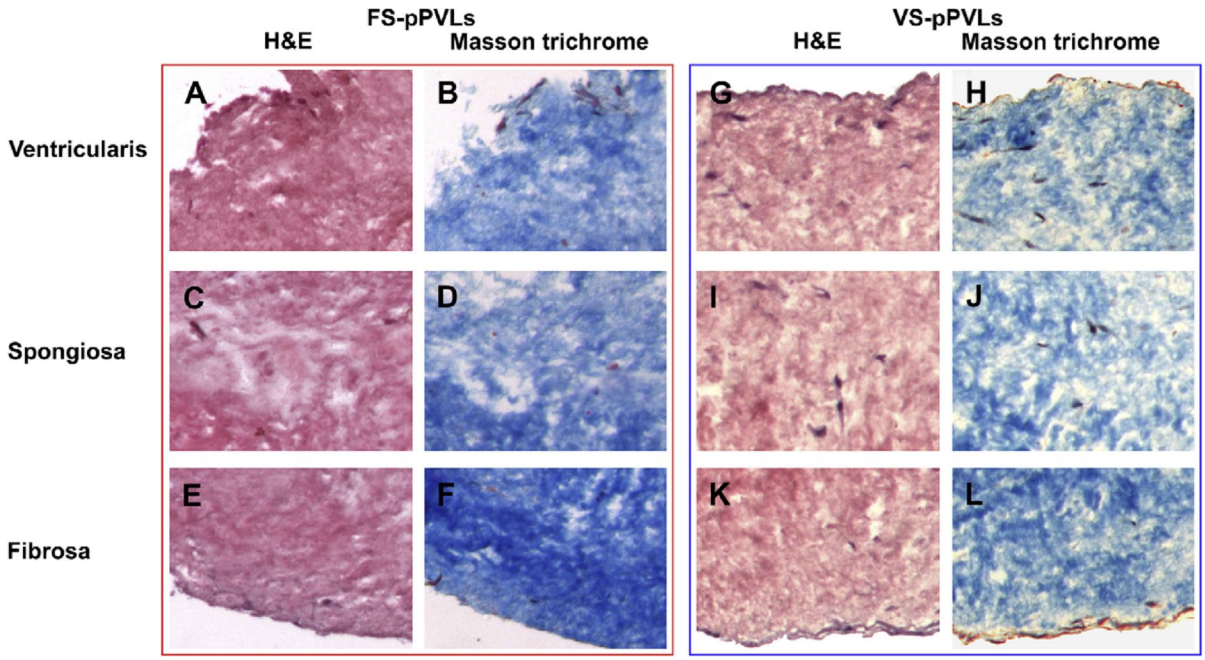


Fig. 3 FS vs. VS procedures. hBM-MSCs, statically seeded on the *ventricularis* layer (blue line), were better able to adhere and spread in the *spongiosa* interstitium than cells on the *fibrosa* one (red line; magnification 320x).

An examination of histological patterns for porcine *fibrosa* or *ventricularis* cell seeding produced slightly different results: while an endothelial-like covering (not always continuous) was achieved in all conditions, cell penetration in the scaffolds was higher in VS samples, resulting in a fairly homogeneous repopulation of the three leaflet layers (Fig. 3, panels A, C and E for FS; G, I and K for VS). Masson's trichrome confirmed these aspects and revealed tissue thickening in the *spongiosa* layer (Fig. 3, panels B, D and F for FS; H, J and L for VS). No signs of calcification could be appreciated in seeded leaflets (von Kossa's staining not shown). Immunohistochemical analysis corroborated the notion that a proper differentiation of hBM-MSCs into valve-specific phenotypes could be achieved in the porcine leaflets, i.e. EC, fibroblast and myofibroblast markers were identified, but there was a very low expression of fully differentiated SMC proteins, such as SM-MyHC.

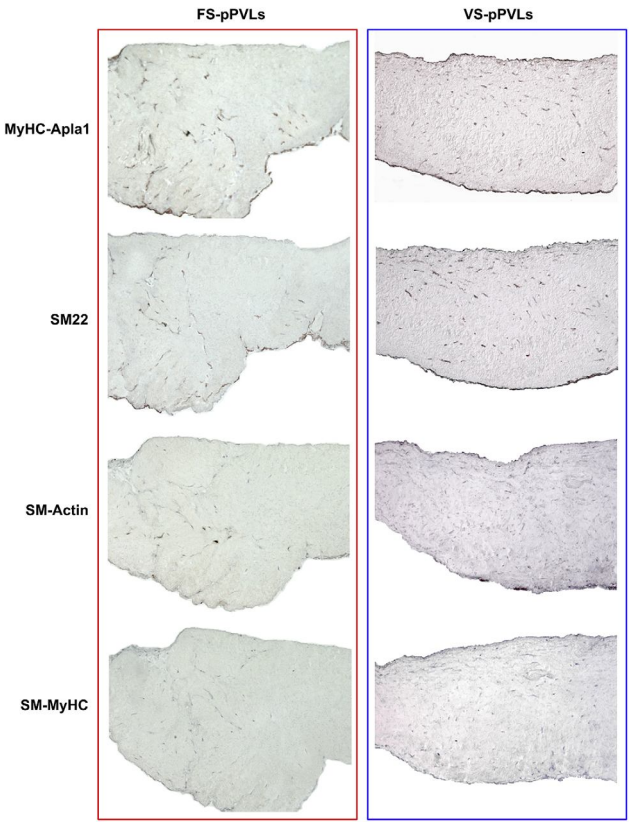


Fig. 4 Immunohistochemical analysis on FS (red line) and VS (blue line) porcine specimens. Contractile proteins were detectable among fibroblasts in both treatments, especially in VS (magnification 65x).

This approach also enabled adhesion, phenotypic distribution, proliferative capacity and apoptotic events that hBM-MSCs can undergo, to be evaluated. Porcine FS demonstrated a lower cell attracting potential than VS counterparts (see Table 2 for a detailed quantitative analysis). Cell spreading, estimated by counting Hoechst$^+$ cells, was greater in VS- than in FS-pPVLs ($p < 0.01$). Differences in phenotypic conversion were noted for the endothelial markers CD31 ($p< 0.01$) and vWf (p = NS). Apart from a MyHCApla1$^+$ fibroblastic phenotype displayed by the vast majority of cells (p =NS), the expression of proteins of smooth muscle lineage, such as SM22, SM α-actin and SM-MyHC, varied: it was significant for the last two, and for VS in particular (Fig. 5, panels A, C, E and G for FS, and B, D, F and H for VS). There was also evidence of a process of matrix element formation involving pro-collagen I and aggrecan cellular synthesis (p = NS; not shown). Importantly, some cells still expressed the stem markers SSEA4 (p =NS) and OCT3/4 ($p < 0.01$). The expression of osteopontin and osteocalcin (marker of calcifying cells) differed between FS and VS, but not to any significant degree. The capacity for survival of hBM-MSCs populating porcine leaflets was also studied. Proliferating, phospho-histone H3$^+$ cells were found in both tissues, but in higher numbers in VS than in FS (p= NS), while the opposite situation was seen among cells embarking on an apoptotic program (p=NS). We decided then to mimic an *in vivo* ''xenogeneic'' vs. ''allogeneic'' setting by testing *in vitro* the potential of hBM-MSC colonization for porcine (fresh) and human (cryopreserved) decellularized VS (Table 2 at the end of the Chapter).

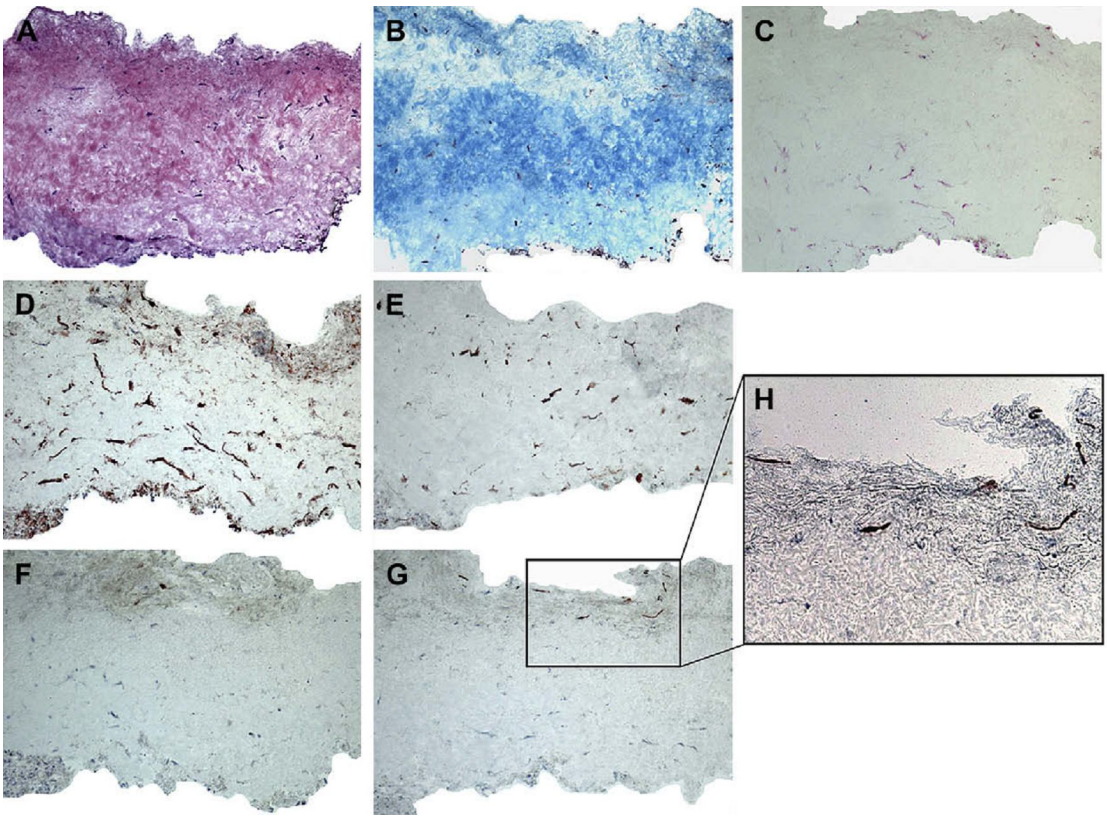


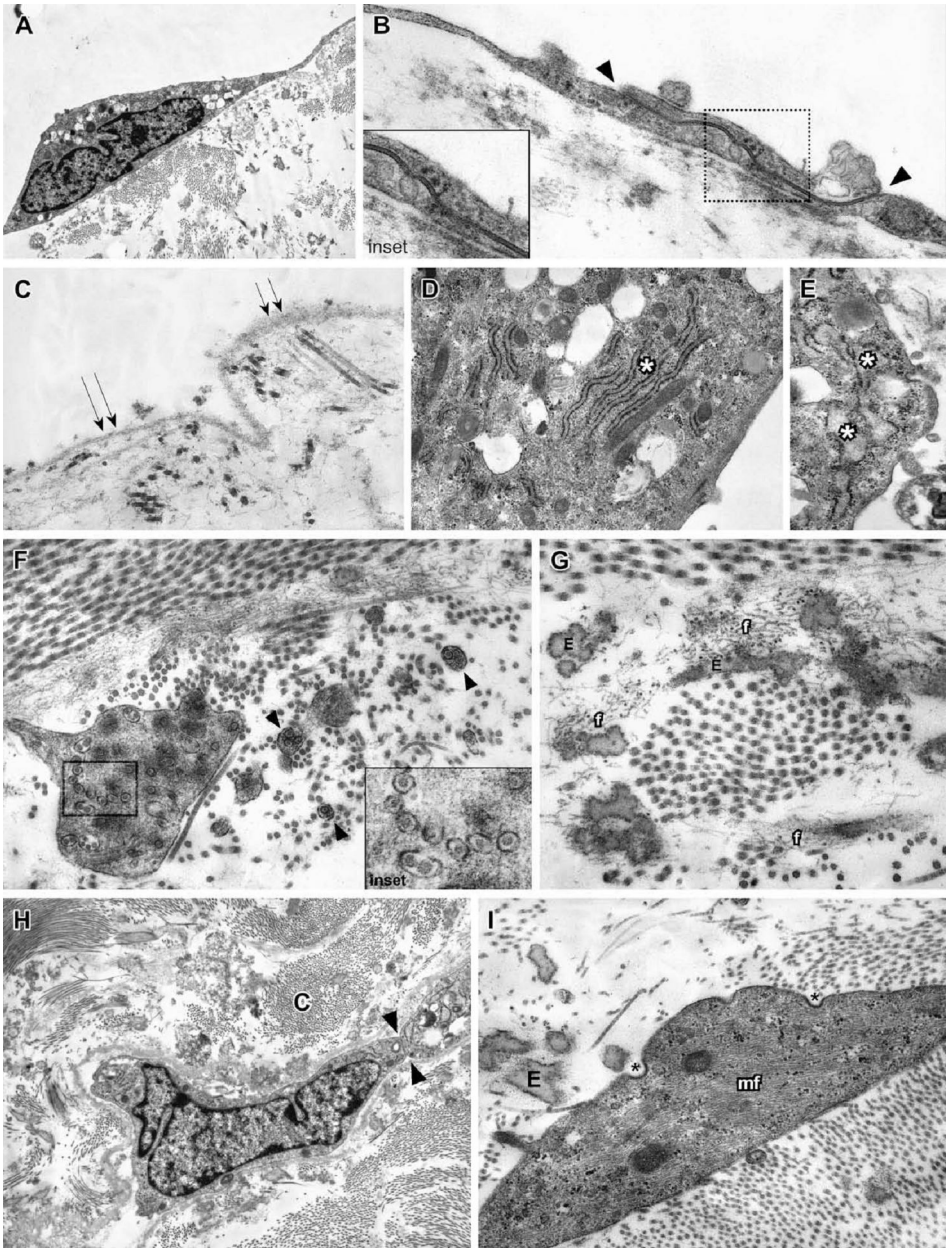
Fig. 5 The ''allogeneic'' setting of engineered cell-to-matrix leaflets. hBM-MSCs were seeded onto the *ventricularis* layer of hPVLs with a good repopulation profile and no calcification events, as seen in serial tissue sections (A–C; magnification 65x). Typical valve markers were analyzed: fibroblast and myofibroblast epitopes were revealed in all three leaflet layers MyHC-Apla1, SM22 and SM-Actin (D–F; magnification 65x). SM-MyHC, expressed by differentiated and mature SMCs, was rightly present in the *ventricularis* amid elastic fibers (G, magnification 65x and in H, magnification 320x).

By comparison with pig VS specimens, the corresponding human ones revealed a greater cell adhesion ($p < 0.01$; see Fig. 5, panels A and B), with no evidence of histopathological changes (Fig. 5C). With regard to the phenotypic conversion, a higher number of cells expressed EC markers, such as von Willebrand factor (p = NS) and CD31 ($p < 0.01$). Fibroblast protein MyHC-Apla1 was well represented by the homotypically-seeded cells (p= NS) (Fig. 5D). Among these fibroblast-like cells, there were contractile elements positive for SM α-actin (p= NS) and SM22 ($p < 0.01$; Fig. 6E and F), with a greater smooth muscle differentiation ($p < 0.01$), as determined from the distribution of SM-MyHC$^+$ cells in the *ventricularis* (Fig. 5, panel G and inset H). Pro-collagen I and aggrecan production did not seem to diverge significantly from the heterotypic VS combination. Osteopontin positive cells increased, while osteocalcin-expressing elements diminished (for both p =NS). The markers of pluripotency, SSEA4 and OCT3/4, persisted in a limited cell number (p= NS). In the ''allogeneic setting'', there was a superior proliferation ($p < 0.01$) accompanied by a lower apoptosis index ($p < 0.01$).

3.3.4 Ultrastructural analysis

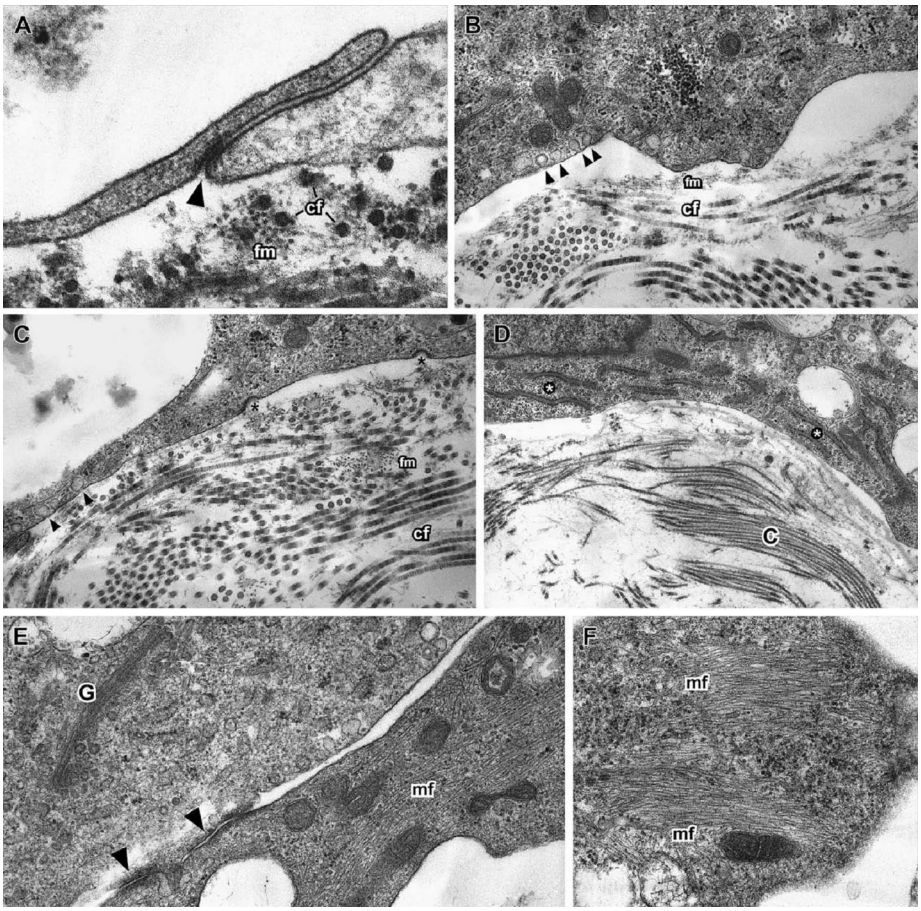
Additional information on the differentiation profile of adhered hBM-MSCs was obtained by analyzing ultrathin sections of engineered valve leaflets. Repopulated PVLs of human origin (from cryopreserved valves; Fig. 6) and from porcine tissue (from fresh specimens; Fig. 7) exhibited well-preserved ECM fibrous components, i.e. collagen fibrils retained their typical D-band pattern, unaffected by the consequences of degradation, such as swelling or shrinkage (Figs. 6A–C, F–I; 7A–D). No clearly distorted/disrupted elastin fibers or fibrillin microfibrils were encountered, while the presence of occasional fragments might correlate with minimal degradation of the fibroelastic valve supporting network. Monolayers of flat endothelium-like cells, lacking in basal lamina, covered ample areas of *ventricularis* and *fibrosa*, (Figs. 6A, B; 7A–D), but lay in close proximity with an amorphous, flocculent layer resembling the basal lamina of the original endothelium cells, possibly retained during valve treatment with detergents. This was more evident when nude leaflet areas were observed (Fig. 6C). In endothelialized regions, most of these cells were interconnected by mature tight junctions (Fig. 6B) or immature cell junctions (Fig. 6A), showing caveolae or clathrin-coated endocytic pits at basal plasma membrane level (Fig. 7B and 7C).

Fig. 6 Ultrastructural analysis on bioengineered hPVLs. (A) Endothelium-like cell adhering to the stromal surface on the *fibrosa* layer; note the retention of preserved collagen fibril bundles. Magnification 3000x. (B) Wide tight junction (arrowheads) between two endothelium-like cells; notice the pentalayered membrane (inset). Magnification 27,000x; inset 43,000x. (C) An acellular tract of *fibrosa*; note coating with a layer resembling basal lamina (double arrows) and inner D-banded collagen fibrils. Magnification 28,000x. (D) RER in a fibroblast-like cell (asterisks). Magnification 35,000x. (E) RER dilated cisternae in a fibroblast-like cell (asterisks). Magnification 80,000x (F) A fibroblast-like cell; observe co-axial collagen fibril-forming channels and cytoplasmic processes. Inset: seven adjacent channels containing one collagen fibril and a two-fibril-containing channel. Magnification 25,000x; inset 48,000x. (G) Electrondense elastin fibers surrounded by fibrillin-microfibrils (f). Magnification 30,000x. (H) Immature intercellular junction between two interstitial cells (arrowheads) within a framework of unaltered collagen fibril bundles (C). Magnification 5000x. (I) Co-oriented bundles of microfilaments (mf) in an elongated interstitial cell; clathrin-coated pits (asterisks). E: an elastin fiber surrounded by fibrillin-microfibrils. Magnification 17,000x. In the following page



Fibroblast-like cells interspersed within the matrix scaffolds were often endowed with abundant rough endoplasmic reticulum (Figs. 6D and 7D), that was frequently composed of dilated cisternae (Fig. 6E), and well-developed Golgi apparatuses (Fig. 7E). In addition, cells engaged in active collagen fibrillogenesis, as shown by the presence of numerous so-called ‘‘fibril-forming channels’’ [21, 22], were also detectable.

Fig. 7 Ultrastructural analysis on engineered pPVLs. (A) Immature intercellular junction (arrowhead) between two endothelium-like cells covering *ventricularis*; cf collagen fibrils; fm: fibrillin microfibrils. Magnification 75,000x. (B) Presence of caveolae (arrowheads) along the basal plasmalemma of an endothelium-like cell; cf D-banded collagen fibrils; fm: fibrillin microfibrils. Magnification 23,000x. (C) Clathrin-coated pits (asterisks) and caveolae (arrowheads) along the basal plasmalemma of an endothelium-like cell; cf D-banded collagen fibrils. Magnification 23,000x. (D) Presence of RER (asterisks) in an interstitial cell; C: collagen fibril bundle. Magnification 10,500x. (E) Two developing intercellular junctions between two interstitial cells, the lower one resembling a smooth muscle cell; G: Golgi apparatus; mf: microfilaments. Magnification 27,000x. (F) Bundles of microfilaments (mf) in an interstitial cell. Magnification 26,000x. In the following page



These extracellular compartments were (1) located within both cell bodies and cell processes, (2) shaped as single fibril-containing narrow channels or wider tubular recesses containing two or more fibrils, and (3) coaxially arranged (Fig. 6F). Early, electrondense elastin fibers surrounded by fibrillin-microfibrils were also observed (Fig. 6G). In the cytoplasm of other fibroblast-like cells, thin bundles of microfilaments and immature intercellular junctions (Fig. 6H), reminiscent of ongoing myofibroblast differentiation, were also visible. Finally, several interstitium-populating cells were characterized by an elongated shape and revealed developing intercellular junctions and clathrin-coated pits as well as abundant bundles of co-oriented microfilaments and mitochondria (Figs. 6G; 7E and 7F), thus resembling nearly mature SMCs, even if short basal lamina tracts were only detectable on their surfaces. On the whole, these ultrastructural data are consistent with the immunohistochemical findings, i.e. the phenotypic potential of hBM-MSCs for fibro-myofibroblast, EC- and SMC-like cell transitions.

Table 1
Immunophenotypic characterization of hBM-MSCs as determined by flow cytometry and on cytocentrifuged cells.

hBM-MSCs before static seeding: flow cytometry and cytocentrifugation		
Antigen	Percentage of positive cells	
CD45	0.39 ± 0.55	/
CD34	0.93 ± 0.44	Negative
CD117	3.06 ± 0.33	Negative
CD133	/	Negative
HLA-DR	1.37 ± 0.10	/
HLA-ABC	91.42 ± 0.19	88.21 ± 0.69
MHC II	/	Negative
CD73	98.03 ± 0.23	/
CD105	99.74 ± 0.34	/
CD29	95.74 ± 5.49	95.93 ± 3.57
CD44	93.33 ± 3.92	/
CD90	95.94 ± 0.28	90.63 ± 2.44
Stro1	/	22.66 ± 6.64
GFAP	/	16.38 ± 1.96
Nestin	/	27.14 ± 3.52
NGFr	/	31.78 ± 1.36
SSEA4	/	26.44 ± 16.39
OCT3/4	/	32.18 ± 3.68
CD146	76.74 ± 11.14	/
CD54	/	40.30 ± 4.33
Flk1	/	27.58 ± 1.58
Vimentin	/	90.74 ± 8.49
SM α−actin	/	9.14 ± 0.83
MyHC-Apla2	/	30.79 ± 2.81
MyHC-Apla1	/	40.32 ± 6.85
SM22	/	15.43 ± 2.04
SM-MyHC	/	3.15 ± 0.28
Smoothelin	/	Negative
CD31	0.67 ± 0.29	/
vWf	/	16.25 ± 2.73
Pro-collagen I	/	38.88 ± 7.96
Aggrecan	/	31.65 ± 5.47
Osteopontin	/	33.10 ± 1.11
Osteocalcin	/	Negative
Osteonectin	/	Negative
Tenascin	/	Negative

Values (means ± SD, in %) were obtained from three different sets of experiments. /=not tested.

Table 2
Quantitative analysis on *in vitro* tissue-engineered heart valve leaflets.

hBM-MSCs statically seeded onto p- and hPVLs: Repopulation, valve cell phenotypic conversion and capacity for survival after 30 days					
	FS-pPVLs	***VS-pPVLs***	***VS-hPVLs***	***p1***	***p2***
Adhered cells					
Total cell nuclei	473.11 ± 46.13	622.40 ± 43.05	768.72 ± 47.45	$p < 0.01$	$p < 0.01$
Differentiating cells					
EC markers					
CD31	204.99 ± 24.72	285.20 ± 15.44	356.51 ± 15.68	$p < 0.01$	$p < 0.01$
vWf	222.82 ± 23.46	276.29 ± 18.38	329.77 ± 30.61	NS	NS
Fibroblast marker					
MyHC-A_{pla1}	463.46 ± 5.82	525.85 ± 46.73	570.41 ± 38.44	NS	NS
SMC proteins					
SM α-actin	134.43 ± 39.23	197.56 ± 33.74	190.88 ± 42.06	$p < 0.01$	NS
SM22	169.34 ± 28.20	204.99 ± 38.63	338.68 ± 24.26	NS	$p < 0.01$
SM-MyHC	–	49.91 ± 4.08	124.78 ± 30.87	$p < 0.01$	$p < 0.01$
ECM synthesis					
Pro-collagen I	212.82 ± 45.92	285.20 ± 12.23	231.73 ± 27.20	NS	NS
Aggrecan	285.20 ± 6.88	311.94 ± 40.31	309.98 ± 14.90	NS	NS
Stem cell markers					
SSEA4	62.37 ± 32.02	142.60 ± 19.98	178.25 ± 33.62	NS	NS
OCT3/4	17.83 ± 6.17	37.43 ± 5.35	50.80 ± 14.15	$p < 0.01$	NS
Other cell components					
Osteopontin	338.68 ± 15.37	347.59 ± 46.31	383.24 ± 40.84	NS	NS
Osteocalcin	17.63 ± 6.73	11.39 ± 2.67	6.48 ± 3.17	NS	NS
Proliferating cells					
Phospho-Histone H3	97.06 ± 25.94	150.53 ± 17.38	190.91 ± 25.67	NS	$p < 0.01$
Apoptotic cells					
Apoptag	86.90 ± 18.98	60.16 ± 11.50	43.58 ± 10.96	NS	$p < 0.01$

For each parameter, values represent the number of positive cells per mm^2, expressed as mean + SD. ***p1*** and ***p2*** refer to p values between ***FS-*** and ***VS- pPVLs*** and ***VS- pPVLs*** and ***VS- hPVLs***, respectively. – = negative; NS = not significant.

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- *ISACB 11th Biennial Meeting* – Bordeaux, France, 17-20 September 2008:

Iop L, Renier V, Naso F, Piccoli M, Gandaglia A, Pozzobon M, Marchini M, Spina M, De Coppi P, Sartore S, Gerosa G. *In vitro* Tissue Engineering of Decellularized Leaflets with Human Mesenchymal Stem Cells: the influence of matrix fiber anisotropy on cell engraftment. (Poster presentation)

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Chapter 4

In vivo Allogeneic Heart Valve Tissue Guided Regeneration

An alternative approach to heart valve tissue engineering

4.1 Introduction

The capability to instruct cells to a correct differentiated function is for sure a prerogative of extracellular matrix, which plays an important role for their integration by providing specific signals to acquire the proper cell phenotype. In the classical tissue engineering vision, once eliminated the allogeneic or xenogeneic cell component through a decellularizing treatment, it is possible to employ the fiber mesh as a template for the repopulation with human stem cells in prospective to create autologous-like substitutes. Another working hypothesis to obtain valvular devices is likely given by the organ regeneration guided by the extracellular matrix, i.e. the implant of naked natural scaffolds that *in vivo* are able to recruit recipient's cells for the reconstruction of the tissue. Prospectively positive aspects associated with this approach should be identified in the possibility to skip the *in vitro* steps of cell seeding and chemo-mechanical conditioning, conceiving the animal as a natural bioreactor. Moreover this method should offer the opportunity to create tissue banks, from which to draw at the moment of clinical need.

It is certainly from this foundation that investigation on the field has particularly increased in the latest years. Among the first experimental evidences, biomaterials have been successfully introduced as pure patch of collagen I even in the therapy of ischemic myocardium: once applied to the interested tissue, the collagen sponge is able to recruit progenitors and less undifferentiated cells which in turn or alone are able to fully colonize it and start a cardiovascular transdifferentiation [1]. And before, it is noteworthy to remember that these patches, either synthetically obtained or cell-purified from biological tissues, have been used as haemostatic or for skin reconstruction with excellent results [2]. A further surprising element for a positive consideration of this approach has been given by Campbell and Colleagues, who were able to obtain a tubular cell construct by implanting a biomaterial tube in an animal peritoneal cavity: the newly formed tissue, pulled from the tube, had the anatomical and histological appearances of a quite mature blood vessel and therefore it could be considered for sure an optimal vascular substitute [3]. An aspect to highlight for all these approaches, as previously mentioned, is the use of the body as natural bioreactor, providing a real physiological conditioning able to allow the maturation of the interested tissue and by-passing the difficultly controllable procedure of cell-seeding and chemo-mechanical stimulation *in vitro*.

Actually, applications of this research concept in the heart valve field are still limited. Interestingly, the Japanese group of Nakayama et al. has produced a trileaflet valved-shaped construct based on 'in-body tissue architecture technology'. The approach exploits, as in the case of Campbell et al., the biological response of the body to a foreign material, by implanting silicon rods in the subcutaneous tissues. In a period of 4 weeks, these grafts are first 'embedded' by granulation-like tissue, then removed and fuse each other for correct valvular shaping and reinserted to complete the cell covering. At the end of the process, the particular arrangement given by the researchers allows to

eliminate the artificial materials used and to attain a trileaflet valve conduit [4]. A real approach of tissue guided regeneration in orthotopic position is furnished by the extensive work of Konertz and Colleagues, who, moving from the classical paradigm of tissue engineering, compared the two methods using an allogeneic decellularized valve for the reconstruction of the right ventricular outflow tract in sheep and ascertained there was no need to seed the scaffolds prior to implantation, seen the good repopulation up to six months [5]. As further step, they developed a xenogeneic model, both pig-to-sheep and even pig-to-human again to substitute the autologous pulmonary valve transferred in aortic position during Ross intervention [6]. In 2007, they published the medium-term results of the RVOT reconstruction with decellularized allografts in Ross aortic valve substitution in 68 patients. In particular, they evaluated the *in vivo* hemodynamics and *ex vivo* tissue analysis of allogeneic scaffolds, decellularized either with deoxycholic acid or sodium dodecyl sulphate methods, where the second one was judged to lower late gradients after 12 months [7]. Up to 4 years they observed very low mortality (1, 4 %), a good valve function, fully comparable with the cryopreserved allografts used as control, and a progressive engrafting –even if discontinuous. Recently, the same group published the results of decellularized heterografts and cryopreserved homografts in the sheep model up to nearly ten months: in the comparison, the first appeared to better repopulate with fibroblastic- and endothelial-like cells differently from the less recellularized homografts [8]. Decellularized allogeneic cusps were used by the same Authors for the correction of Fallot's tetralogy in pediatric patients [9]. Another group compared the performances of valve homografts, either cryopreserved and/or decellularized, verifying a reduced calcification tendency in the sheep transplanted with decellularized matrices [10]. In another work corroborating the feasibility of this approach, two groups of heart valves, cryopreserved or decellularized and treated with an anti-calcinosis devitalisation (digitonin and ethylenediaminetetraacetic acid), were tested in dogs by substituting an aorta fragment with the non-coronary sinus of the cusp allografts: the Authors stated the lack of immunologic infiltration in the decellularized inserted specimens in contrast to the cryopreserved ones, even if in both cases no engrafting of recipient 's cells was observed [11]. At the same time, however, a few reports indicate an 'unexpected failure' of decellularized graft between the right ventricle and the pulmonary artery [12].

Nevertheless, for a general overview, it is necessary to move back from a pre-clinical evaluation of this regenerative approach to some bench tests and to early *in vivo* animal models, by focusing on the biocompatibility characteristics of the newly produced biological materials. Decellularization has been thought to eliminate all the antigenic epitopes due to the complete cell removal, but it is not improbable that the used process might be able to provoke an exposure of previously masked epitopes or even induce micromodifications in the matrix fibers, recognized as non-self by the immune system (see Chapter 2, *Immunogenicity of xenogeneic cells and extracellular matrices*). A recent paper by Zhou and Coll. discussed upon the effects of different decellularizing treatments on

ECM preservation, thrombogenicity and immunogenicity, by testing the last ones with an *in vitro* direct contact with human blood. While sodium deoxycholate-based method induced no ECM disruption and complete decellularization, to all the proposed methods, including also the use of sodium dodecyl-sulphate, trypsin/EDTA or trypsin-detergent-nuclease, the thrombogenic and immunological response was surely higher than to the glutaraldehyde-fixed specimens [13]. A confirm to these observations is supported by another work published in the same year: through a quantitative approach based on immunoblotting technique, Arai and Orton were able to demonstrate the detection of soluble protein antigen still maintained in bovine pericardium and porcine heart valves after decellularization with sodium dodecyl-sulphate and sodium deoxycholate [14].

Even in our experience, we observed a minute, but present response to decellularized aortic roots syngenically transplanted in the peritoneum cavity of immunocompetent rat, hypothesizing as responsible of these events the induction of fine molecular ECM alterations by the decellularizing treatment (paper in preparation).

4.2 Materials and methods

TRICOL decellularized porcine α-Gal negative aortic roots were implanted in pulmonary position in 4 Vietnamese pigs (age 8-12 months) in a long-term animal model up to 14 months. Control animals received the same interventional treatment, but no substitution of their right ventricular outflow tract occurred with its resection-reimplantation. Valve function was monitored through a two-dimension echocardiography at 15 days and then monthly until animal sacrifice. Explanted roots were subdivided in three parts, each composed by the chimerical vessel wall (recipient's native pulmonary artery and donor's decellularized aorta) with the valve sinus and the respective leaflet (each belonging to the implanted aortic root) in order to analyse the tissues with different magnifications techniques using both light and electron transmission microscopy.

In this Chapter, light microscopy analysis on explants will be considered.

4.2.1 Tissue and cell analysis

Different specimens were harvested both near the suture zone and the sinus level of the implanted vessel wall. These samples and an half of the leaflet underwent OCT inclusion and cryocooling for further histological and immunohistochemical tissue analysis.

Haematoxylin-eosin, Masson's Trichrome and Von Kossa were performed on 7-μm serial cryosections using standard protocols. Indirect immunohistochemistry had been applied to research the following epitopes expressed by:

Calcification aspects		Osteopontin, OP (Abcam, Cambridge, UK)
		Osteocalcin, OC (Abcam, Cambridge, UK)
		Osteonectin (Developmental Studies Hybridoma Bank, Iowa)
Inflammatory and immunological response		Pig CD45 (VMRD Inc., Pulmann, WA, USA)
		Pig T-cells (VMRD Inc., Pulmann, WA, USA)
		B-cells (Abcam, Cambridge, UK)
		MHC II (VMRD Inc., Pulmann, WA, USA)
		Natural Killers, NK (Abcam, Cambridge, UK)
		Mast Cells (Abcam, Cambridge, UK)
Differentiated cells		MyHC-Apla1 (type A non-muscle myosin heavy chains; **)
		Vimentin (clone V9, Dako, Dakopatts, Denmark)
		Smooth muscle Actin, SM-Actin (clone 1A4, Sigma, St.Louis,MO)
		Smoothelin (Abcam, Cambridge, UK)
		Calponin (**)
		Desmin (Sigma, St. Louis, MO)
		Von Willebrand Factor (Dako, Dakopatts, Denmark)
ECM preservation and neosynthesis		Collagen I (clone Col1; Sigma St. Louis, MO)
		Procollagen I (clone M38; Developmental Studies Hybridoma Bank, Iowa)
		Elastin (Sigma St. Louis, MO)
Stem cells	***Embryonic***	SSEA4 (Chemicon, Temecula, CA)
		OCT4 (Santa Cruz, Santa Cruz, CA)
	Haematopoietic	CD34 (Dako, Dakopatts, Denmark)
	Neural	Nerve Growth Factor, NGFr (Abcam, Cambridge, UK)
		Nestin (Abcam, Cambridge, UK)
		Glial fibrillary acidic protein, GFAP (Chemicon,Temecula, CA)
	Mesenchymal	CD29 (Integrin β1; VMRD Inc., Pulmann, WA, USA)
		CD44 (Homing receptor; VMRD Inc., Pulmann, WA, USA)
		CD105 (Endoglin; Abcam, Cambridge, UK)

(**) Sartore S, Franch R, Roelofs M, Chiavegato A. Molecular and cellular phenotypes and their regulation in smooth muscle. Rev Physiol Biochem Pharmacol 1999;134:235–320)

In addition to these antibodies, a specific marker for cell proliferation was used (Phospho-Histone 3; P-Histone3, Ser 10; Upstate, Lake Placid, NY). Primary antibody binding was revealed through HRP-conjugated anti-mouse and anti-rabbit IgGs (Dako) as secondary antibodies. Controls were performed with non-immune IgGs instead of the primary antibodies. The substrate revealing the affinity antibody reaction was 3-amino-9-ethylcarbazole (Sigma). Nuclear counterstaining was obtained with Harris's haematoxylin (Sigma). Antigen distribution was evaluated using a Leica light microscope and image acquisition was obtained with a Leica DC300 digital videocamera (Leica).

Primary cultures of valvular interstitial cells, adventitial and medial cells were also obtained from the leaflet remaining part and the central vessel region between the suture and sinus zones. For this purpose, *adventitia* was carefully stripped from the vessel, minced and cultured in complete medium containing α-MEM (Sigma), 20% FBS (Gibco, Carlsbad, CA), 1% L-Glutamine and 1% Penicillin-Streptomycin (Sigma). Leaflet surfaces and vessel lumen were scraped to remove the endothelium, minced in fragments of about 2-3 mm^3, incubated with a sterile-filtered digestion solution

composed of collagenase I (125 units/ml; Sigma), elastase (8 units/ml; Fluka) and soybean trypsin inhibitor (0,375mg/ml; Sigma) for 30 minutes at 37°C. Fragments were collected in an OPTILUX non tissue culture Petri dish (Falcon BD Bioscience, San Diego, CA) and cultured in the complete medium described before. Cells spread from pre-digested fragments after 7-10 days. Cell morphology was studied using a phase contrast Leica DM IRB microscope (Leica, Wetzlar, Germany) connected to a Canon Power Shot S40 camera. After first passaging, cells were plated onto coverslips and analysed immunohistochemically after fixation in 2% p-formaldehyde in PBS pH 7.2. A double immunofluorescence was performed on the coverslips by incubating with FITC-conjugated SM-Actin (clone 1A4; Sigma) and the following antibodies directed versus Pig CD45, MyHC-apla1, Smoothelin, Calponin, Smooth muscle Myosin (SM-Myosin; [15]), OP, OC, $SSEA_4$, OCT_4, β-1 integrin (CD29). The secondary AlexaFluor594-conjugated goat Fab' to mouse IgGs (Molecular Probes, Carlsbad, CA) and anti-rabbit TRITC conjugated goat IgGs (Chemicon) were used as secondary antibodies. Cells nuclei were identified by Hoechst staining (Hoechst 33258; Sigma). Distribution of antigens was studied using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and images were acquired by using a Leica DC300F digital videocamera (Leica, Wetzlar, Germany).

4.3 Results

4.3.1 Tissue and cell characteristics of the regenerated roots

Ultrasound scanning revealed that leaflet motility of the implanted root was quite preserved although a thickness increase could be appreciated in respect to control animals. Doppler analysis identified a progressively decreasing transvalvular turbulent flow, whose peak gradient reached 15–25 mmHg (with a range mean gradient of 9–16 mm Hg) at 14 months from implant, versus 1,87-2,05 mmHg; range peak gradient: 3,45-4,05 mmHg for the controls.

Ex vivo analysis of the explanted tissues documented a started repopulation process, but almost the major part of the medial layers of the vessel scaffolds appeared to be still acellular (Fig. 1).

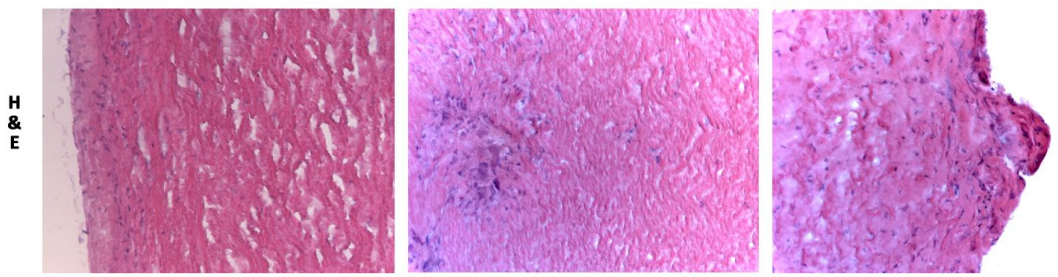


Fig. 1 Classical histology of an *in vivo* regenerated vessel wall at 14 months of implantation. As for the following images, the three panels correspond respectively to the *intima*, *media* and *adventitia* layers. Cell infiltration occurred mostly in *intima* and *adventitia*, whereas *media* appeared recellularized only in some areas. Note the mild *intima*l thickening. Magnification: 200x.

A slight *intima*l thickening was commonly observed in all specimens. Some microcalcifications could be visualized along the elastic fibres of the non-repopulated *media* above all in the areas nearer the suture points. Cell-associated calcific events could be detected rarely (Fig. 2) and in fact the positivity for osteocalcin was minute and confined to the *media*.

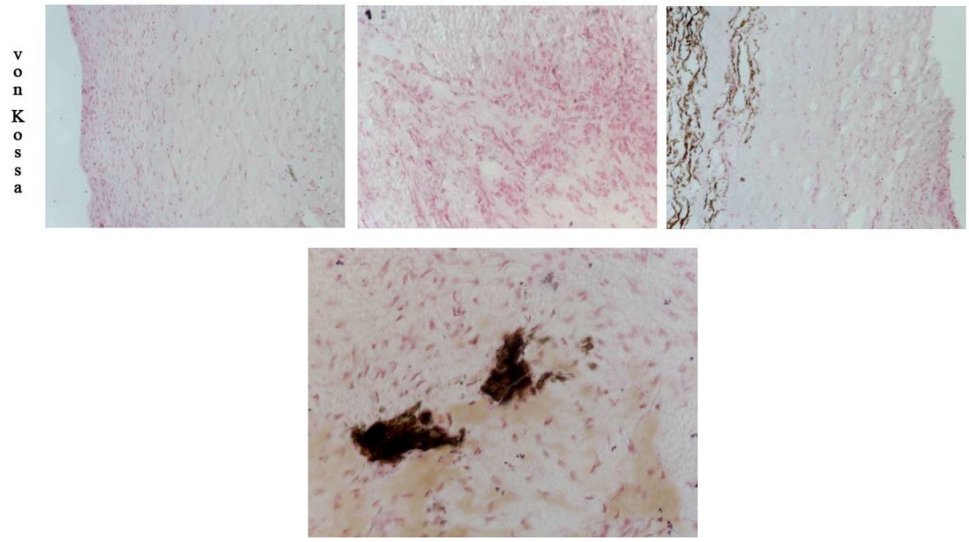


Fig. 2 Von Kossa staining. Extensive calcifications were not encountered. In some specimens, the deposition of hydroxyapatite-like structures verified along the medial-adventitial border where elastic fibres were not covered by engrafting cells and act as nucleation site, probably for their sticky activity (Magnification: 100x). Cell-driven microcalcifications could also be appreciated mainly in the repopulated *media* regions near the anastomosis in between the host pulmonary artery and the implanted root (Magnification: 200x). A probable explanation of this phenomenon is the proximity to the suture points where often fibrotic degeneration might occur.

At 14 months, the inflammatory response seemed to be limited only to the regions newly vascularized both in *media* and in *adventitia* (not shown).

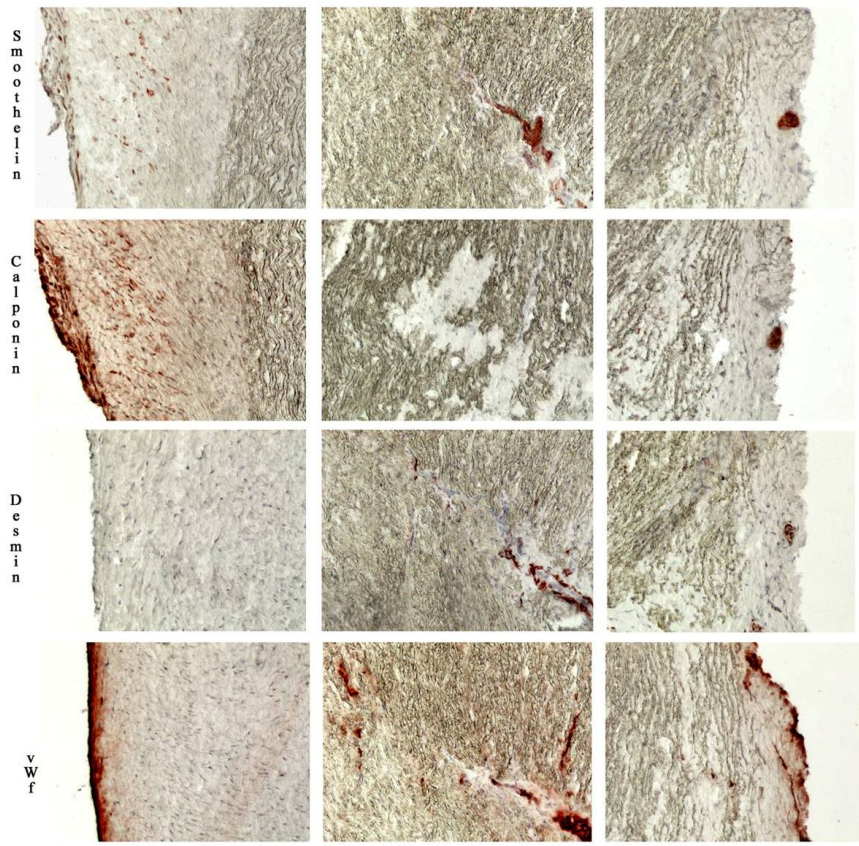


Fig. 3 Evaluation of the expression of typical smooth muscle proteins (smoothelin, calponin and desmin) and endothelial factor (von Willebrand Factor).
New fully mature arteriolar and capillary vessels could be observed both in *adventitia* and in *media* underlying an active angiogenic and arteriogenic process, from which the regenerated tissue could gain nutrition and oxigenation.
Smooth muscle cells were rarely present in repopulated *mediae*.
Magnification: 200x

The event of neovascularization was particularly important in these samples and tended to acquire a fully mature phenotype, seen the expression of mature markers of the smooth muscle, as smoothelin, calponin and desmin. *Vasa vasorum*-like vessels could be observed in addition to capillaries positive for the endothelial von Willebrand factor (Fig. 3).

However, there was rare positivity for smooth muscle elements in the *media* as expected and

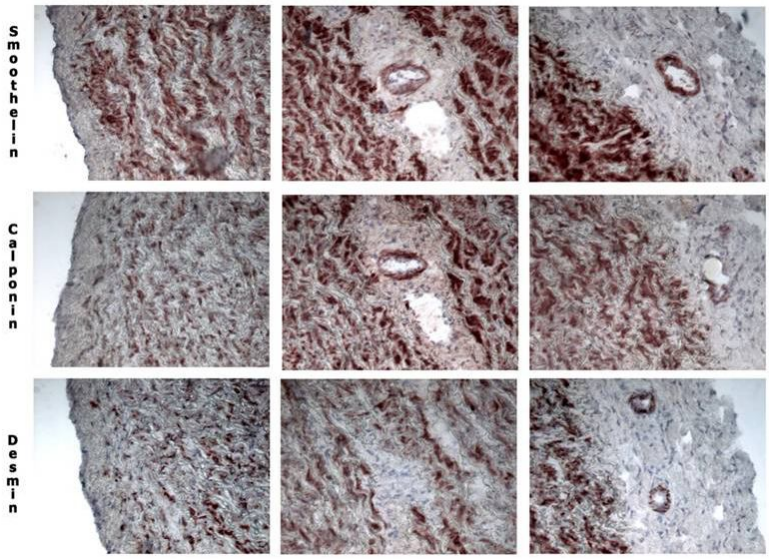


Fig. 4 Evaluation of the smooth muscle distribution in a pulmonary wall of a control animal. Mature smooth muscle elements can be found both as medial fibres and as constituents of the arteriolar wall of the *vasa vasorum.* Magnification: 200x

observable in control specimens (Fig. 4), while most of the colonizing cells expressed fibroblastic markers, as vimentin and non-muscle myosin, often acquiring a contractile phenotype for sm-actin positivity (Fig. 5).

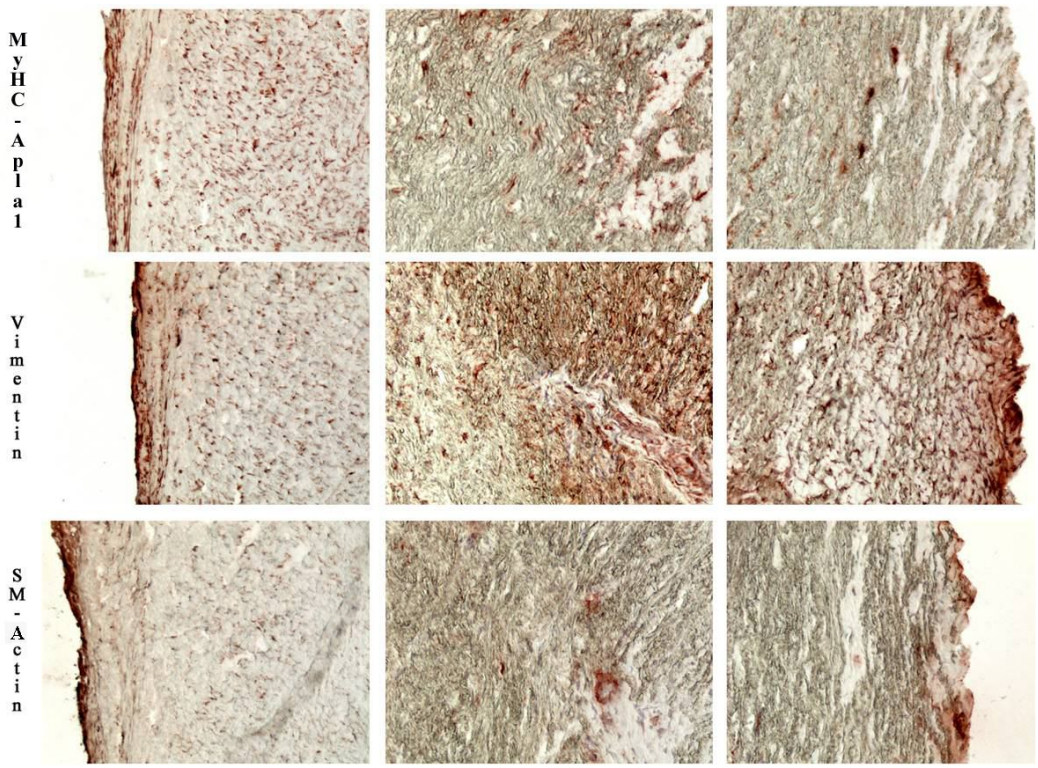


Fig. 5 Fibroblastic phenotype of the cells engrafting the regenerated wall. Vimentin and platelet myosin were positive for all repopulating cells. A subset of fibroblasts expressed smooth muscle actin, leading to hypothesize a conversion to a more differentiated phenotype. Magnification: 200x

While the donor's matrix appeared maintaining a well preserved fibre distribution, cells populating the entire wall seemed particular active in the synthesis of new collagen elements (Fig. 6).

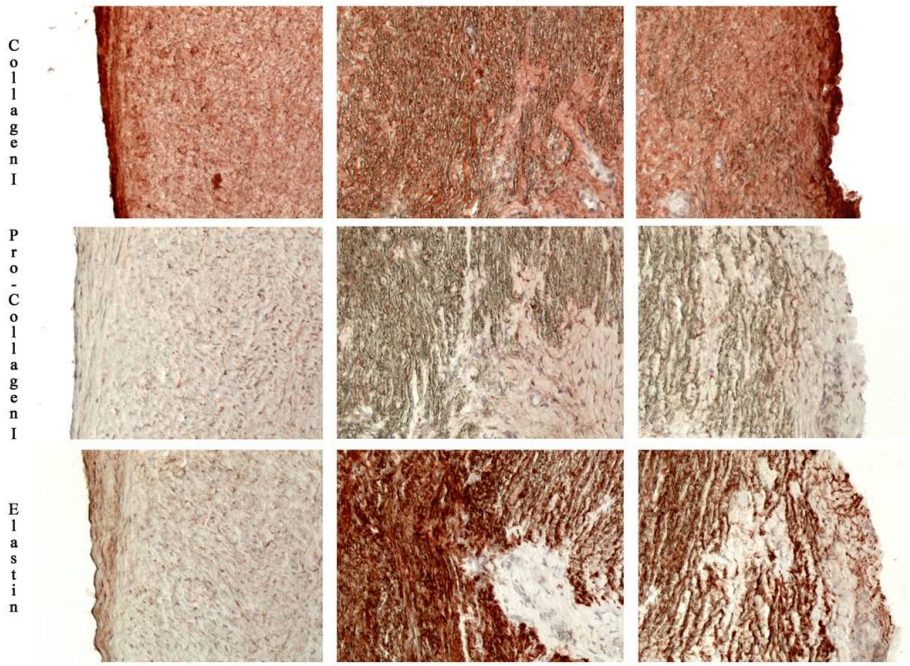


Fig. 6 ECM preservation and neosynthesis. Collagen I and elastin seem to be well preserved, as also confirmed by ultrastructural analysis (data not shown). The synthetical activity of collagen production was highly represented in the regenerated wall, as demonstrated by the positivity for procollagen I in engrafting fibroblasts (Magnification: 200x). Transmission electronic microscopic observations consistently confirmed this pattern and moreover underlined an active synthesis of elastin through the visualization of immature eulaninic fibres (data not shown).

Stem marker expressing cells were found scattered in the whole wall, indicating an engrafting process, probably played by circulating progenitors. Expression of the embryonic SSEA4 and OCT3/4, as like as of the mesenchymal homing receptor CD44 (receptor for the chemokine SDF-1) and β-1 integrin CD29 could be frequently verified among the more differentiated fibroblastic colonizing cells (Fig. 7).

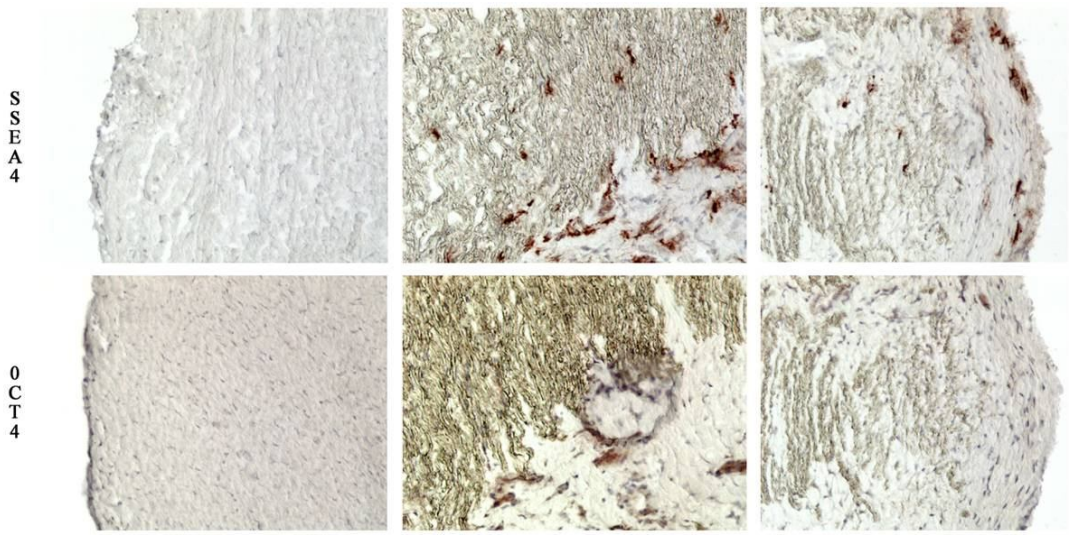


Fig. 7 Stem cell pattern of regenerated walls. A discrete expression of embryonic stem cell markers could be evaluated in the medial and adventitial layers, evoking a still active engraftment process at 12-14 months from the implant. Magnification: 200x

Repopulating cells demonstrated also an evident proliferative activity (Fig. 8).

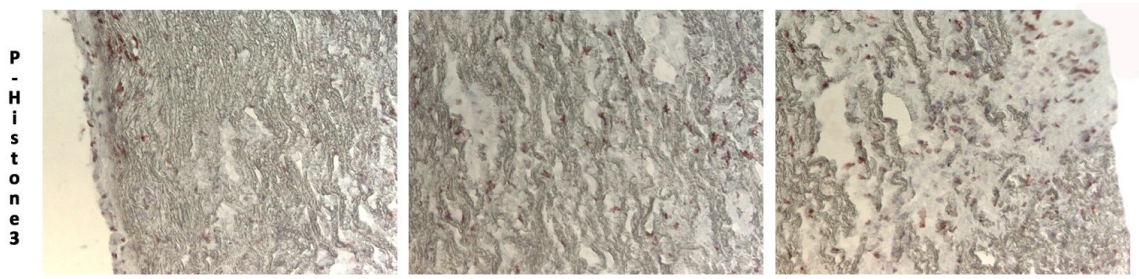


Fig. 8 Proliferative state of the colonizing cells. The large majority of cells present in the intimal, medial and adventitial *tunicae* expressed at the explant-time point a high expression of phospho-histone 3, used in the experimental analysis as indicator of proliferation. Magnification: 200x

Even the explanted leaflets revealed a tendency to cell repopulation, but the observations could not always be univocal as for the walls. The more cellular zones were proximal to the vessel *annulus* (Fig. 9). Von Kossa positivity was rarely detected in the cusp stroma (Fig. 9), however in one case a strong osteocalcin could be observed (data not shown).

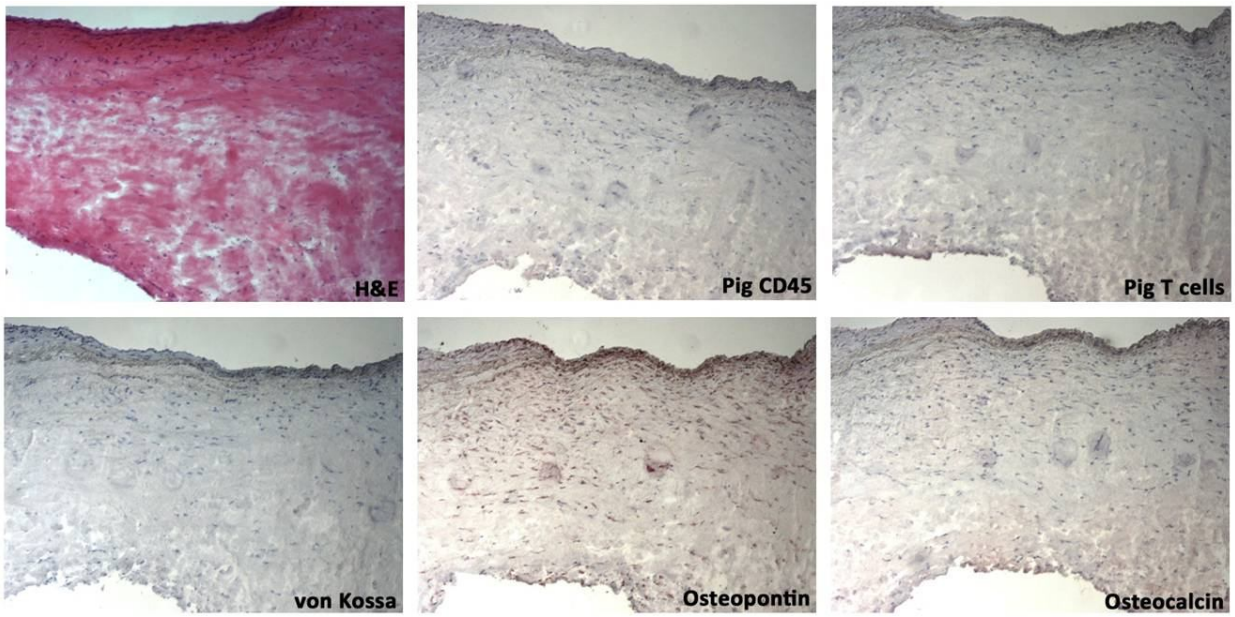


Fig. 9 General characteristics of the regenerated valve leaflet. In this sample, a good repopulation could be observed along the leaflet with no adverse events, both of inflammatory and calcific nature (Magnification: 70x). These statements could not be ascribed however to all the evaluated cusps especially in terms of engrafting index and inflammatory response, due to infection causes.

The proliferation rate is particularly high even in the leaflet specimens and many cells expressing the proliferative marker were also positive for the stem cell mesenchymal integrin CD29 (Fig. 10).

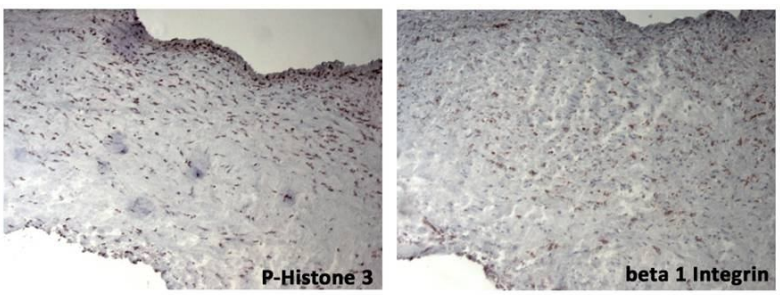


Fig. 10 Phospho-histone 3 and beta-1 integrin expressions. The positivity for the proliferative and mesenchymal stem cell markers tend to overlap in the regenerated leaflets. Magnification:70x.

In an animal, part of the projection was interested by an infiltration of cells with quite large dimensions, resembling macrophages and possibly evoking a response to a bacterial infection.

Primary cultures obtained from the *adventitia* and the *media*, as like as from the cusp, revealed two main phenotypes, which could be clearly identified in the same culture for the medial layer. These morphological and immunophenotypical cytotypes presented a strict similarity to immature smooth muscle-like or to bone marrow mesenchymal stem cell-like porcine populations, as visible in Figs. 11 and 12.

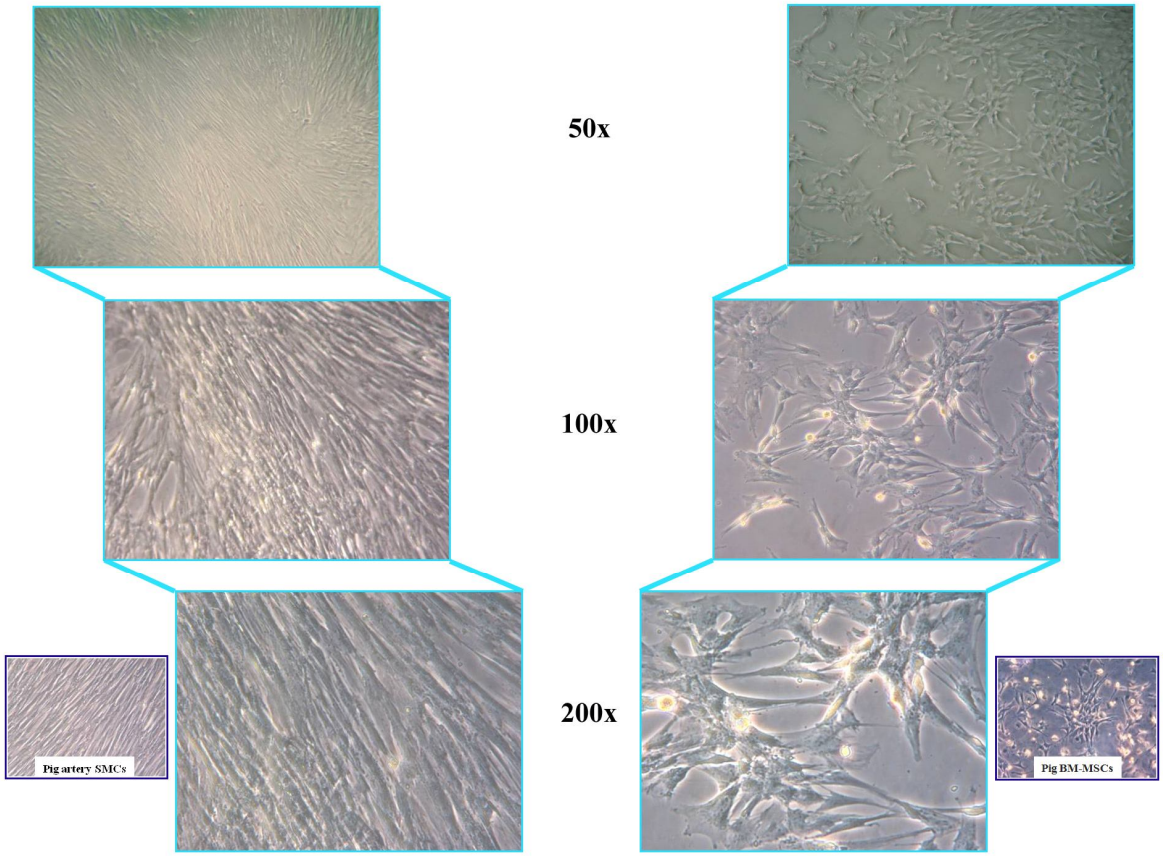


Fig. 11 Morphological phenotype of the primary culture obtained from the regenerated wall. Two main morphologies could be observed during culturing, with fine analogy to mature artery smooth muscle cells and bone marrow mesenchymal stem cells, either of porcine origin. Magnifications: 50x, 100x, 200x.

The large majority of the cells harvested from the *media* layer revealed the presence of classical cytoskeletal proteins involved in the contractile apparatus, indeed the high positivity for sm-actin and MyHC-apla1 together with the scarce expression of smoothelin, calponin and sm-myosin probably indicates the engagement of a smooth muscle transdifferentiation by the colonizing myofibroblasts. Other aspects to note are the absence of pig leukocyte antigen CD45 and the rare detection of osteocalcin in these cells. Some of the myofibroblastic cells expressed the stem cell epitopes SSEA4, OCT4 and much more CD29, as visible in Fig. 12.

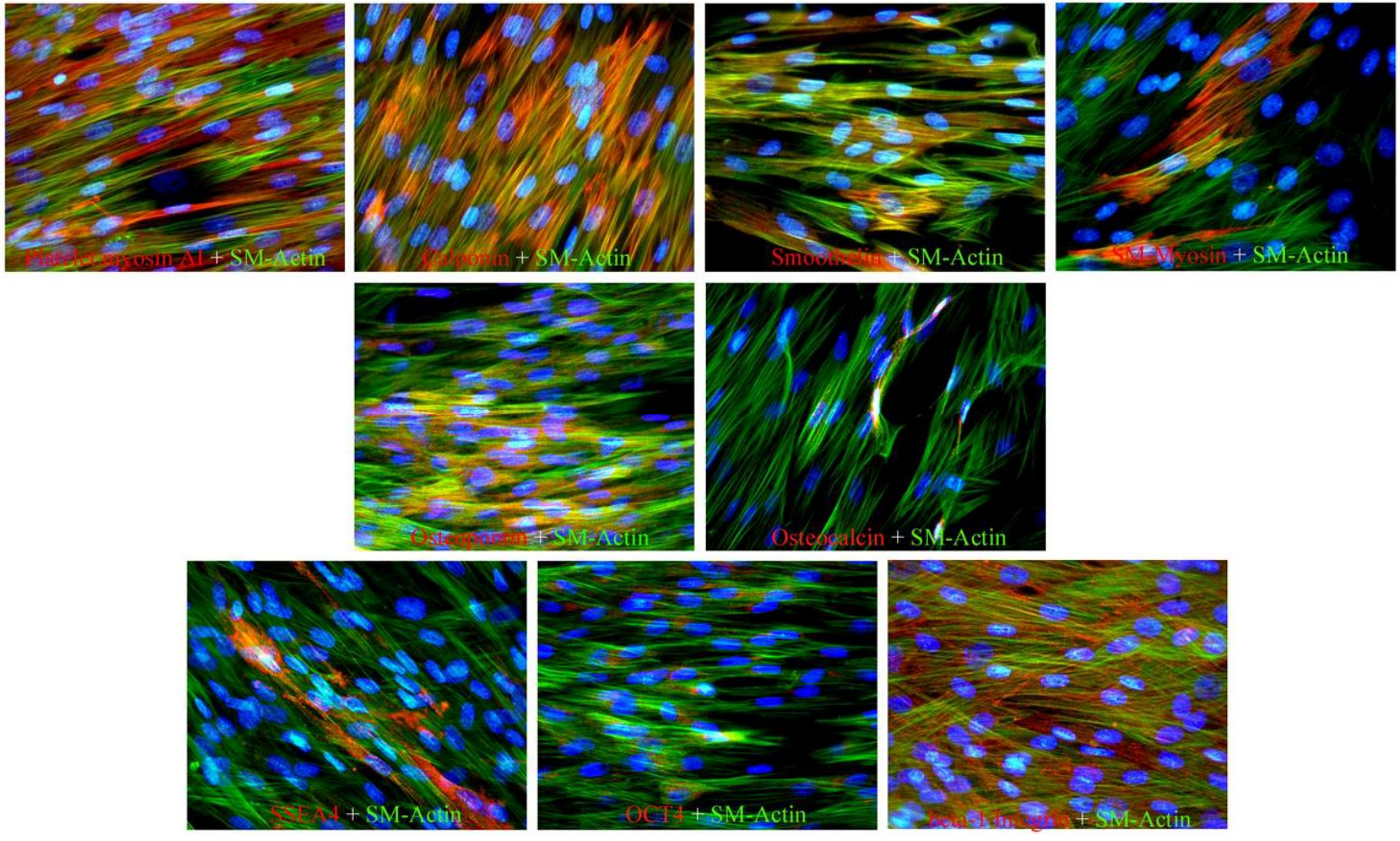
A summary of the immunocytochemical results obtained from the analysis of the regenerated tissues-primary cultures could be visualized in Table 1:

Table 1 Immunophenotype of primary cultures derived from explanted tissues

Antigens	Medial cells	Adventitial cells	Valve Interstitial cells
CD45	-	-	-
Non muscle myosin	+++	+++	+++
SM-Actin	+++	+++	+++
Calponin	+	+/-	+/-
SM-Myosin	+	++	+
Smoothelin	+	+/-	+/-
SSEA4	+/-	+/-	+/-
OCT3/4	+/-	+/-	+/-
CD34	+/-	-	-
CD29	+++	++	+++

Legend: - negative; +/- 0-25%; + 26-50%; ++ 51-75%; +++ 76-100% positive cells

Fig. 12 Immunophenotype of the cell population extracted from the regenerated vessel wall. The double immunofluorescence with FITC-conjugated sm-actin allows studying the cell maturation status: while sm-actin (in green) could be generally detected among cultured cells, its co-expression with smoothelin or sm-myosin (in red) was less usual. Rare was the positivity for osteocalcin, consistent with the observations of tissue analysis. A wide immunodetection of beta-1 integrin (CD29) could be evaluated. Magnification: 400x. In the following page


Platelet Myosin-AI + SM-Actin
Calponin + SM-Actin
Smoothelin + SM-Actin
SM-Myosin + SM-Actin
Osteopontin + SM-Actin
Osteocalcin + SM-Actin
SSEA4 + SM-Actin
OCT4 + SM-Actin
Beta-1 Integrin + SM-Actin

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This study has been presented at the *5th Biennial Meeting of the Society for Heart Valve Disease (Joint meeting with Heart Valve Society of America)* in Berlin, Germany, 27-30 June 2009 as:

Iop L, Gandaglia A, Bonetti A, Marchini M, Spina M, Basso C, Thiene G, Gerosa G. *In vivo* Spontaneous Tissue Regeneration Of Allogeneic Decellularized Aortic Valves. (Oral presentation)

Chapter 5

Stem Cell Populations in Adult Heart Valve Leaflets

identification, isolation and characterization in Valve Allografts and Surgical Specimens

5.1 Introduction

Lack of function and end-stage organ insufficiency represent medical concerns with higher social and economic relevancies. In the cardiac field, the continuous increase of valvular pathologies mostly finds etiological basis on degenerative pathological conditions in the industrialized World or can be associated to unrecognized infective endocarditis in the developing countries [1]. Actually the problem is selectively faced through a cardio-surgical approach: every year, almost 300000 interventions of valve substitution are executed [2]. From the 1950s, more than a hundred of device models has been proposed, being essentially identified as mechanical valves, xenogeneic and allogeneic biological valves and pulmonary autografts. Although these substitutes have substantially contributed to ameliorate the natural history of the valvular pathology, factors as long-life anticoagulation therapy, increased infective and immunological risks provoke a limited temporal durability *in vivo*. These limitations tend to affect the outcomes of the surgical reconstructions primarily for the pediatric patients with congenital cardiac defects (i.e. *truncus arteriosus*) [3].

In this sense, as already discussed, the interdisciplinary approach of cardiovascular tissue engineering could offer in the future a valid alternative pathway to solve such dramatic problems, by the potentiality to create a vital construct quite similar to a native and healthy cardiac valve both in functionality and characteristics [4]. In the designing process of the ideal valve substitute, it is necessary to consider not only rescue but also maintenance of function once in the patient. To this aim, it is fundamental to understand which kind of *in vivo* events are able to provoke a reduction or even at worst a complete loss of preservation in both native and biological commercial valves. Structural deterioration is the major cause of failure for bioprostheses, i.e. porcine aortic valves, mounted or not on flexible stents, and bovine pericardium-based valves, both fixed and preserved in glutaraldehyde.

After almost 15 years *in vivo*, the fifty per cent of the implanted porcine valves encounters complications that reduce stability and hemodynamics performances. The main pathological process, faced by the bioprosthetic as well as native tissues, is the cusp calcification which can provoke reflow or stenosis due to leaflet thickening. Intrinsic calcific deposits can also verify concomitantly with extrinsic events, as like as those following thrombi and endocardic vegetations. Determinants of valvular mineralization include factors related to host's metabolism (younger patients present a more accelerated calcium metabolism), chemo-physical implant structure (glutaraldehyde fixation) and mechanical aspects (high hemodynamic stress). In the bioprosthetic substitutes, dystrophic calcification is linked to the reaction of the extracellular calcium to the phosphate of the cell membrane (phosphatidil- serine and choline) [5, 6] and/or of nucleic acids in a connective tissue, devoid of cell viability, due to the cytotoxic effects of the glutaraldehyde crosslinks. These events are correlated also to the failing control activity of the calcification

pathway, which is present in vital tissues and in normal conditions. As already seen, natural effectors of this control mostly are non collagenous matrix proteins, among which osteopontin and osteocalcin. Pushing the balance towards osteocalcin by *in vitro* cell treatment with inflammatory stimuli or hyperseric phosphate [7] means an increase of the calcification propensity and therefore, even the probability of valve failure increases if the concept is extended at an *in vivo* systemic pathological micromilieu.

Genetic factors have also to be evaluated in heart valve substitute maintenance because they could contribute to the pathobiological description of the valvular dysfunction: it is in fact probable the hypothesis for which the complex signalling pathways of valvulogenesis could be reactivated in the adult to produce a pathologic phenotype.

The valvulopathies on degenerative, inflammatory or genetic basis frequently show a quite common response to injury, characterized by the accumulation of extracellular matrix (ialuronic acid and chondroitin-6-sulphate) and valve interstitial cells (VICs), in addition to inflammation and/or calcification. Myofibroblasts, highly represented in the cell population of pathologic valves, share high analogy with embryonic mesenchymal cells, because they express smooth muscle actin, platelet endothelial cell adhesion molecule (PECAM), synthesize the chemokine SDF-1 (Stromal Derived Factor-1) by VEGF (Vascular Endothelial Growth Factor) induction, are able to migrate, proliferate and remodel extracellular matrix. Potentially, it could be speculated that these cells are the fruit of an incomplete endothelial-mesenchymal transdifferentiation from a specific subset of adult endothelial cells. *In vitro* analysis demonstrated the TGFβ-2 unresponsiveness (Transforming Growth Factor β-2) of vein and capillary endothelial cells, differently from that one evidenced by the endothelial circulating progenitors chemiotactically recruited in the valve tissue [8].

To this aim, different research groups demonstrated the existence in the adult VIC population of a homeostatic regeneration, mediated by hematopoietic stem cells [9-11]. A direct demonstration of this modality *in vivo* was provided by the group of Visconti and Colleagues by transplanting lineage-negative (Lin^-), c-kit^+, Sca-1^+, $CD34^-$ hematopoietic cells, transgenically marked by the enhanced green fluorescein protein (EGFP), into a lethally irradiated syngeneic non-EGFP mouse: injected cells were able to migrate and were found in valvular tissues too [9]. Bona fide confirmation that this phenomenon occurs even in men could not clearly be obtained, but some researchers arrived separately to similar conclusions regarding the human cardiac valves. In particular, the work of Schowasch et al. put on evidence the presence of dendritic cells, T-lymphocytes, endothelial progenitor cells and macrophages predominantly localized in the valvular *fibrosa* [10]. In the same years, another paper focused on the detection of the hematopoietic stem marker CD117 in both degenerative aortic valves and bioprostheses, and through different histochemical techniques inferred to this positivity the presence of mast cells. As the same Authors suggested, the

implications for this cell occurrence are to be strictly evaluated for durability of both native valves, but also of bioengineered grafts [11].

In the adult, genetic mutations, but also the presence of a pathological valve microenvironment could induce these stem cells or progenitors towards a differentiation lineage with adverse effects on the maintenance of valvular integrity. Therefore, a complete knowledge of the different stem populations together with a clearer comprehension of the physiological and pathological stimuli influencing them could allow establishing which cell components are to be used in the preparation of tissue engineered valve substitutes and on which targets it is to interact to prolong the *in vivo* durability of the graft.

5.2 Materials and Methods

5.2.1 Human sampling and tissue analysis

Valve leaflets were obtained from two different sources: allografts obtained by donors, died of extracardiac deaths or for non natural causes, and cusps excised from pathological heart valves. Aortic valve conduits (n=27; aortic valve leaflet= Vao), with annexed mitral anterior leaflet (n=27; mitral valve leaflet= Vm)- and in some cases pulmonary roots of the same subject- have been provided by a local Tissue Bank and classified in five groups based on the age of the donor (11-20 yrs, n=5; 21-30 yrs, n=7; 31-40 yrs, n=1; 41-50 yrs, n=8 and 51-60 yrs, n=6).

Aortic cusps were removed during cardiac interventions of valve substitution for valve stenosis or insufficiency, while mitral valve leaflet fragments were obtained after surgical remodeling in case of floppy degeneration (n=12, comprehensive of aortic and mitral samples).

Specimens were embedded in OCT (Tissue Tek, Sakura, Japan), cryocooled in liquid nitrogen and stored at -80°C until further processing. Serial 7-µm cryosections of cusp tissue were analyzed with standard protocols of classic histology (Haematoxylin & Eosin, Mallory's trichrome) and histochemistry for the detection of lipid infiltration and calcification (Oil Red O and von Kossa). Immunohistochemical tests for the research of differentiated cell proteins, calcifying cytotypes, inflammatory cells and stem cells of various lineages were also performed: a complete list of used antibodies is provided in the following table, where the affinity antigen is indicated:

Calcification aspects	Osteopontin, OP (Abcam, Cambridge, UK)
	Osteocalcin, OC (Abcam, Cambridge, UK)
Inflammatory cells	Human CD45 (Dako, Dakopatts, Denmark)
	Human CD14 (clone Tuek 4; Monocyte marker; Dako, Dakopatts, Denmark)
	Human CD68 (Macrophage marker; Dako, Dakopatts, Denmark)
	S100 (Chemicon, Temecula, CA)
	Human Granulocytes (Abcam, Cambridge, UK)
	Mast Cells Tryptase (Mast cells; Abcam, Cambridge, UK)

Differentiated cells		MyHC-Apla1 (type A non-muscle myosin heavy chains; ******)
		Vimentin (clone V9, Dako, Dakopatts, Denmark)
		Smooth muscle Actin, SM-Actin (clone 1A4, Sigma, St.Louis,MO)
		Smooth muscle myosin (SM-Myosin; ******)
		Human CD31 (clone JC70A; Dako, Dakopatts, Denmark)
		Von Willebrand Factor (Dako, Dakopatts, Denmark)
Stem cells	***Embryonic***	SSEA4 (Chemicon, Temecula, CA)
		OCT4 (Santa Cruz, Santa Cruz, CA)
		Nanog (Abcam, Cambridge, UK)
	Hematopoietic	Human CD34 (Dako, Dakopatts, Denmark)
		Human CD117 (Dako, Dakopatts, Denmark)
		Human CD133 (Abcam, Cambridge, UK)
	Neural	Nerve Growth Factor, NGFr (Abcam, Cambridge, UK)
		Human Nestin (Abcam, Cambridge, UK)
		Glial fibrillary acidic protein, GFAP (Chemicon,Temecula, CA)
		B-catenin (Abcam, Cambridge, UK)
	Mesenchymal	Human CD29 (Abcam, Cambridge, UK)
		CD44 (Homing receptor; VMRD Inc., Pulmann, WA, USA)
		Human CD90 (Abcam, Cambridge, UK)
		Human CD105 (Endoglin; Abcam, Cambridge, UK)
		CXCR4 (Abcam, Cambridge, UK)

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5.2.2 Cell isolation and *in vitro* characterization

5.2.2.a Valvular Interstitial Cells (VICs) primary cultures using stem cell culturing procedures: Immunocytochemical and FACS analysis

Primary Valvular Interstitial Cells were harvested from the surgical specimens and from two allografts used as controls (donors' median age=23±3 yrs). After complete removal of the endothelial lining by gentle scraping, cusp tissue was minced in 2-3 mm^3 fragments and incubated with a digestion mix composed of collagenase I (125 units/ml; Sigma), elastase (8 units/ml; Fluka) and soybean trypsin inhibitor (0,375mg/ml; Sigma) for 20 minutes at 37°C. Protease activity was blocked with fresh foetal bovine serum (FBS). Fragments were collected in an OPTILUX non tissue culture Petri dish (Falcon BD Bioscience, San Diego, CA) and cultured in complete medium (αMEM (Sigma, St.Louis, MO), 20% FBS (Gibco, Invitrogen, Carlsbad, CA), 1% L-Glutamine and 1% Penicillin-Streptomycin (Sigma)). Cells spread out from the pre-digested explants 7-10 days after treatment and maintained in culture in semi-confluence. Cell morphology was studied using a phase contrast Leica DM IRB microscope (Leica, Wetzlar, Germany) connected to a Canon Power Shot S40 camera.For immunophenotype determination, cells at the second passage were cytocentrifuged using a Shandon Cytospin 4 centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA) and fixed in 2%

PFA in PBS and incubated with primary antibodies to membrane and cytoplasmic epitopes present on stem cells and progenitors (SSEA4, OCT3/4, human CD117, human CD34, human CD133, human CD90, CD271 (NGFr), human CD29 (Integrin β1), human CD105, CD44, Flk-1 (VEGF-R2, Santa Cruz, Santa Cruz, CA), human Nestin and human HLA-ABC (Chemicon, Temecula, CA) and revealed with Cy2 conjugated goat Fab' to mouse or rabbit IgGs (Chemicon) as secondary antibodies. Cell nuclei were stained with Hoechst (Hoechst 33258; Sigma). Antigen distribution was studied using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and images were acquired using a Leica DC300F digital videocamera (Leica, Wetzlar, Germany).

Flow cytometry was performed on 2^{nd}-3^{rd} passage cells, detached with citrate buffer (Sigma), rinsed and re-suspended in PBS, pH 7.2 (Sigma) at a concentration of $5x10^5$ cells/100 ml, directly stained with 10 µl of FITC fluorochrome-labeled anti-human CD29 (Immunotech, Marseille, France), CD44 (Immunotech), CD31 (Immunotech) and PE-conjugated anti-human antibodies to the progenitor markers CD45 (Immunotech), CD34 (Immunotech), CD73 (BD Biosciences), and CD105 (Beckman Coulter, Fullerton, CA). Cytometric analysis was completed using a COULTER Epics XL-MCL cytometer (Beckman Coulter) and data were processed using EXPO™ 32 ADC software.

5.2.2.b Endothelial, smooth muscle, adipogenic and osteogenic transdifferentiation evaluation of Valve primary cultures

Cell lineage differentiation was assessed by stimulating passaged cells with induction media specific for endothelial cells (Endothelial cell growth medium; PromoCell, Heidelberg, Germany) and smooth muscle cells (DMEM with 10% FBS, 5% horse serum [Invitrogen], and 50 mM hydrocortisone [Sigma]), which normally populate heart valve leaflets. Adipogenic and osteogenic trandifferentiations were also investigated, plating cells at a density of 4 $x10^3$ cells/cm^2 on tissue culture dishes (Falcon, BD) and stimulating them for 21 days with an adipogenic medium (low-glucose DMEM supplemented with 10% FBS, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 mM insulin, 200 mM indomethacin [Sigma]) or an osteogenic mix (low-glucose DMEM supplemented with 100 nM dexamethasone, 10 mM beta-glycerol phosphate, 50 mM ascorbic acid 2 phosphate [Stem Cell Technologies Inc., Vancouver, BC]). After stimulation, cells were fixed in 2% PFA and analyzed by immunofluorescence for the primary antibodies anti-vWf, anti-CD31, anti-SM α-actin and anti- SM-MyHC. Mouse or rabbit Cy2 conjugated-IgGs were employed in the secondary antibody incubation and Hoechst for nuclear counterstaining. Evaluation of the adipogenic conversion was obtained by staining fat vesicles in treated cells with Oil Red O (Sigma), while von Kossa was applied to verify possible osteogenic induction. Mayer's hematoxylin (Sigma) was applied to mark cell nuclear material. Stained cell coverslips were observed by a Leica light microscope and images were acquired with a Leica DC300 digital videocamera (Leica).

5.3 Results

5.3.1 Allograft- and pathological heart valve leaflet tissue characteristics

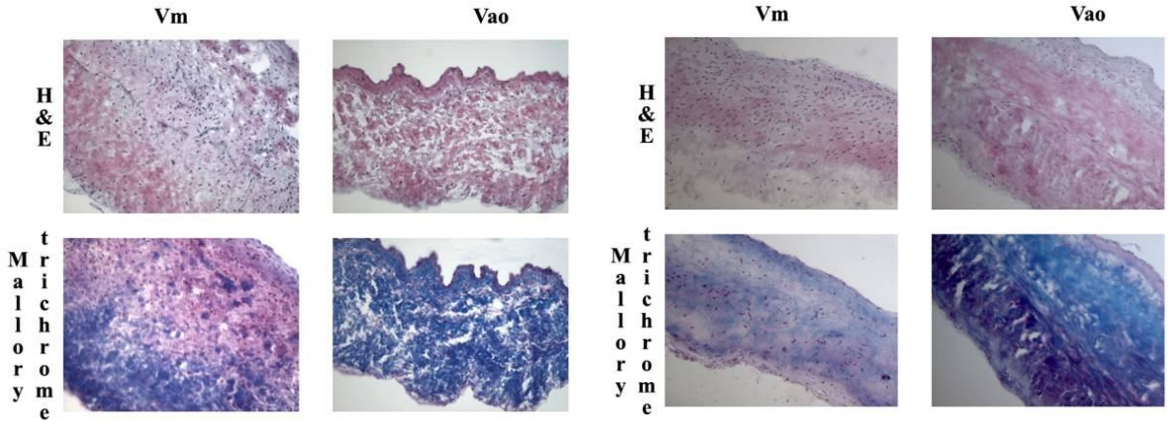


Fig. 1 Histological examinations of mitral (Vm) and aortic (Vao) leaflets belonging to allografts of the 20-30 yr- and 50-60 yr-age groups. During the analysis, this image disposition would be always maintained. While the younger subjects' s valve leaflets display a quiet normal trilayered distribution, the older ones tend to lose this structure and often are infiltrated by cholesterols in *fibrosa* or lipids in *ventricularis*, as visible in haematoxylin-eosin and Mallory's trichrome (magnification: 70x).

The observation of histopathological findings, such as lipid deposits, tends to become more frequent in allografts with increasing donor's age, moving from simple lipid droplets in the first years of age to organized formations of cholesterol crystals in the last age group.

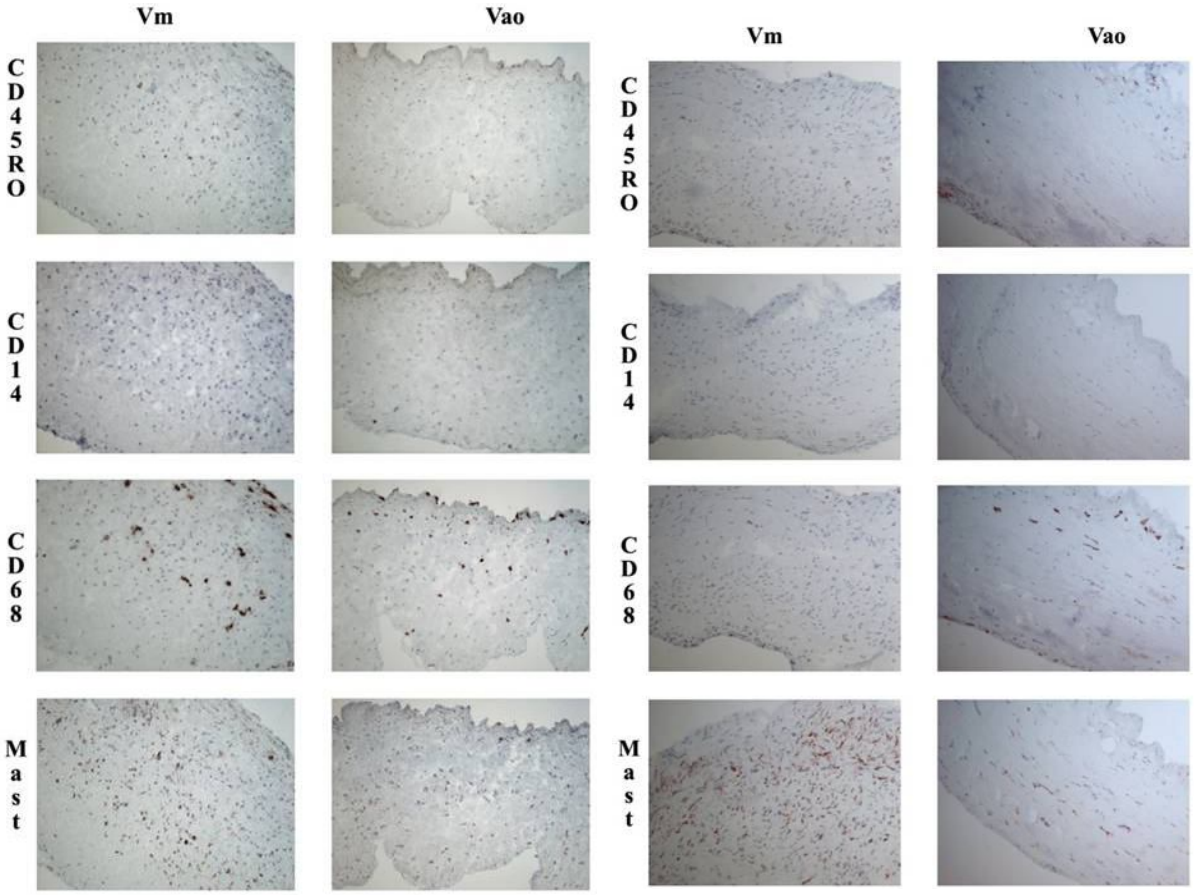


Fig. 2 Immunohistochemical detection of inflammatory markers. CD45RO, CD68 and Mast cell tryptase were frequently observed in the valve specimens of both age groups: in the younger one, their expression was scattered and homogenous in all leaflet, likely to identify a protective function, as that observable in the healthy myocardium. In lipid-infiltrated cusps, these markers are distributed in precise areas probably to limit the pathological trend (magnification: 70x). In the previous page

The effect of this infiltration is a decreased cellularity in the interested cusps. Calcifications are found more rarely and with restriction to some cases, while frequent, albeit low, the presence of the early calcification marker osteocalcin. In terms of inflammation, whatever the age considered, there is always a component of cells expressing CD45RO, CD68 and tryptase (Fig. 2).

This detection reveals the presence of general leukocyte cells, but also macrophages and mast cells which have a mild and homogeneous distribution in leaflets without histopathological reliefs or can tend to localize specifically in some areas of the affected samples.

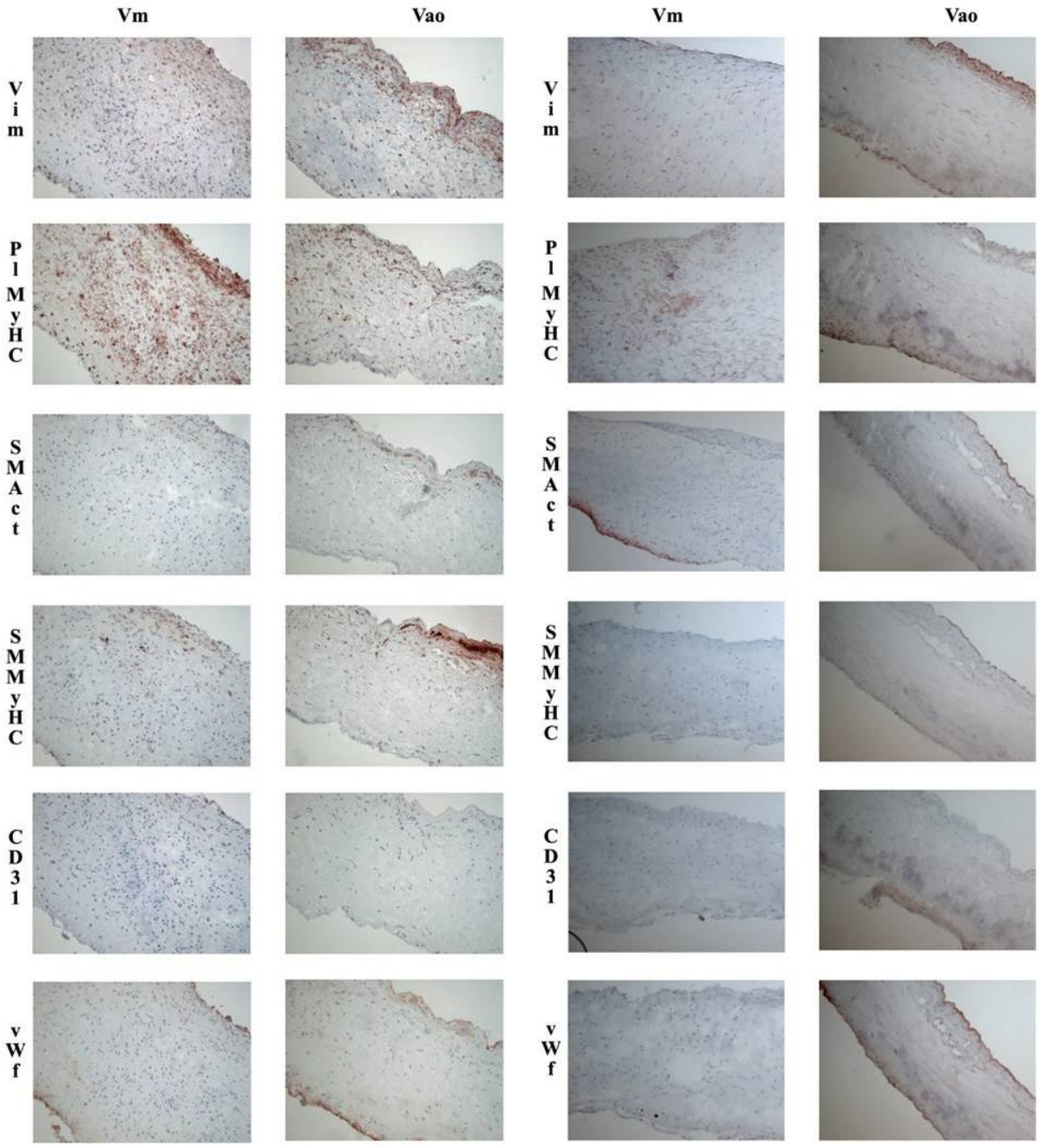


Fig. 3 Differentiated cells present in the leaflets. As already described by our group [12], semilunar but also atrioventricular healthy leaflets are characterized by the presence of widespread fibroblasts, a smooth muscle cell population only populating the *ventricularis* and endothelium at the outer surfaces (even if manipulation can often damage the lining and remove it). In compromised specimens, there is a little tendency to observe sm-actin activated fibroblasts. Note the lipid infiltration in the *ventricularis* and the anomalous bluish staining in correspondence to the cholesterol deposition in *fibrosa* of the 50-60 yr- Vao allograft. Magnification: 70x. In the previous page

Fibroblasts, populating the cusps, may show an activated phenotype, SM-actin positive, only in certain cases, while express for the large majority the typical markers vimentin and non-muscular myosin (Fig. 3). $CD31^+$ and/or VWF^+ endothelial cells may be found in the innermost regions of some samples, while the expected positivity on the surface is not observed, probably because of endothelium exfoliation during cryopreservation procedures (Fig. 3).

Stem markers are variously expressed by the valve cells with different degrees of homogeneity of distribution. The hematopoietic marker CD34, as previously demonstrated [13], is expressed at high levels: almost all the interstitial cells show positivity with the exception of the populations of the *ventricularis* layer (Fig. 4).

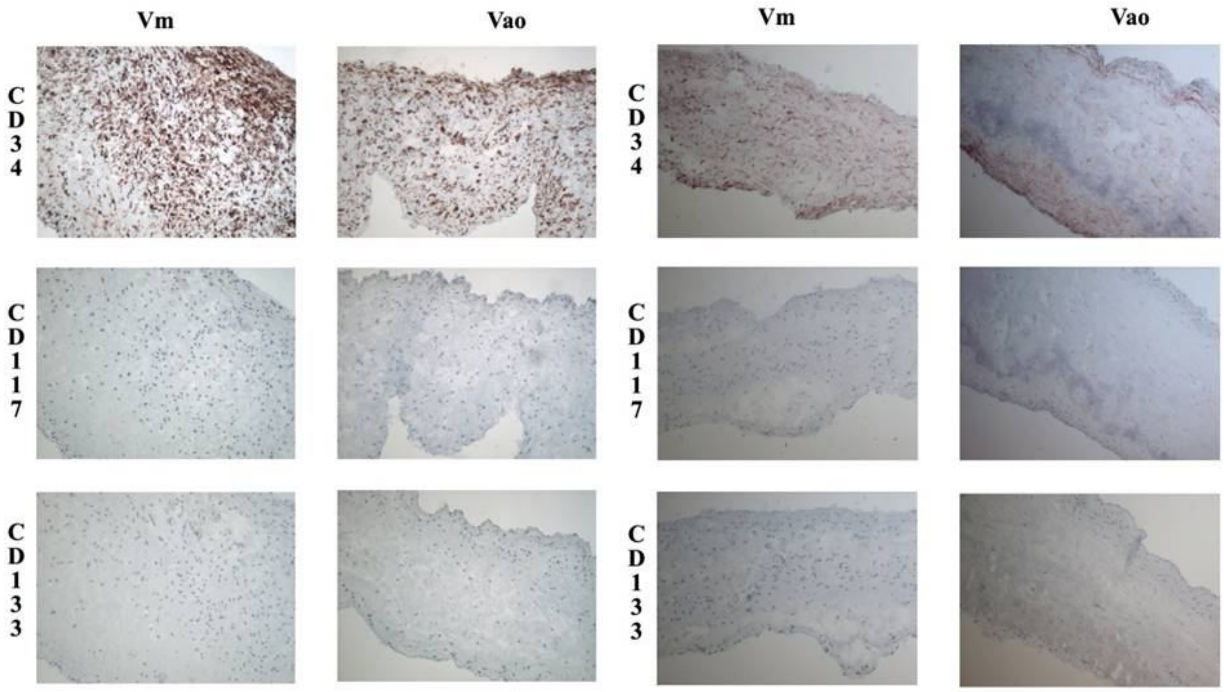
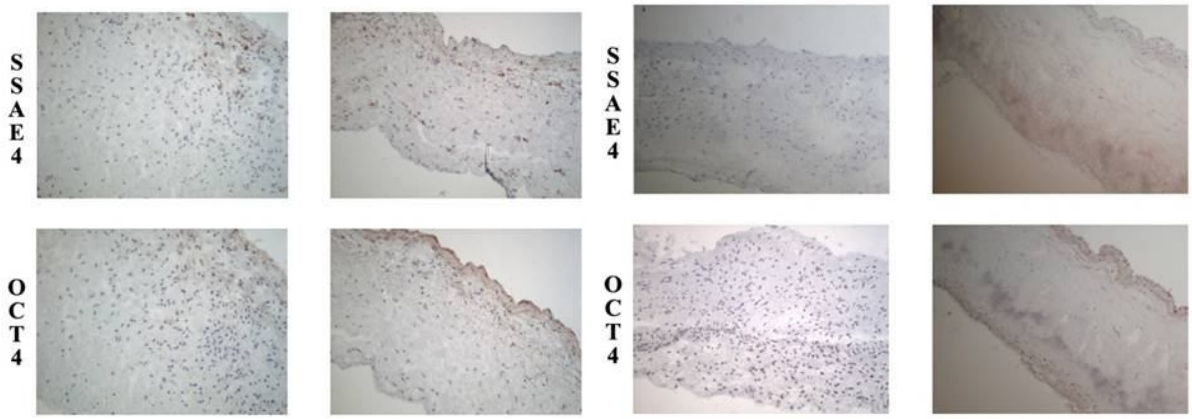


Fig. 4 Hematopoietic stem cell markers expressed in the Vm and Vao leaflets. CD34 is widely positive in all the cusps, but not on the surface of some *ventricularis* cells. Note the highly positive cell cluster in the *fibrosa* of the 50-60 yr- Vao allograft. Other considered epitopes are rarely expressed. Magnification: 70x

Embryonic markers present in the adjacent myocardium only after injury, are expressed at low levels in the cusp tissue: SSEA4 with more even distribution while OCT3 / 4 more tending to localize in the elastin regions (Fig. 5).

Fig. 5 Embryonic stem cell markers in the Vm and Vao leaflets. While SSEA4 tends to have a scattered and homogenous distribution, OCT4 is mainly expressed at the *ventricularis* of the younger donor's leaflets. Note the highly positive cell cluster in the *fibrosa* of the 50-60 yr- Vao allograft for both markers. Magnification: 70x. In the following page



CD90 and CD29 are among the more expressed mesenchymal markers, whereas CXCR4, i.e. SDF-1 factor receptor, is rarely present in *spongiosa* (Fig. 6).

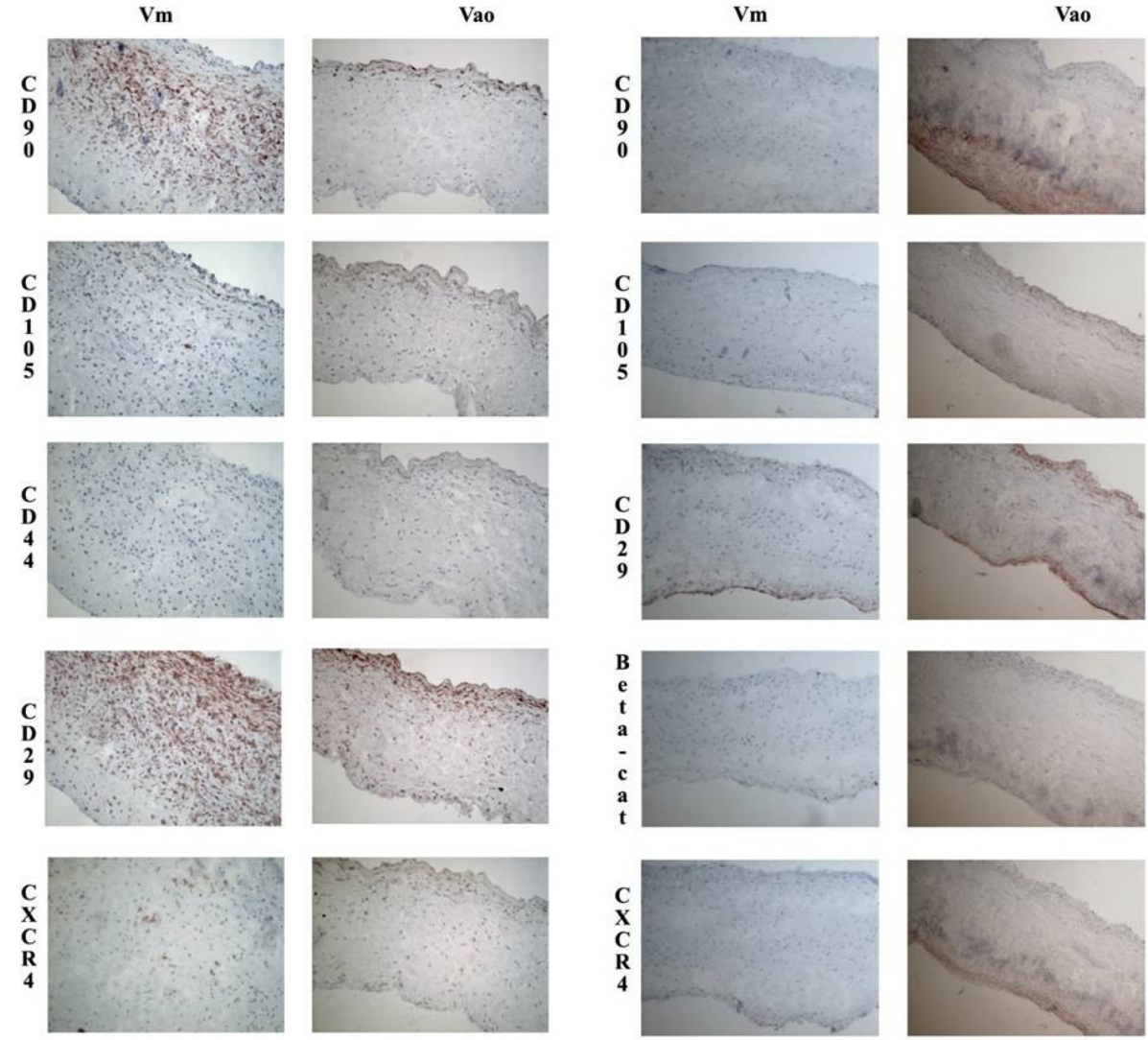


Fig. 6 Mesenchymal stem cell markers in the Vm and Vao leaflets. CD90 and CD29 are highly detectable in the younger donor's leaflets, but it is not infrequent to observe positivity for other markers as CD105 and CXCR4. Note again the high marker distribution in the *fibrosa* cell cluster of the 50-60 yr- Vao allograft for both markers. Magnification: 70x

As for the hematopoietic CD34 and the mesenchymal CD29, the expression of the neuronal GFAP is very high especially in the leaflets of the semilunar valves. Other neuronal markers have homogeneous, but sparse localization (Fig. 7).

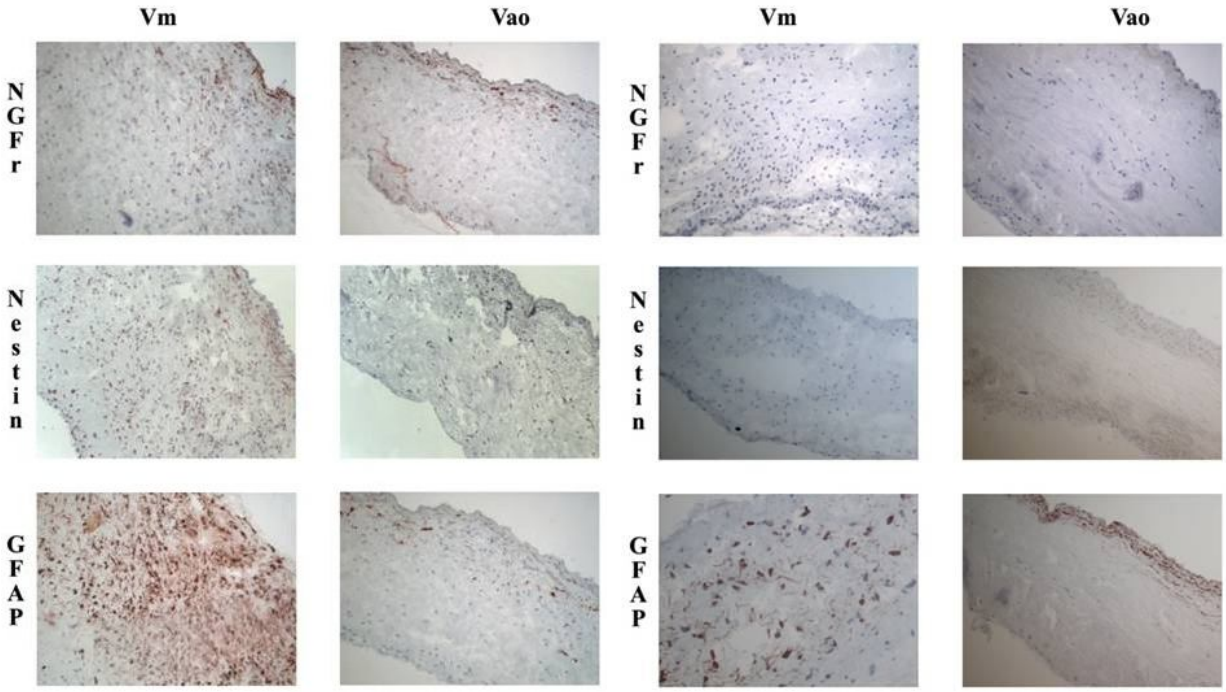


Fig. 7 Neural stem cell markers in the Vm and Vao leaflets. GFAP has a similar distribution pattern to CD29, even if at low intensity. NGFr and Nestin are equally found in these tissues, but at lesser extent. Magnification: 70x

In more compromised specimens, a tendency to cell clustering with semi-clonal connotation can be appreciated nearby the cholesterol depositions in the *fibrosa* layer. These clusters appear to be positive for CD34, SSEA4, OCT4, CD90, CD29 and CXCR4, likely to indicate a recruitment of circulating progenitors. Similar cell groups can also be evaluated in the surgical specimens removed during heart valve substitution (Fig. 8).

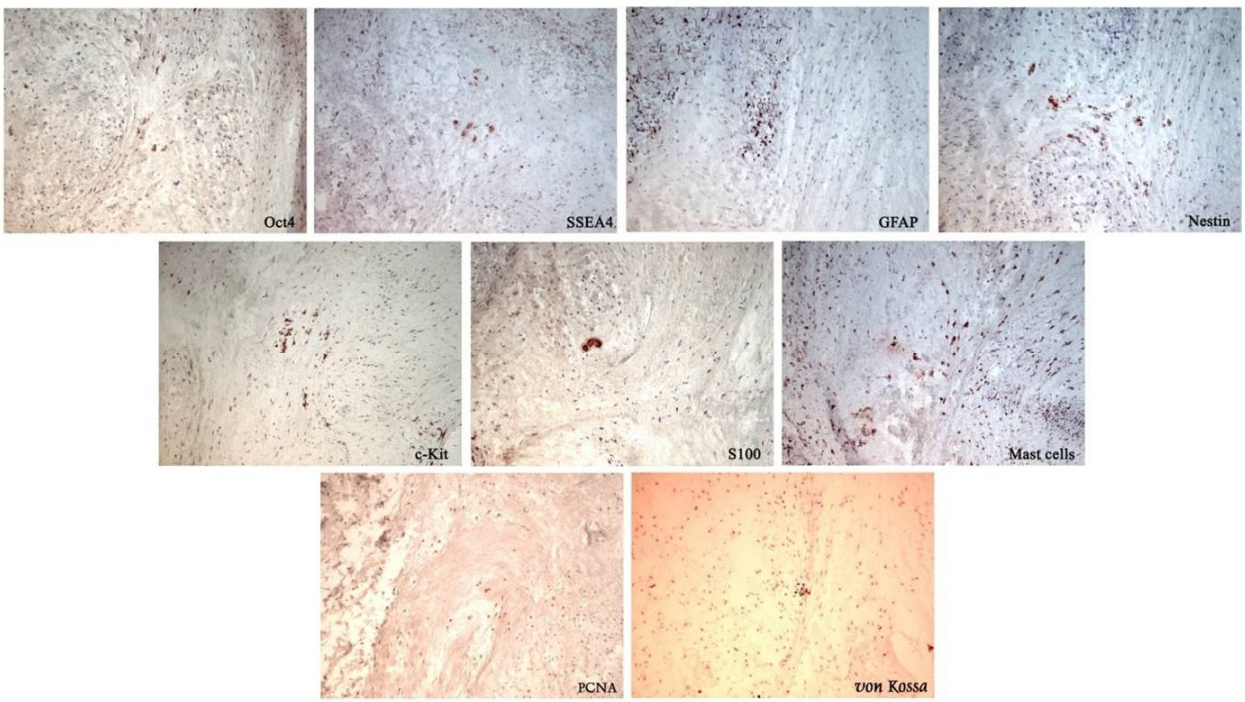


Fig. 8 Immunohistochemical analysis of a floppy mitral valve leaflet excised during surgical remodeling. Cell clusters, already identified in the allograft samples, can be widely detected in the pathological cusps and show positivity for the embryonic, the neural and inflammatory cell markers in an area of active proliferation and rare microcalcifications. Magnification: 100x. In the previous page

From the whole tissue analysis it is possible to draw some conclusions about the expression of stem cell markers in the cardiac valves. First of all, various cell lineages compose the VIC population: in addition to differentiated (endothelial and smooth muscle cells, calcifying cytotypes) and hematopoietic stem epitopes –as reported in the literature- also embryonic, neural and mesenchymal stem markers appear to be widely expressed in all human adult leaflets of the atrioventricular and semilunar valves. Similarly to the differentiated cell epitopes, the stem cell ones tend to localize in a precise topographical distribution.

5.3.2 Characterization of the primary cultures obtained by pathologic and allograft valve leaflets

Cusps obtained from discard after surgical resection of floppy mitral valves, removed from dysfunctional aortic valves or excised from allografts were used for the study of cell populations *in vitro*. The primary cultures, selected for the criteria used to grow bone marrow stem cells, have been characterized in terms of morphology and immunophenotype. Obtained cells were elongated with prominent nucleoli and long cytoplasmic protrusions (Fig. 9).

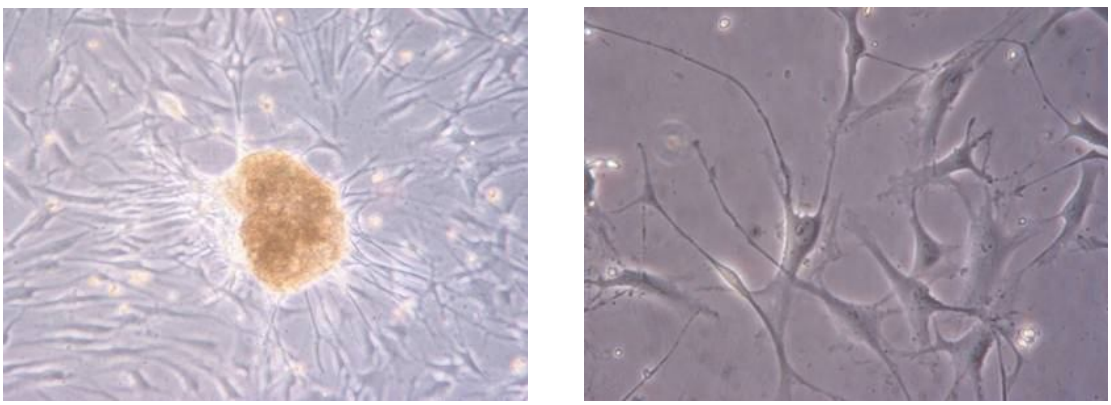


Fig. 9 Morphological aspect of the primary culture obtained from a floppy mitral valve leaflet. Cells appear very elongated with spindle-shaped morphology and multilayer growth. Magnifications: 100x and 200x

Moreover, they showed a tendency to form clusters with growth in multilayer. When extracted from more compromised leaflets, they doubled more slowly. Immunocytochemical analysis revealed the expression of markers such as CD29, CD90, CD105 and Stro1 suggesting a typical mesenchymal stem cell profile. Nestin, NGFr, GFAP, Flk1 and a low percentage of CD34 were even present (Fig. 10).

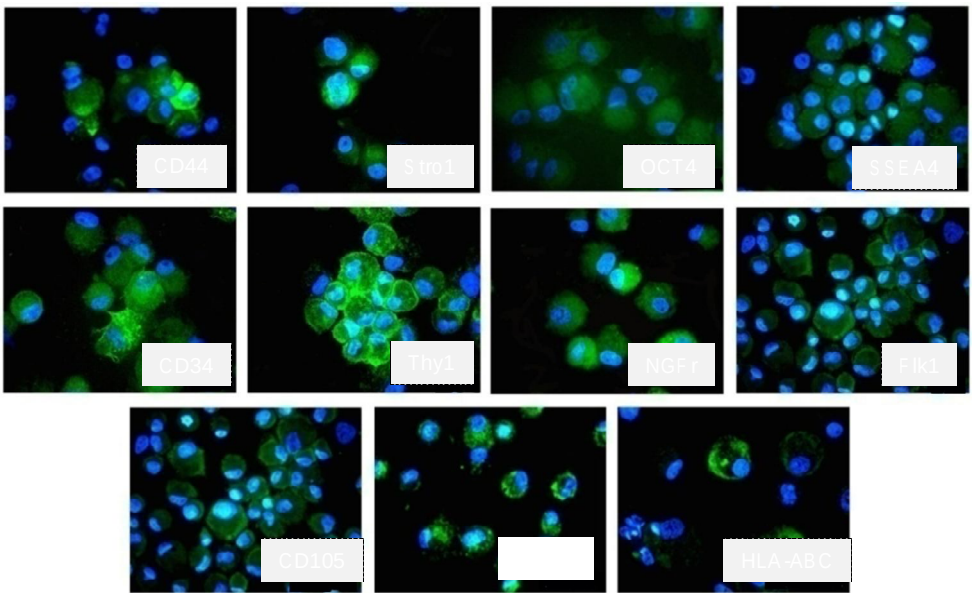


Fig. 10 Immunocytochemical profile of valve leaflet primary cultures. Typical mesenchymal stem cell markers are expressed (CD44, Stro1, Thy1, CD105 and HLA-ABC); the neural NGFr and Nestin, the embryonic SSEA4 and OCT4 are indeed positive at lower level, as the hematopoietic CD34 (Magnification: 400x).

HLA-ABC expression by these cells is particularly appealing in respect to their usage in tissue engineering approach or simply in allografting of native cryopreserved valves. This antigen of the minor histocompatibility complex is a 45 kDa monomorphic epitope with human species-specificity and plays a pivotal function in allomodulation, therefore only the cells expressing it are like to avoid rejection when allogenically transplanted. Even the cytofluorimetric tests identified these cells with a mesenchymal phenotype, positive for the expression of typical lineage markers, as CD29, CD44, CD73 and CD105, although differently from bone marrow stem populations, almost the 8-10% of them expresses the hematopoietic epitope CD34 (Figs. 11 and 12). Similar results can be appreciated both in mitral and aortic leaflet-derived -primary cultures. Cell populations are identified as pure valvular interstitial cells for the quite null expression of endothelial markers, even if they can gain a slight expression of CD31 by extensive culturing probably due to the plastic rigid contact.

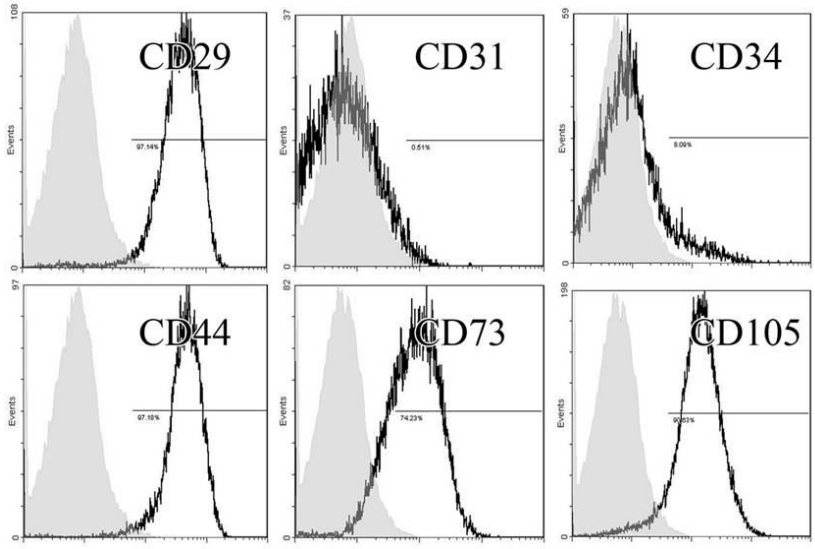
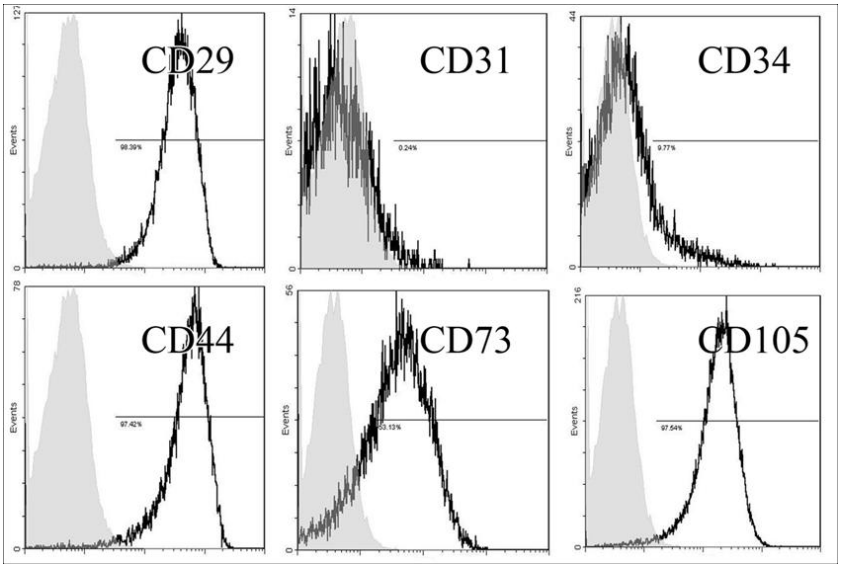


Fig. 11 and 12 FACS analysis on mitral (above) and aortic (in the following page) valve primary cultures. Cells express the membrane epitopes CD29, CD44, CD73 and CD105 at high event percentage, but a hematopoietic contamination was present. The two populations appear to be quite similar at the cytometric tests.



Treatment with stimulating media has delivered positive outcomes for endothelial and smooth muscle conversion (data not shown). Oil Red O revealed the presence of many cells undergoing an adipogenic transdifferentiation (Fig.13).

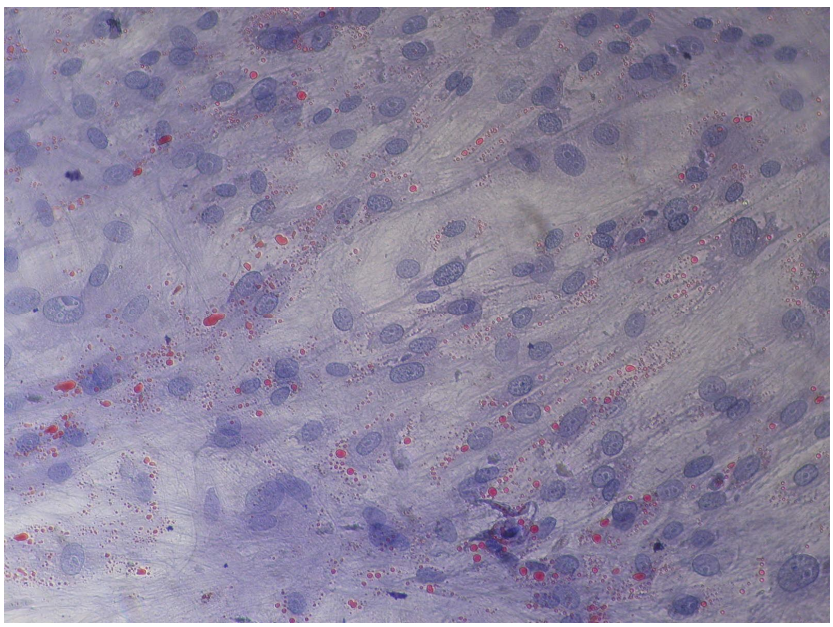


Fig. 13 Oil Red O on allograft mitral valve primary cultures stimulated with adipogenic medium. Magnification: 200x

Rare cells instead show a tendency to osteogenesis -at least at the cell biology level- both in the allograft and pathological valve populations (Fig. 14).

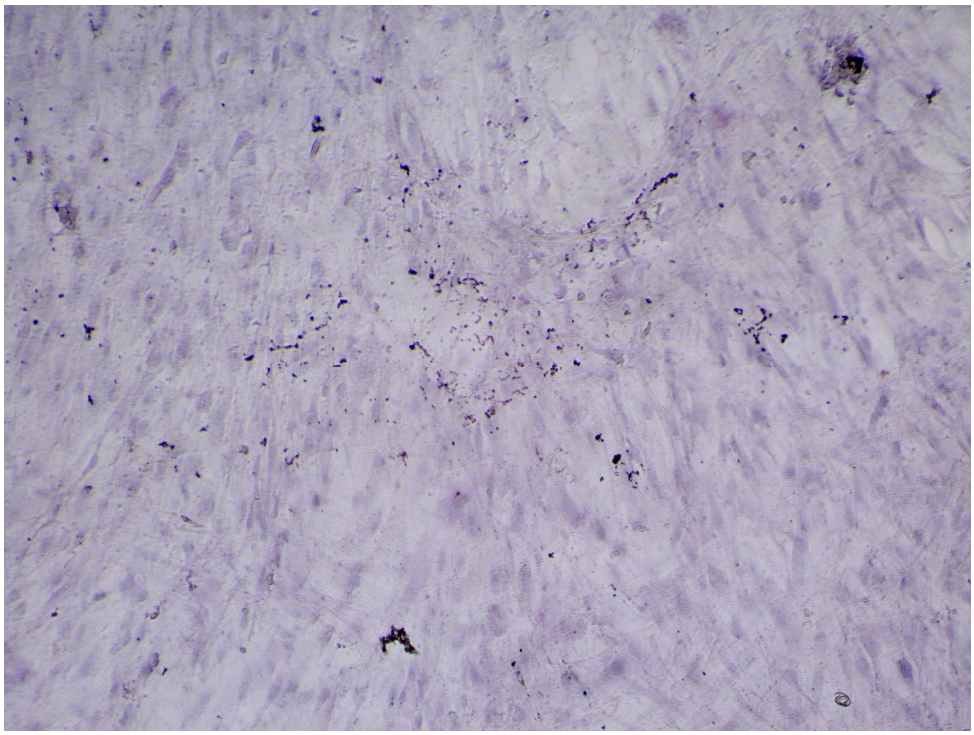


Fig. 14 Von Kossa staining on allograft mitral valve primary cultures stimulated with osteogenic medium. Magnification: 100x

This last statement leads to two different considerations. At first, these cells are characterized by a low responsiveness to powerful osteogenic stimuli: it should be probable therefore that they are not able to prime an osteogenic program. Only a pro-calcific stimulation could definitely ascertain if they show propensity to calcification and hence could actively participate or cause valve degenerative events. Moreover, the application of molecular biology techniques would help to understand if there has been a genetic switch towards an osteogenic expression. The second reflection, which is closely associated with the one already proposed, concerns the multipotentiality of these cells, which can be identified as partially committed stem cells or better valvular interstitial cell progenitors.

5.4 Chapter References

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This study has been presented at:

- *World Conference on Regenerative Medicine* – Leipzig, Germany, 29-31 October 2009

Iop L, Basso C, Rizzo S, Piccoli M, Callegari M, Paolin A, De Coppi P, Thiene G, Sartore S, Gerosa G. Stem Cell Populations in Human Heart Valves: Identification, Isolation and Characterization in Valve Homografts and Surgical Specimens. (Oral presentation)

- *70° Congresso della Società Italiana di Cardiologia* – Rome, Italy, 12-15 December 2009

Iop L, Basso C, Rizzo S, Piccoli M, Callegari M, Paolin A, De Coppi P, Thiene G, Sartore S, Gerosa G. Stem Cell Populations in Adult Human Heart Valves. (Poster presentation)

Chapter 6

Discussion

General overview and comments

6.1 The ideal valve substitute

Prior to discuss the results evaluated on the different approaches for the creation of valve substitutive devices, it is essential to define the peculiar characteristics of the ideal model.

Harken identified in these 1o rules the principles of correct valve function played by the perfect construct:

1. It has to close rapidly (less than 0.05 seconds);
2. It must remain closed during the appropriate cardiac cycle phase;
3. It has to be easily implantable;
4. It has to be lodge in an anatomic position;
5. It has to demonstrate stability and time durability;
6. It has to offer a good life quality for the treated patient;
7. It must not offer resistance to the blood flow;
8. It has to display specific physics and geometrical conditions;
9. It must not be able to provoke thrombotic events;
10. It has to be chemically inert and unable to damage the corpuscular blood elements [1].

By evaluating these commandments, it is possible to enlighten that the major focus of the Author is the satisfaction of the mechanical performance by the replacement device.

In the era of biomechanics [2], it is no more possible to conceive the biological activity separated from the mechanical behavior of the valves and Harken's principles have to be implemented with other rules to respect in the design of the vital and functional biological substitute.

Feature to optimize	Conventional (Mechanical, bioprosthetic)	Tissue engineered
Closure of leaflets	Rapid and complete	Rapid and complete
Size of orifice area	Less than that of natural valves	Better
Mechanical properties	Stable	Stable
Surgical insertion	Easy and permanent	Easy and permanent
Risk of thrombosis	Yes, especially mechanical valves, which require anticoagulation, causing vulnerability to hemorrhage	No; endothelial surface to inhibit thrombogenesis
Risk of structural dysfunction	Degradation of synthetic materials rare with mechanical valves Tissue degradation and calcification of leaflets with bioprosthetic valves	Resistant to degradation and calcification
Risk of Infection	Ever present	Resistant to infection
Viability	No	Yes, able to repair injury, remodel, and potentially grow with patient

Table 1 Characteristics of the ideal valve substitutes as proposed by Mendelson K and Schoen FJ. Modified from [3]

General features of the newly formulated valves should conceive nonthrombogenicity, infection resistance and above all cellular viability (Table 1), but depending of the kind of biomaterial used, also biocompatibility, lack of adverse immunoreactions and controlled dissolution to avoid an early failure of the construct *in vivo*.

Further indications for this purpose should be found from an evaluation of the entire World cardiopathic population [3, 4]. There are two main categories that might take beneficial from a tissue engineered or regenerated valve. The first one is represented by pediatric patients with cardiogenic malformations [5]: the incapability of growth demonstrated by the actual replacement devices submits these subjects to continuous surgical interventions with the aim to maintain a correct cardiac function, but at the same time by increasing the peri-operative mortality risks. In these patients and in young ones, in particular, calcium metabolism is very active and can seriously compromise the stability of the valvular tissue. The second population interested by valve failure, whose numerosity is growing for increased life expectancy, is formed by elder subjects, where aging has provoked loss of cell and ECM integrity on behalf of fibrosis and calcification events. Hence, the new valves have to be produced in a quality and number able to assure the actual epidemiological demands. In addition to cell viability the new bioengineered constructs have to grow and remodel with the patient. Also the manufacture costs have to be considered in order to allow a large-scale usage and economic accessibility [6].

6.2 Heart Valve Tissue Engineering versus Heart Valve Tissue Guided Regeneration

A bioengineered valve that relies on the multipotent differentiation potential of hBM-MSCs could be the best choice for the complete repopulation of natural or artificial scaffolds suitable for successfully valvular substitutions. In fact, the *in vitro* analysis of how competent hBM-MSCs are for this task, prior to their *in vivo* application in an animal model, has shown that these cells can indeed differentiate, after stimulation (Fig. 1C, Chapter 3) or direct contact with matrix elements (Figs. 4–7, and Table 2, Chapter 3), into appropriate valve cell lineages (fibroblasts, myofibroblasts, SMCs and ECs). hBM-MSCs display a repertoire of molecules that may be relevant to their adhesion and penetration in decellularized scaffolds (see Fig. 1B and Table 1, Chapter 3), including b1-integrin (which plays a pivotal role by mediating cell–ECM interactions) and CD54, CD105 andCD44 (which act cooperatively in cell homing via binding to hyaluronan, the major non-protein glycosaminoglycan of the ECM [7], the main components of which (pro-collagen I and aggrecan) are also synthesized by hBM-MSCs). Although such properties are inherent in these cells *in vitro*, the efficiency of their attachment and survival when seeded on the matrix, and subsequently transplanted into an animal model, is likely to depend ultimately on the fibrous supporting network and its associated molecules. The *in vitro* observed dissimilarities in proliferation and differentiation patterns in *fibrosa* vs. *ventricularis* settings could be related to regional differences in blood pressure/volume which concern the ascendant aortic wall [8]. Though these gradients do not have the same magnitude in the pulmonary circulation, a similar flow behavior is reported for both right

and left outflow tracts, where the different fluid mechanics on the ventricular vs. artery side influences the local distribution of the main ECM constituents (elastin and collagens), as well as the least represented proteins (laminin and fibronectin) [8]. In fact, the proteoglycans versican, decorin and biglycan have a significantly higher combinatorial distribution in the *ventricularis* with a decreasing staining intensity towards the *fibrosa*, while hyaluronan is strongly expressed in the *spongiosa*. This layer-specific distribution reflects properly the anisotropic mechanical microenvironment and, hence, the unique function played in this respect by each ECM element, i.e. the compressive resistance offered by hyaluronan, the critical roles performed by decorin and biglycan in tension and elastogenesis and yet the important contribution of decorin in collagen fibrillogenesis [9]. In this regard, Sales and colleagues [10] investigated the effects produced by different protein precoatings of elastomeric biomaterials (glycerol sebacate) on EPC phenotypic regulation. Coating with fibronectin, collagen types I/III or elastin drives the differentiation of engrafted EPCs towards a heterogeneous antigenic profile in CD31, vWf and SM a-actin expression. Among the various experimental conditions tested, elastin-precoated scaffolds notably show a higher cellularity when colonized with these cells and this result is consistent with our finding of an additional SM-like differentiation of hBM-MSCs in the elastin-rich *ventricularis*. By enzymatic decellularization and dynamic seeding with ovine arterial cells, Schenke-Layland et al. also found that cells expressing markers of SMC lineages segregate in the *ventricularis* of engineered porcine pulmonary valve leaflets [11]. Elastic fibers, which are insoluble in the valvular ECM, consist of macromolecules of cross-linked elastin surrounded by fibrillin-rich microfibrils, containing the RGD cell adhesion motif, which is capable of interacting with cell integrins, thus regulating cytoskeleton reorganization, cell proliferation and synthetic activities [12]. VGVAPG hexapeptide in tropoelastin is also able to induce similar phenotypic changes by promoting a proliferating smooth muscle commitment in fibroblasts, ECs, chondrocytes and, even, in contractile vascular SMCs [13, 14]. In the hetero- vs. homotypic cell seeding onto decellularized porcine and human valve leaflets, an even greater potential for SMC and EC differentiation is achieved when hBM-MSCs are applied in the homotypic combination with the *ventricularis* side of the pulmonary leaflets, suggesting, here again, that this particular matrix expresses local conditions to favor the appropriate cell conversion for this valve layer, i.e. myofibroblasts and SMCs. Occasionally, such phenotypic conversions seem to be incomplete in as much as some stem cell markers persist along with proteins of SMC lineage among the homing hBM-MSCs. It thus seems more appropriate to identify such cells as SMC-like cells, since it is hard to say whether this pattern is transient or permanent. In the process of ECM architectural reconstruction operated by hBM-MSCs especially into human matrices, it is noteworthy that – even by static conditioning – some cells are priming a novel collagen fibrillogenesis (Table 2 and Fig. 7F, Chapter 3). Actually, the formation of collagen ‘‘fibril-forming channels’’, substantiating the synthesis of new pro-collagen components, nearly recapitulates the mechanism by which fibroblasts

regulate the morphogenetic steps in embryonic tissues. The complete achievement of a fully differentiated cell phenotype demands, however, a specific local *milieu* for cell growth, possibly containing a suitable cocktail of growth factors, with prolonged culture times and appropriate biomechanical stimulation [15]. Less certain is the interpretation of the differential colonizing potential observed in the ''xenogeneic'' (hBM-MSCs and porcine fresh PVLs) and ''allogeneic'' (hBM-MSCs and human cryopreserved PVLs) combinations, being potentially attributable to decellularization effects on treated matrices. Most collagen fibrils and elastic fibers exhibit well-preserved ultrastructural features (Figs. 6 and 7, Chapter 3) – a situation consistent with our previous results [16–18] – therefore the gross distribution of collagen and elastin is left unchanged with respect to the native ECM [19]. These ultrastructural patterns are also confirmed for the human cryopreserved specimens (Fig. 6, Chapter 3), in striking contrast with previous studies for which cryopreservation has been reported to cause severe alterations, including disintegration of most collagenous structures of cryopreserved porcine pulmonary roots [20].

These evidences about the decellularized valve scaffolds make them interesting for their use on a tissue guided regeneration-based approach. This procedure is not fully contemplated by the classical paradigm of tissue engineering, because it only employs the biomaterial as a guide for successive cell engraftment *in vivo*. It is surely appealing to avoid the difficult phase of cell seeding and construct conditioning and the positive results verified by many groups [21-25] make it an alternative and apparently simpler attempt to rescue valve function. Once decellularized, aortic valvulated roots have been allogenically implanted in pulmonary position in a minipig evaluation model. Echocardiographic monitoring, performed periodically until the sacrifice of the animals (almost 14 months), generally observes a decreasing transvalvular gradient and the absence of graft fibrosis. The analysis of the explanted valves revealed an engrafting process, which is not always able to reach the medial layer of the regenerated vessel (Figs. 1, 3 and 5, Chapter 4). Cells able to colonize the implanted nude matrix display different phenotypes, among which the expression of stem cell and fibroblast markers is particularly high (Figs. 5 and 7, Chapter 4). However, these cytotypes difficultly reach a mature character (Fig. 3, Chapter 4). A very positive note is given by the high vascularization process concerning the entire wall both at the *media* and *adventitia tunicae* (Fig. 3, Chapter 4). This phenomenon demonstrates appearance of both neoangiogenesis and neoarteriogenesis, with fully mature capillaries and arterioles resembling the typical *vasa vasorum* of the adventitial layer. It is certain that the presence of new vascular elements can favor the provision of nutrients and oxygen for the neighboring cells, conferring vitality to all the interested tissues. Tissue vitality is also confirmed by the high proliferative activity registered in all vessel layers and by the dynamic biosynthesis (Fig.6 and 8, Chapter 4). However the zones with less recellularization or vascularization can encounter calcification, rarely correlated to cells, but due to mineralization on nude collagen and elastic fibers (Fig. 2, Chapter 4). Also the leaflets appear to be interested by a

repopulation phenomenon not always homogenous in all the leaflet length. Mineral deposition is rarely observed and the main cell phenotype is fibroblastic with high proliferation rate and positivity for mesenchymal stem cell epitopes. The immunocharacterization of the obtained primary cultures is consistent with the tissue observations and in particular allows inferring the nature of the engrafting cell source: from the same explant tissue two different morphologies can be identified with high similarity to mesenchymal stem cells of bone marrow origin and smooth muscle cells. Hence the repopulation phenomenon might have origin from a systemic bone marrow cell mobilization and a local smooth muscle cell spreading from the adjacent anastomized pulmonary artery. Especially the first event is often observed after any surgical intervention, by facilitating the diffusion of a plethora of cytokines and growth factors which might have contributed to the creation of new small blood vessels and favored the recruitment/motility of the same adjacent smooth muscle cells [26].

The motivations for an incomplete cell repopulation after 0ne year of natural conditioning *in vivo* are however not easy to understand. Probably, some consequences of the decellularizing treatment, i.e. the partial loss of glycosaminoglycans and chondroitin sulphate might alter the stabilization of the tertiary and quaternary structures of some ECM macromolecular aggregates. Further experiments are needed to establish whether the preparation of fresh porcine leaflets/roots may require additional treatment with detergents to get rid of ‘‘contaminating’’ protein residues, if a parallelism is established with the human specimens of the *in vitro* approach. The microscopic and ultrastructural examinations would support however the conviction that most of the cellular debris has been removed.

Although the experimental results of these different studies indicate the achievement of an early-stage construct differentiation, it is particularly encouraging to state that both approaches seem to be valid for the construction of a new vital substitute. In this sense, to ameliorate the maturation of the newly formed tissue, different strategies can be employed on the premise of the absolute need of a chemo-mechanical stimulation. The stable chemical addition to the decellularized matrix of growth factors or anti-calcification treatments could enhance recruitment and spreading of cells, by circumventing adverse events. In the prospective to employ such biomimetic strategies, composite scaffolds should represent a valid future alternative to the alone biopolymer or decellularization-related methods.

6.3 Evaluation of alpha-Gal on non-human mammalian heart valves

Given the scarcity of organ donations, it will be more and more necessary to rely on the readily available animal organs to create new valve alternatives. The structural and functional resemblance of porcine and human heart valves has led to the pig being chosen as the ideal donor for

bioprostheses, but also bovine pericardium finds indication to this aim. Tissue-engineered constructs from allogeneic or xenogeneic valve scaffolds covered with autologous, multipotent progenitor cells are considered the new frontier for valve replacement, indeed the same matrices, devoid of cells, could be directly employed *in vivo*. Until now, adverse immune reactions to valve substitutes from (glutaraldehyde-fixed) xenogeneic or allogeneic scaffolds have been attributed to cellular components of heart valves (for instance, alpha-gal epitopes expressed by porcine endothelial cells [27]), or to the glutaraldehyde [28]. Alpha-Gal is not only expressed at the endothelial level of the leaflet surface covering, but also by the endothelial cells which coat the vascular lumen of the arterioles at the cusp base (Figs. 1-4, Chapter 2). Even some stromal cells appear to display the antigen in their cell membranes (Figs. 2E-G, Chapter 3). For future applications in this field, it therefore appears promising that scaffolds employed in these studies are immunohistochemically devoid of alpha-gal antigens (Fig. 6B, Chapter 2; Fig. 2G, Chapter 3). Particular attention should be addressed to the validation of the techniques used to reveal the antigen. The isolectin BSI-B4 commonly suggested in the literature for alpha-gal detection gives reliable results only for native specimens, but it displays an aspecific affinity when applied to TriCol decellularized leaflets. BSI-B4 creates an affinity binding respect to the glucidic molecules contained in alpha-Gal epitopes. It is not hence the stable link that instead can be achieved through the employ of a highly specific antibody probe, as M86.

On the other hand, it may be that detergent procedures used for heart valve decellularization could affect the antigenic stability of the collagen-elastic matrix (or induce cryptic epitopes) in scaffolds from xeno- and (to a lesser extent) allogeneic donors [29–31]. Ongoing studies at our laboratory on immunocompetent and immunodeficient rats transplanted with intact or decellularized porcine leaflets and type I collagen patches in the peritoneum cavity suggest that treatment with detergents makes the valve matrix more immunogenic, probably due to fine molecular alterations of the ECM system (publication in preparation). Using *in vitro* assays, Rieder et al. ascertained that decellularized porcine pulmonary valve conduits are able to attract more monocytic cells than their human counterparts [32]. The same group also demonstrated that human polymorphonuclear leukocyte activation, subsequent to the contact of decellularized porcine matrices with human plasma, can be blocked by inhibitory elements extracted in the decellularization process [33], thus suggesting that an appropriate matching of the host (scaffold) to the donor (patient's cells) is of paramount importance for the successful outcome of tissue engineering experiments.

6.4 Stem cell populations in adult human heart valve leaflets

The investigation on the stem cell component populating the heart valve leaflet has deserved a quite central attention in the last five years [34-38]. Endothelial progenitor cells, $CD117^{+}$or $CD34^{+}$cells, but

also mast cells have been found in normal and pathological specimens, confirming once again the continuous remodeling process interesting heart valve cusps [39]. In fact, an engrafting of circulating cells has been demonstrated [40, 41], indicating that it is quite impossible to give a character of residency to the entire valvular population, either of endothelial or interstitial cytotypes.

The study on the interstitial aortic and mitral population here proposed (Chapter 5) evidences the presence of stem cells of different lineages, i.e. hematopoietic (Fig. 4, Chapter 5), embryonic(Fig. 5, Chapter 5), neural (Fig.7 , Chapter 5) and mesenchymal (Fig. 6, Chapter 5) both in young and older donors' allografts and in pathological cusps (Fig. 8, Chapter 5). The distribution of the analyzed markers is not homogenous, indeed some of them in particular, i.e. OCT4 and GFAP, display a precise topographic expression. Aging compromises cellularity and favors the accumulation of lipids. Primary culture from these specimens reveals typical aspects of mesenchymal stem cells at the morphological and immunophenotypical level (Figs. 9-12, Chapter 5) with ability to endothelial, smooth muscle and adipogenic differentiation (Fig. 13, Chapter 6), but scarce osteogenic induction (Fig. 14, Chapter 5).

These features find partial confirmation in a work by Latif et al. who demonstrated a close similarity between total VIC population and mesenchymal stem cells of bone marrow origin [42]. In contrast to this paper, the valvular interstitial progenitor population here isolated displays an absolutely higher positivity for typical mesenchymal stem markers, for instance while the differentiated VICs express CD105 at a nearly 10%, these progenitors exhibit an almost 90% event percentage.

The distribution of mesenchymal stem cells in the postnatal organism is correlated to their existence in the perivascular niche with ancillary role to the hematopoietic progenitors [43]. In this case, however, vascularization cannot be contemplated in a normal valve tissue, unless the natural lodging of the valve in direct blood contact could be regarded as a niche-like structure. It is not to be excluded a contribution from the neural crest population to the tissue and cell positivity for neural stem markers.

Some aspects of this study have to be enlightened for a further comprehension of the biology and pathobiology of the heart valve.

Differently from any other cardiovascular tissue, as the adjacent myocardium, the hematopoietic marker CD34 is expressed by almost 90% of the entire interstitial valve population (Fig. 2, Chapter 5), as evidenced in the work of Barth and Colleagues [38]. Inflammatory cell markers have been found positive even in the less compromised specimens, i.e. the younger donor's leaflets (Fig. 2, Chapter 5). The meaning of this presence in a quite normal tissue is not known, but it could be that the continuous mechanical stress imposed to the thin leaflet tissue, together with the direct exposure to blood, needs a sentinel function exerted by macrophages and mast cells. Another interesting hypothesis could be proposed by observing the double nature of the macrophage cell. In effect a phenotype committed to tissue regeneration has been discovered among the typical

protective functions played by these cells. For the similarity with the T2 helper lymphocytes, these macrophages have been named M2 and can be characterized by the positivity for IL-10 and mannose receptor (MR) [44]. In valve specimens the positivity for CD68, a broad macrophage marker, is associated to the expression of MR and IL-10, as visible in Figure 1.

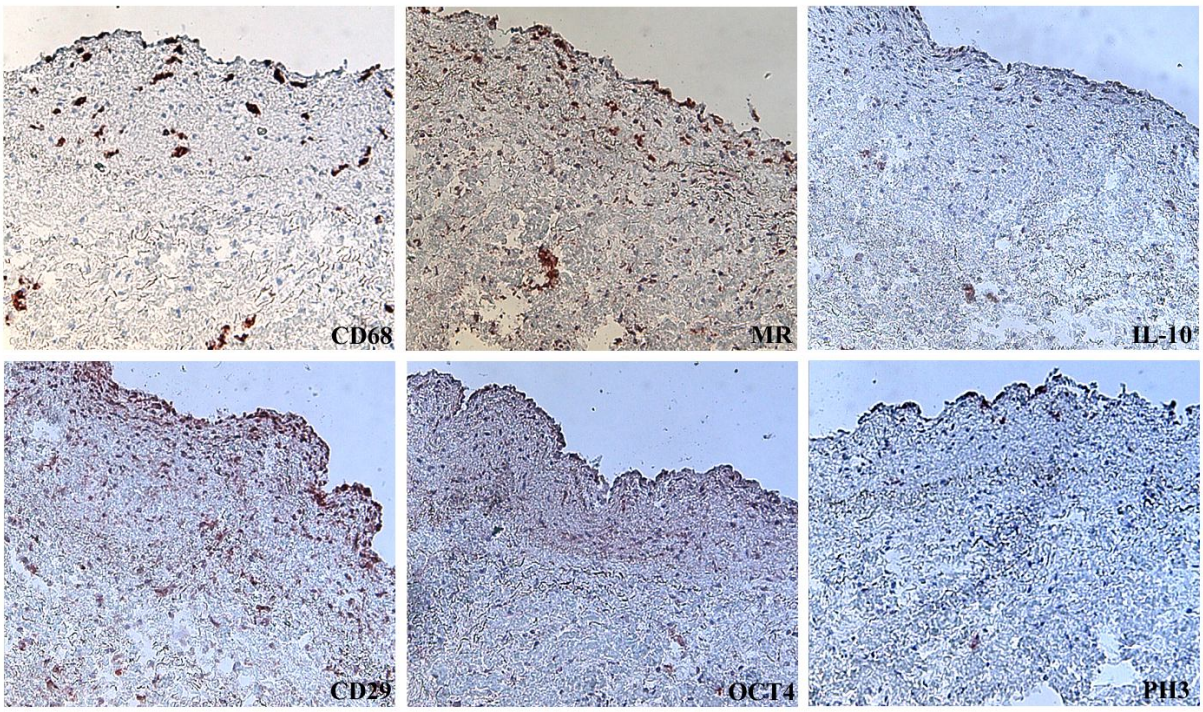

Fig. 1 Immunohistochemistry on mitral leaflet excised from a 26 yr-old donor. Note the strong positivity for the mannose receptor (MR) and IL-10, typical markers of M2 macrophages. Magnification: 200x

Even if further studies are needed to confirm the phenomenon, the expression of these M2 markers in the cusp tissue- especially of the younger donors where no cholesterol or calcifications are documented-, is a further prove to substantiate the stem properties of the valvular interstitial cells. In addition, IL-10 is also ascribed to have a key role in immunomodulation, another important property of the mesenchymal stem phenotype [45]. A full demonstration of this ability in the valvular interstitial progenitors cells isolated in this study should hence be provided in the future, even if this condition might be highly probable, as it effectively is for the more differentiated population [46, 47].

Another work recently described the presence of mesenchymal progenitors in the valve tissues but with a different approach based on primary cultures submitted to different lineage differentiation, without any immunocharacterization or topographical identification *ex vivo* [48]. The Authors state the 'osteogenic calcific potential' of the cells they isolated. In the present work, the valvular progenitors show a limited osteogenic transdifferentiation at least at the cell biology level and molecular examinations will be useful to determine if the genetic pathway has been activated after the osteogenic stimulation. It is certain difficult to image in term of valve tissue preservation that all these cells do possess a calcific potential, but it cannot be excluded that a more permissive phenotype to calcific stimuli exists as for the differentiated VICs [49]. Hence, a direct treatment with

pro-inflammatory and hyperphosphatemic conditions should be useful to define a possible contribution of valvular interstitial progenitor cells to the adverse event of calcification.

A possible explanation of the early failure of implanted allografts should be extrapolated from the analysis of the elder donors' specimens. The usage of allogeneic, but already compromised substitutes for endothelial damage or cholesterol deposition is surely able to influence in a negative way the durability of the graft. Currently, the qualification of the mechanical and tissue valve integrity is established at the moment of harvesting from the donor through a gross macroscopical examination and it is not uncommon to observe fatty streaks at the ascendant aorta and at the base/commissures of the leaflets especially with aging, as provoked by actual life style. A suggestion from this study is therefore the establishment of a new and strict grading system, through which Tissue Banks could provide healthy-like allografts for replacement with a higher chance to integrity maintenance once in the patient.

6.5 Chapter references

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Research Products of the Doctoral Activity

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Poster and Oral Communications

Iop L, Basso C, Rizzo S, Piccoli M, Callegari M, Paolin A, De Coppi P, Thiene G, Sartore S, Gerosa G. Stem Cell Populations in Human Heart Valves. *70° Congresso della Società Italiana di Cardiologia* – Rome, Italy, 12-15 December 2009 (Poster presentation)

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