Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Metodologie di Ricerca Nelle Malattie Vascolari
Scienze Chirurgiche. Progetto N.1

Ciclo XXIV

Settore Concorsuale di afferenza: MED/22

TITOLO TESI

Cell-free cryopreserved arterial allografts from multiorgan donors: a new strategy to fabricate artificial blood vessels suited for peripheral vascular surgery.

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Esame finale anno 2012

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INTRODUCTION

1. CARDIOVASCULAR DISEASE

Cardiovascular disease, including coronary artery and peripheral vascular disease, is the leading cause of morbidity and mortality in word population. Peripheral arterial disease results in the manifestation of many serious conditions, including critical limb ischemia (CLI). CLI is the end-stage of lower extremity PAD in which severe obstruction of blood flow results in ischemic rest pain, ulcers and a significant risk for limb loss (Fig.1).

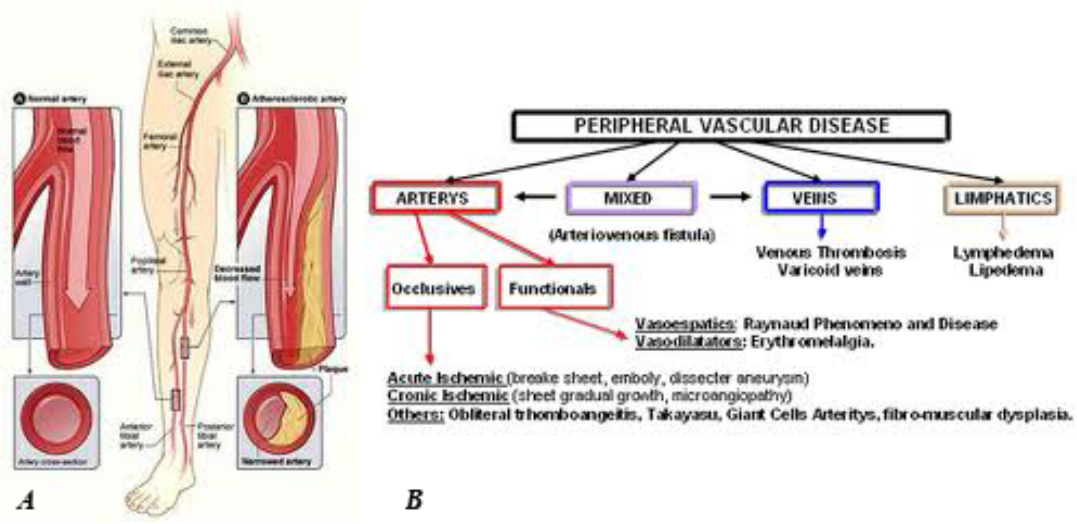

FIG.1 Peripheral Artery Disease (PAD). Anatomic location (A); Peripheral Vascular Disease diagram.

The clinical aim is the revascularization of occluded vessels to permit a functional reestablishment of blood flow. If not revascularized, patients with CLI can lose limbs or acquire other potentially fatal complications due to gangrene progression or sepsis. Common approaches and therapies used to treat PAD include changes in life style, drugs, or bypass surgery.

A bypass surgery involves implantation of infrainguinal bypass to replace the diseased segment and to redirect blood flow around the blocked portion of the artery (Fig.2).

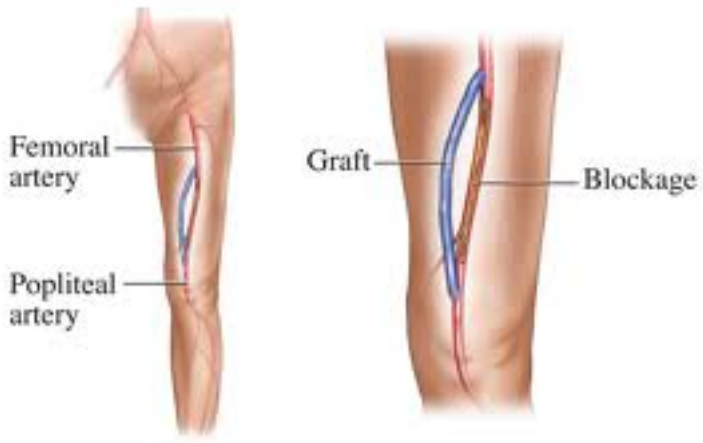

FIG.2 Replacement of diseased vessel

There are three main classifications for a biological bypass graft: autografts, allograft, xenograft.

Autografts utilize ones own vessels such as saphenous veins, mammary arteries and radial arteries. The chance of an immune response is low since the graft is from the host's body. Unfortunately this approach requires multiple surgical procedures and is often limited by the quality and availability of autologous vein (Mirelli M. et al, 2005).
In most cases the patient suffers from a number of pre-existing cardiovascular diseases and therefore may have atherosclerotic build up rendering any possible autologous vessels non-viable for grafting.

Allograft, the vessel is donated from an individual of the same species. The institution of Cardiovascular Tissues Bank opened new frontiers in cardiovascular surgery, since it offers the possibility to have a large availability of cryopreserved allograft from multiorgan donors with different calibre and length to be used whether autologous material is not available or not adequate.
However, the use of cryopreserved allograft is often unsatisfactory. The major disadvantage seems the immunological reaction caused from the persistence of cells components. The immunological reaction contributes to the degradation dilatation end consequent rupture of the graft (Chiesa R. et al, 2002).

Xenografts are synthetic vascular prosthesis that can be scaled up for high-throughput clinical uses. These types of graft are commonly used in peripheral bypass procedures for the implantation of large diameter graft, since they act as good conduits in high-flow low-resistance conditions (Bosma J. et al, 2012).

For years synthetic graft have been made of poly(ethylene terphalates) [Dacron™] and expanded poly(tetrafluoroethylene) [ePTFE Teflon™]. Both of these materials have been used clinically for peripheral applications and have become well characterized and clinically tested with successful outcomes for large diameter (>6 mm) vessels (Peck M. et al, 2012).

In the contrary, almost all vascular conduits with a smaller diameter (<6mm) fail in 5 years, because of intimal hyperplasia, the increase of the number of the cells that form the inner layer of vessels, or atherosclerotic degeneration, plaque formation in the inner surface, thrombosis and consequent complications such as stenosis, sclerosis and vessel obstruction (Teebken OE. et al, 2002).

To summarize, the use of either a synthetic or natural graft has specific individual limitations resulting in a high demand for alternative treatments (Table 1).

	Autograft	**Allograft**	**Xenograft**
Examples	Saphenous veins, mammary arteries and radial arteries.	Cryopreserved artery and vein segment; Great saphenous vein.	Synthetic vascular prosthesis made of Dacron and expanded PTFE.
Availability	Limited; diameter <1to>6mm	Good; diameter 4-6mm	Very good; diameter 6 to >30mm
Long-term results	Almost complete healing; rare events of aneurysm or degeneration; intimal hyperplasia.	No complete healing; degenerative disease; calcifications.	Intimal hyperplasia, thrombosis, obstructions.

Table1. Characteristics of vascular replacement methods

1.1 Cardiovascular System

The circulatory system in adult human is a closed system characterized by a double and complete circulation. It is composed of the heart and blood vessels, including arteries, veins, and capillaries (Fig.3). The heart is the key organ in the circulatory system. As a hollow, muscular pump, its main function is to propel blood throughout the body. Arteries carry blood from the heart to the peripheral district, deeper than veins and they branch out in numerous vessels with a decreasing calibre (from 25 mm in aorta to 0.2 mm in small arteries). Capillaries are very small, with a thin wall composed of a single layer of endothelial cells and create a huge net that permit gaseous exchange between blood and tissues. Veins carry blood from periphery to the heart, they have a major calibre and near the heart their wall structure is almost similar to arteries even if less elastic.

1.1.1 Artery composition

Arteries wall is constituted by 3 concentric laminas: Internal Laminae (intimal), Medial Laminae (mesoartery) and External Laminae (adventitia). Between those laminae, as bounds, there are 2 other laminae of elastic tissue, called inner elastic and outer laminae

Intimal Tunica

The most internal layer, in close contact with flowing blood; it is constituted by single line of endothelial cells that are overlooking to the longitudinal axis of the vessel. Endothelial cells lean on a thin layer of loose connective tissue, or sub-endothelial laminae, rich in elastic fibres that clump together forming the inner elastic lamina.

Medial Tunica

The intermediate layers. It is constituted of smooth muscle cells in a matrix rich in elastic fibres, collagen and proteoglycan; those components are always present in the vessel wall, their amount is different according to the area in which they reside. In the medial tunica the activation of the sympathetic system determine both contraction and consequent reduction of vessel calibre (vasoconstriction) and relaxation and consequent

increase of vessel diameter (vasodilatation).

Adventitial Tunica

The most external layer, it is a loose connective tissue rich in collagen fibres sprawling orientated, just few inflammatory, smooth muscle cells and adipocytes. It can be considered a connective sheath with a restraint role. In large and medium calibre vessels the adventitial tunica is more developed to hold in his thickness vasa vasorum (small vessels drizzling and feeding vascular wall) and nerva nervorum (vegetative sympathetic fibers controlling mainly the smooth muscle fibres of the medial tunica).

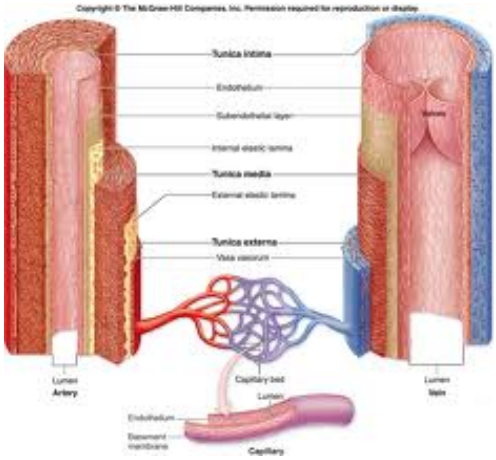

FIG.3 Vessels composition and difference

From the heart to peripheral capillaries the blood flows through a network of arteries with a decreasing diameter; distinguished by their size and structural characteristics in large-calibre elastic artery, medium-calibre muscular artery and small-calibre artery.

1.2 Vascular Tissues

1.2.1 Endothelial Tissue

Endothelial tissue is a mesenchymal-derived connective tissue, similar to the endothelial layer of the interior surface of vessels. Endothelial cells are very flat, have a central nucleus and with a diameter of about 1-2 μm. They form flat, pavement-like patterns on the inside of the vessels and at the junctions between cells there are overlapping regions that help to seal the vessel. Endothelial cells contain just few intracellular organelles (Golgi apparatus, mytochondria, endoplasmatic reticulum and free ribosome), numerous pinocytosis vesicles to carry nutrients through the endothelium, and the characteristic Weibel-Palade electrondense granules. Those secretory granules have a role in the coagulation as they contain the von Willebrand factor (VIII factor of the coagulation). Endothelium was erroneously considered as a simple covering layer, but now it is known that elaborating several active molecules acquire the ability to modulate the activity of the vessel wall (Nitric Oxide; Endothelin) and to cooperate with blood cell and coagulation proteins in contact with the luminal surface. Besides endothelial cells have an important role in the inhibition of the formation of thrombosis or hyperplasia, and are essential to guarantee homeostasis and the maintenance of vascular integrity. Endothelium is in direct contact with blood flow and a target for both mechanical and hormonal signals, it is fundamental in the control of microcirculation arteries function. Shear stress and Tense stress are the two mains mechanical forces induced from blood on the vessel wall. Shear stress is caused by the laminar flow attrite mainly on endothelial cell; Tense stress is caused by the hydrostatic pressure into the vessel. The final effect is the modulation of contraction relaxation phenomena in the vessel depending on the blood flow.

1.2.2 Muscular tissue

Smooth muscle is an involuntary non-striated muscle, found within the tunica media layer of arteries and veins. Smooth muscular tissue is composed by spindle-shaped cells with a central nucleus and characterized by abundant sarcoplasm and a less content of

myoglobin protein. SMCs don't present transversal muscular stripes because myofilaments, which run along cells major axis, are disposed in an irregular way.

1.2.3 Connective tissue

Connective tissue is the most different of the four tissue types with a wide variety of functions. It ranges in consistency from the gel-like softness of areolar connective tissue to the hardness of bone. Connective tissue (CT) forms an extensive compartment in the body and can be considered as the "glue" that holds the body together. Connective tissue consists of cells and extracellular material secreted by some of those cells. Thus, unlike the other basic tissues (epithelia, muscle, nervous), the cells in CT may be widely separated from one another within the extracellular matrix. In many types of connective tissue, the matrix-secreting cells are called fibroblasts assigned to formation and maintenance of the matrix. An abundance of other cell types with different functions may also be present like defence (macrophages, mast cells, lymphoid cells) and specific functions (adipocytes). Fibroblasts are the principal cells of connective tissue. They are responsible for the secretion of all types of fibres (collagen, reticular, elastin) and the complex carbohydrates of ground substance. A single fibroblast is believed to be able to secrete all the extracellular components, both sequentially and simultaneously. In histological preparations, only the nucleus of the fibroblast can be identified, the cytoplasmatic processes blend in with the surrounding collagen. After the biosynthetic activity fibroblast become fibrocytes.
There are three types of fibres secreted by connective tissue cells: collagen fibres, reticular fibres, and elastic fibres. The abundance and preponderance of different types of fibres varies in different CTs.

Collagen fibres: The most common fibre type is the collagen fibre. These are flexible fibres with a high tensile strength. At light microscope (LM), they appear as wavy lines of variable width and indeterminate length.

Reticular fibres: Reticular fibres are closely related to collagen fibres. They are made of type III collagen fibrils (sometimes in association with type IV collagen). The

individual fibrils that constitute the reticular fibre are of narrow diameter and typically do not bundle to form thick fibres.

Elastic fibres: Elastic fibres are thinner than collagen fibres and are arranged in a branching pattern to form a three dimensional network. They give tissue the ability to cope with stretch and distension. Elastic fibres are interwoven with collagen fibres in order to limit distensibility and to prevent tearing (FIG.4).

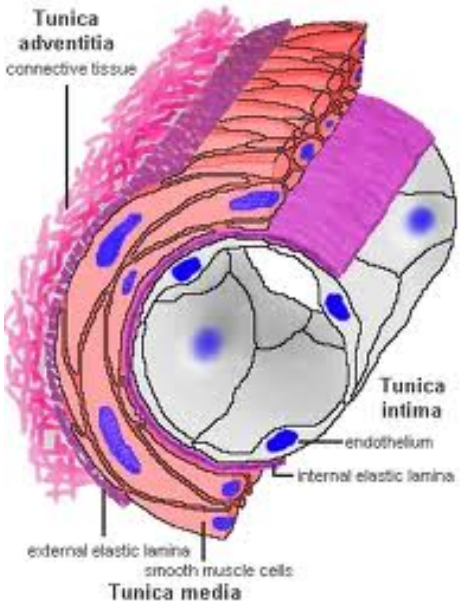

FIG.4 Diagram of vascular tissues

2. REGENERATIVE MEDICINE

Regenerative medicine and tissue engineering is an emerging and growing multidisciplinary field, involving knowledge in biology, medicine biotechnology and bioengineering, and focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including disease, trauma, congenital defects and aging. This field holds the promise of using the body's natural healing process to regenerate damaged tissues and organs, previously irreparable or to accelerate and secure the healing process with stem cell therapy and by the association with constructs apt to promote natural repair mechanism of biological tissues or to replace them. Tissue engineering has an important role in the development of regeneration field, thanks to the association between engineering and biological knowledge it is possible to realize specific constructs (FIG.5). A construct has to satisfy two important requisites. The scaffold, a biological structure that allows cells and extracellular matrix (ECM) growth and tissue assembly, and the cellular component able, in vitro and in vivo, to adhere and grow after seeding into the scaffold.

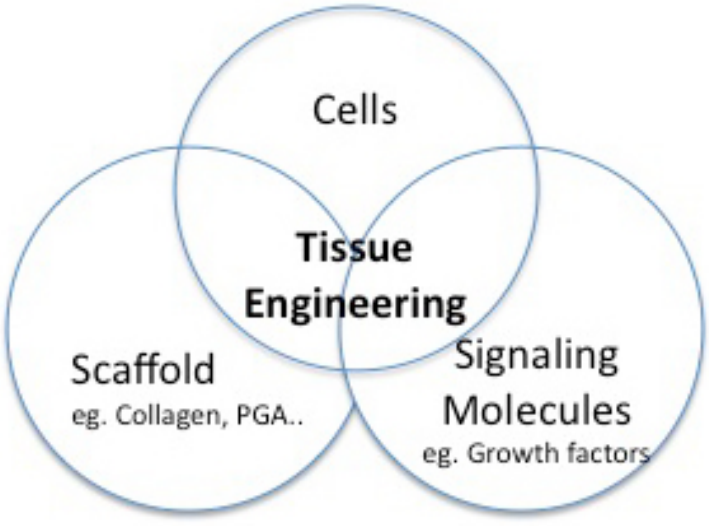

FIG.5 Triage of Tissue Engineering

Scaffolds must be biocompatible-materials, they can be natural or synthetic, with different structures according to the specific application they are made for. Scaffolds should hold cells, permit their adhesion in order to promote cell proliferation and differentiation. Instead cells are the active component in the construct, after their adhesion they start to proliferate, synthesize and depose specific matrix of the interested organ.

2.1 Highlights of Regenerative Medicine and Tissue Engineering

Alexis Carrel, a French surgeon and biologist, is considered the father of modern cardiovascular and transplant surgery. His visionary studies on cell culture and ex vivo organ preservation and growth anticipated concepts regarding tissue engineering and regenerative medicine. His investigations and his collaborative friendship with the aviator and engineer Linbergh brought in 1930 to the development of the first perfusion pump, which allowed living organs to exist outside of the body during surgery, opening the way to organ transplantation. Their invention was a crucial step in the development of perfusion systems for cardiac and transplant surgery, and for bioreactors currently used in regenerative medicine and tissue engineering investigations. Tissue engineering and regenerative medicine remained quiescent for the next 30 years after Carrell's death, during this time, several studies permitted the translation of findings from the bench to the bedside in the field of organ transplantation, which may be considered as one of the first forms of cell therapy in the history of medicine. Vacanti et al. in 1988 published one of the first tissue engineering experimental studies. In this study, were seeded fetal and adult rat and mouse hepatocytes, pancreatic islet cells, and cells from the small intestine onto synthetic scaffolds. These scaffolds were made of synthetic polymers organized into fibres networks that reproduced the extracellular matrices present in all organs that allows cells to remain viable by diffusion, promotes vascular ingrowth, and permits cellular proliferation. The following years were characterized by several variations and implementation of this principle and numerous tissues were constructed and implanted (Orlando G. et al, 2011). In the early 1990s tissue engineering principles combined with the increasing studies of adult stem cells and ESC

(Deans et al, 2000, Pittenger et al, 1999) unified these apparently distinct fields of science in one concept, the regeneration of living and functioning body parts destined to replace diseased or damaged cells, tissues, or organs. More often the terms "regenerative medicine" and "tissue engineering" are used as synonymous, but it's important to note that "regenerative medicine" indicate a field in the health sciences that aims to replace or regenerate human cells, tissues, or organs establishing normal function. This process may require cells, natural or artificial scaffolding materials, growth factors, or combinations of all three elements. "Tissue engineering" is a field of science that result by a combination of cells, engineering and materials methods and biochemical factors to improve or replace biological functions (FIG.6). Tissue engineering was also been defined as "understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use" (MacArthur et al, 2005).

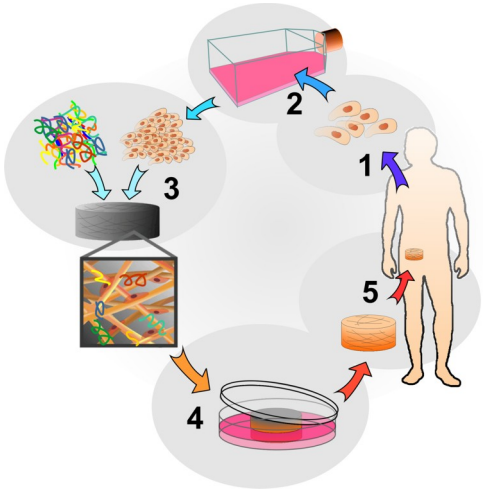

Fig.6 Example of tissue engineering as applied to various organ system

2.2 Tissue Engineered Blood Vessels

The main goal of a Tissue Engineered Blood Vessel (TEBV) is to create an autologous vascular graft for bypass surgery. However engineered blood vessel are also use in preclinical device evaluation. Tissue engineering manipulates material properties to

produces a unique configuration to mimic a specific tissue.

2.2.1 Highlights of Tissue Engineered Blood Vessels

The first report of vascular tissue engineered graft was in 1986 when Weinberg and Bell developed a vessel composed primarily of biological components: bovine smooth muscle cells (SMCs), and fibroblasts on a layer of collagen (Song Li et al, 2011). The collagen and SMCs were jelled together using a casting media to create a tubular lattice. After a week a Dacron sleeve was placed around the exterior of the tissue engineered 'media' and seeded with fibroblasts to enhance mechanical properties of vessel and mimic the adventitia. After another two weeks were seeded endothelial cells and left in culture for one week. After this period the central mandrel was removed and they obtained a tubular structure similar to a vessel. The mechanical strengths along with the scaffold structure of this vessel mimicked the mammary muscular artery, representing the first TEBV with increased physiological relevance and durability.

This first experiment was followed by several others studies, Foxhall et al., for example, worked with different coatings to promote endothelization. Endothelial cells were seeded on the luminal surfaces of Dacron coated with collagen and fibronectine the result was a surface that significantly increased the proliferation of endothelial cells. The obtained scaffold had similar in-vitro properties as a mammalian muscular artery and could be used to study the cellular interactions with a vascular ECM.

In 1998 L'heureux et al. developed a construct utilizing a simple collagen sheet on which were seeded human vascular SMCs, the sheet was put around a small mandrel to represent the media of a vessel. Then a similar sheet seeded with fibroblasts was put around the exterior of the SMCs, to mimic the adventia. Finally, the mandrel was removed and the lumen was seeded with ECs. L'heureux et al. developed a completely biologic vessel and with the potential for natural remodelling and reduction of foreign body reactions, but unfortunately, this model had limited mechanical strength (L'heureux et al, 1998; Shaikh F.M. et al, 2008).

The development of these and several other scaffold types put the bases for the creation of future TEBVs. Many researchers worked to create countless types of tissue-engineered grafts with the purpose to develop a model exportable into clinical

applications for cardiovascular disease.

Through the development of TEBV, several qualities have been identified to be extremely important to the graft function. As said before, the scaffold has to give a comfortable 'living environment' for cells, its structure should have small fibres randomly interconnected to permit the cells to anchor and encourage strong cell adhesion and proliferation. Scaffold must be microstructure that closely mimics the native ECM.

2.3 Synthetic scaffold

Scaffolds play a critical role in tissue regeneration and repair, in particular synthetic polymeric biomaterials act as synthetic frameworks referred as scaffolds, matrices, or constructs. Polymeric scaffolds have unique properties such as high surface-to-volume ratio, high porosity with very small pore size, biodegradation, and mechanical property. They offer distinct advantages of biocompatibility, versatility of chemistry, and the biological properties which are significant in the application of tissue engineering and organ substitution (Brahatheeswaran D. et al, 2011). Synthetic polymers are largely divided into two categories: biodegradeable and nonbiodegradeable. Some synthetic biodegradeable polymers are the family of poly(α-hydroxy esters) such as polyglycolide (PGA), polylactide (PLA) and its copolymer poly(lactide-co-glycolide) (PLGA) (Kakisis J. et al, 2005). Non-degradable synthetic polymers which are most often utilized in a TEBV are poly(ethylene terphalates) [Dacron] and ePTFE. Dacron and ePTFE are both a highly crystalline and hydrophobic material, that reduce the overall elusion of particles and structural integrity. Scaffold properties can be changed by manipulate the components of the polymer structure. This manipulation will contribute to cells interaction with the scaffold and to facilitate cells anchoring (Brett C. et al, 2005; Lovett M. et al, 2010). Even if these synthetic materials have been wildly used in bypass procedures a high rate of thrombosis formation has limited their use as small-diameter vascular grafts. In efforts to overcome these limitations, several tissue engineering approaches have been developed to prepare functional small diameter vessels in vitro (Peck M. et al, 2012). These techniques include culturing vascular cells

on a collagen matrix or other biodegradable synthetic scaffolds, such as polyglycolic acid (PGA). PGA along with other degradable scaffolds can be manufactured to have unique microstructures. Scaffold microstructure alterations may include increased porosity for better cell adhesion, a variety of degradation profiles for drug delivery or integration with the host, as well as specific mechanical properties for improved surgical handling or overall strength improvement (FIG.7). However, these tissue-engineered vascular grafts either lack sufficient mechanical strength or require long culture periods to obtain the mechanical strength that is required for implantation and are thus cost-and labor-intensive (Liqiong G. et al, 2009).

FIG.7 Synthetic graft

2.4 Biological scaffold

Recently, an alternative tissue engineering approach involving the decellularization of native tissues has shown success in a variety of applications. Decellularized tissues are composed of natural extracellular matrix (ECM) and have the advantage of maintaining the structure and mechanical properties of native tissues. The idea of using decellularized vessels in this manner has been around for at least 2 decades, but attempts at recellularizing this material are more recent and are now one of the more common tissue-engineering technologies (Stagemann J.P. et al, 2011).

In 1960 a group of researchers using primary tissues developed a scaffold by cross-linking the tissue, the immunogenic effects was reduced enough to create a viable scaffold source. Even though mechanical properties were lost and cell death occurred. In 1989 Laka et al. decided to use a detergent to break cellular bonds from the ECM,

leaving an acellular, biological graft. From this original paper, many other researchers further developed the decellularization process. There are many applications for which decellularization may be utilized; essentially any tissue that can be isolated can be decellularized. The major benefit of decellularization is not only the potential to create scaffold with ideal microstructure and mechanical properties, but also limit immunogenic effects of a graft and increase the potential tissue sources.

Decellularized biomaterial can be seeded with various cardiovascular cells, including endothelial cells, progenitor cells, and myocardial cells, to generate functional tissues, including blood vessels and other tissues. Additionally, decellularized tissues have the potential for repair, growth, and remodelling in vivo.

3. DECELLULARIZATION

Decellularization is the systematic removal of all cellular and nuclear components from tissues (cytoplasmatic material, membrane pieces and nucleic acid) to leave the complex mixture of functional and structural proteins that form the native ECM

Decellularization itself can partially damage the ECM, the removal process must be gentle enough to minimize its effects on the composition, biological activity, and mechanical integrity of the remaining ECM

Decellularization methods result often in a combination of chemical, physical and enzymatic techniques. Physic treatment as sonication, shaking, frozing-defrozing, are commonly used together with chemical solutions. The combinations between those methods permit the fragmentation of cell membrane and the consequent lost of cell contents.

Enzymatic treatments are usually done with trypsin a solution indicated for the rupture of intercellular and intracellular bound and transmembrane proteins. Fragmentation of membranes is realized with detergents and ionic solutions.

Decellularization protocols must guarantee cell membrane lysis (ionic solutions or physic methods); separation of cellular material from the proteins on ECM (enzymatic treatment or detergents); elimination of cellular rest from matrix structure. Mechanical treatment such as shaking or high pressure can be added to improve decellularization process.

There are several chemical solutions like alkaline and acid treatments, ionic or non-ionic solutions, zwitteronic solutions, hypotonic or hypertonic solutions. All those method can both be useful and damaging, it is important to choose the appropriate method for each tissue we want to decellularize.

Alkaline and acid solutions are very efficient and act as disinfectant against eventual microorganism infection on the construct, but unlikely is also too aggressive for the ECM, in which cause the rupture of peptides as GAG in collagene tissues.

Ionic detergents are efficient to solubilise both cytoplasmatic and nuclear waste materials, but they can damage some proteins too. The most used is Sodium DodecylSulphate (SDS), very efficient for the removal of cellular debris, it doesn’t damage collagene fibres but it can damage GAGs and small proteins.

The most used zwitteronic detergent is CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate) harmful for the ECM but optimum for the decellularization of vessel endothelium as do not damage collagen and elastic fibres. Hypotonic and hypertonic solutions generate osmotic shocks causing cells lysis; this treatment has to be used together with other detergent that eliminate cell debris.

After each decellularization process it is necessary to wash tissues with neutral solution, physiological solution or PBS, to avoid the persistence in the scaffold of cells material compromising the following cellularization.

3.1 Cellularization

Choose the right cell line to seed on the scaffold is the key of tissue regeneration. There are several methods used for cells seeding as injection, simple seeding or induction to migrate in it. Following proliferation and matrix deposition according with the phenotype, determine new tissue formation. Adult Stem Cells (ASCs) gained researcher's interest because they are undifferentiated cells characterized by self-renewal and ability to differentiate in almost all specialized cells types related to the tissue or organ. ASCs reside in all developed organisms, maintaining and repairing tissues in which they reside, as bone marrow, brain, heart, blood vessels etc. (Barrilleaux et al.2006, Gimble et al. 2007). Those characteristics render ASCs possible candidate for transplantation-based therapies. The most important alternative cells source for tissue regeneration especially for vascular tissue engineering are autologous human mesenchymal stem cells (hMSCs).

3.1.1 Mesenchymal Stem Cells

Mesenchymal stem cells were discovered in 1960 by Friedenstein, who identified in bone marrow two population of stem cells: Hematopoietic Stem Cells (HSCs) precursors of blood cells, and Mesenchymal Stem Cells (MSCs) precursors of cells supporting the formation of blood and fibrous connective tissue and bone, cartilage and

fat cells (Friedenstein et al,1974). MSCs are an archetype of multipotent adult stem cell able to differentiate into several mesodermal cells, like chondrocytes, osteocytes, adipocytes, myocytes and bone marrow stromal cells (Deans et al., 2000, Pittenger et al, 1999). Differentiation potential and tolerogenic properties confer to MSCs a promising role in future application in regenerative medicine field. One potential advantage of hMSCs lies in their harvest ease and large availability, they have been isolated from several tissues, including bone marrow (Pittinger et al, 1999), umbilical cord blood (Erices et al, 2000, 2003), peripheral blood, adipose tissue vascular wall (Pasquinelli G. et al, 2010) and placenta (Schdmidt. et al, 2009; Evangelista M, 2008), solving ethical and legal concerns. Moreover hMSCs present advantages over other type of stem and progenitor cells for the other following reasons: (1) long-term crypreservation; (2) extraordinary plasticity, (3) easy genetic manipulation (Moby V. et al, 2010).

hMSCs have a fibroblast-like morphology, they are characterized by adherent growth on plastic and expansion under specific culture conditions, they were defined by a panel of non-specific surface antigens and by their in vitro e in vivo differentiation potential (*Javazon et al., 2004*). The gold standard assay utilized to identify MSC is CFU-F assay that identifies adherent, spindle-shaped cells that proliferate to form colonies (*Friedenstein et al., 1970*). hMSCs, were initially isolated as the plastic adherent fraction of bone marrow (*Friedenstein et al., 1970*). A Percoll density gradient was used to remove unwanted cell types, present in the bone marrow aspirate, and hMSCs were just a small percentage (estimated at about 0.001-0.01%).

Mononuclear cells are isolated with a gradient centrifugation and seed with fetal bovine serum (FBS). The adherent fraction is expanded in a limited number of passages (*Ulloa-Montoya et al., 2005*).

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (*Dominici et al., 2006*) proposed a pathway of antigen to define MSCs:

- CD105 identifies an epitope of endoglin (CD105), the Transforming growth factor beta (TGFβ) receptor III present on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells, it helps the enrichment of

stromal progenitors from bone marrow (*Short et al., 2003*);

- CD73 a glycoprotein, identified by monoclonal antibody SH3 and SH4, involved in B-cell activation (*Short et al., 2003*) and expressed in lymphocytes and endothelial cells.
- CD90 or Thy1: a 25–37 kDa N-glycosylated, glycophosphatidylinositol (GPI) anchored conserved cell surface protein, originally discovered as a thymocyte antigen. Thy-1 can be used as a marker for several stem cells and for the axonal processes of mature neurons. Structural study of Thy-1 lead to the foundation of the Immunoglobulin superfamily.

MSCs are negative for other markers of the hematopoietic lineage including CD34 considered a primitive HSCs marker (a transmembrane protein that defined ˜ 1% of normal bone marrow mononuclear cells including hematopoietic precursors/stem cells and normal endothelial); CD31 (a glycoprotein designed also platelet endothelial cell adhesion molecule-1 (PECAM-1) normally expressed on endothelial cells, circulating and tissue hematopoietic cells including platelets, monocytes/macrophages, granulocytes and B-cells); CD45 (recognizes a family of proteins known as the leukocyte common antigens expressed on the surface of almost all haemato-lymphoid cells and their progenitors) and CD14 or CD11b (an immune cell marker).

Stro-1 is a well know MSC marker, Stro-1 negative cells are not able to form colonies. Stro-1 positive cells can become HSC-supporting fibroblasts, smooth muscle cells, adipocytes, osteoblasts and chondrocytes (*Dennis et al., 2002*). In addition, the expression of Stro-1 distinguishes two populations of MSCs with different location and HSC-supportive abilities (*Bensidhoum et al., 2004*). However, Stro-1 is not specific for these cells and its expression in MSCs is gradually lost during culture expansion (*Gronthos et al., 2003*) so Stro-1 labelling is possible only during early passages.

After their discovery, several studies demonstrated the multilineage differentiation potential of MSCs populations showing their capacity to develop into differentiated mesenchymal phenotypes including bone (*Bruder et al., 1997*), cartilage (*Kadiyala et al., 1997*), tendon (*Young et al., 1998*), muscle (*Ferrari et al., 1998*), adipose tissue (*Dennis et al., 1999*) and hematopoietic-supporting stroma (*Prockop et al., 1997*) and showing also a high degree of plasticity (*D'Ippolito et al., 2004, Zhao et al., 2002*) (FIG.8).

MSCs easy isolation technique and their expansion potential, migratory capacity and immunosuppressive ability made them a popular cell type for regenerative medicine, gene therapy and tissue engineering.

Several studies on animal transplantation shown that ex-vivo expanded MSCs were able to differentiate into cells of the residing tissue, to repair damaged tissue and to restore partially its normal function, generating promising results for the treatments of several illness, including bone (*Mauney et al., 2005*), cardiovascular (*Zimmet et al., 2005*) and brain disease *(Zimmet et al., 2005*). Moreover it seems that MSCs have paracrine activity, the bioactive factors produced can suppress the local immune system, inhibit apoptosis and enhance angiogenesis (*Caplan et al., 2006*).

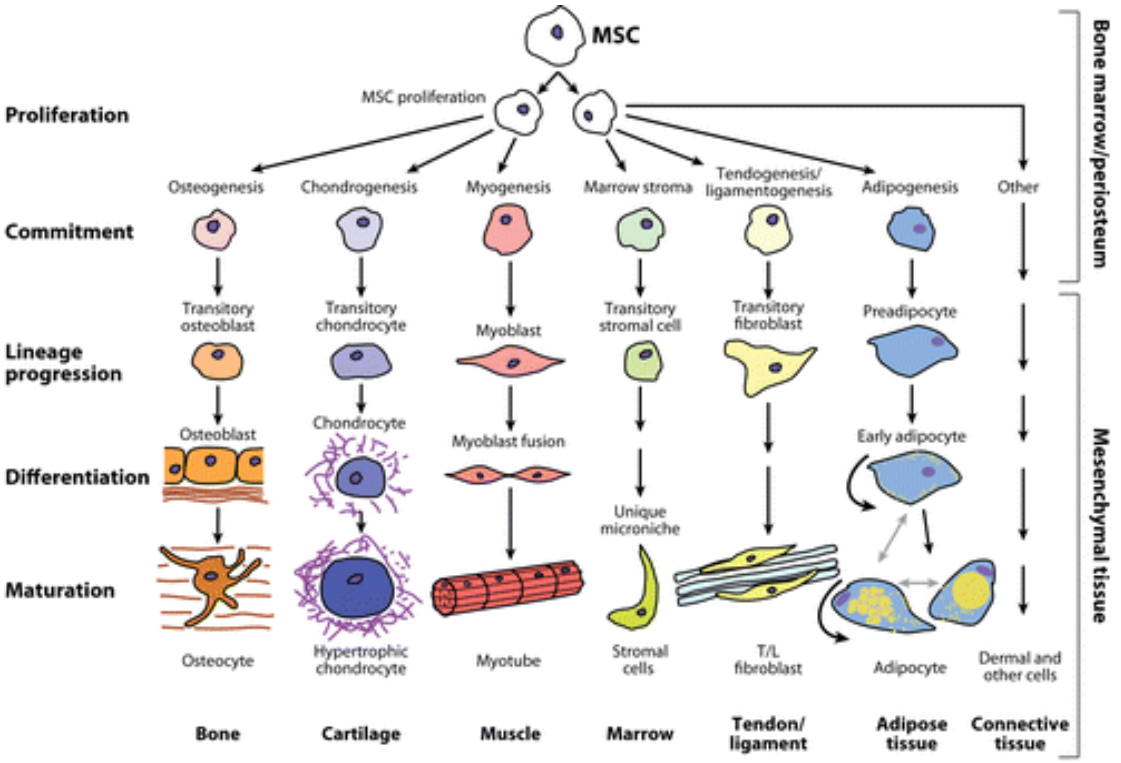

FIG.8 Plasticity and mesengenic process of MSCs

3.2 Tissue-engineering bioreactors

A bioreactor is a system or device with the purpose to simulate an active environment that is close to a biological system. The use of bioreactors in tissue engineering field is widely increasing for the several following advantages: (1) facilitate cell seeding procedures on both sides of a 3D tubular matrix; (2) allow seeding and culturing of different cell types on either side of the tubular scaffold; (3) enhance oxygenation of the culture medium and mass transport (oxygen, nutrients and catabolites) between the medium and the adhering cells; (6) stimulate the cells with hydrodynamic stimuli, favouring the metabolic activity and the differentiation process; (7) allow the achievement and maintenance of sterility and other criteria of Good Laboratory Practice (Asnaghi M. et al, 2009). Bioreactors are normally made of Plexiglas and the system consists of three different chambers: air chamber I, cell culture medium chamber II, and cell-seeding and perfusion chamber III. The tissue-engineered conduit is surrounded and continuously perfused by cell culture medium that is recirculating through a closed loop connected to a reservoir filled with cell culture medium. The whole system can be placed in a regular humidified incubator at 37°C and 5% CO2 and air driven by a simple dual-phase control ventilator, which is placed outside the incubator and connected to the air chamber of the bioreactor by a single silicone tube (FIG.9). To establish various pulsatile flows and biomechanical stresses it's necessary to adjust the stroke volume, the stroke rate, and the inspiration/expiration time of the ventilator. The flow rates ranged from 100 mL/min to 3 L/min, pressures ranged from 3 to 150 mmHg, and shear stress (t) ranged from 1.12 to 32.45 dyn/cm2 (Sodian et al, 2002).

FIG.9 Example of flow-pump-bioreactor

The main advantage in the use of bioreactors in the production on vascular tissue engineered graft is that the perfusion system mimic the physiological flow of cardiovascular system, and more over is possible to produce vascular grafts in a manner that is reproducible, so that not only each graft is the same, but also so that the bioreactor produces the same cell derived graft each time it is used.

EXPERIMENTAL DESIGN

4. AIM OF THE STUDY

This Introduction has described the background for which this thesis is based.
In cardiac or peripheral bypass surgery, diseased arteries are replaced with autologous veins or, less frequently, arteries. However more often patients in need of bypass surgery do not have suitable vessels or absence of veins as replacement due to the systemic vascular disease. In clinical practice are commonly used synthetic vascular prothesis that are good conduits in high-flow low-resistance conditions such as large peripheral arteries, but have difficulty in their performance as small diameter vessel grafts.
Based on published use of decellularized tissues as biologic scaffolds, this thesis explored the development of decellularized human vessels for use as tubular graft.
Cell-free vessels are expected to have more biocompatibility when compared to synthetic vascular conduits; and are optimal natural 3D matrix templates for driving stem cell growth and tissue assembly in vivo.
To investigate the potential use of these cell-free conduits as scaffold in this thesis will be discussed three main aspects: establishment of a decellularization protocol, mechanical characterization of the decellularized scaffold, and finally re-cellularization of the scaffold.

The following is a summary of the specific aims pursued in this thesis.

- Aim 1: Establish a decellularization protocol for the complete decellularization of human arteries and evaluate the success of the decellularization protocol using histology.
- Aim 2: Determine the ability of the decellularized scaffold to maintain the native mechanical properties by assessing the ECM matrix with TEM analysis.
- Aim 3: Develop a protocol to recellularize the scaffold, by evaluating the right cell type and preconditioning it in appropriate bioreactors.

5. MATHERIAL AND METHODS

5.1 Decellularization Protocol Development

All vessels, fresh and cryopreserved, were harvested from heart beating (HB) and non-hearth beating (NHB) donors at the Regional Bank for Cardiovascular Tissue of the Sant'Orsola/Malpighi Hospital. The chosen segments were femoral arteries and veins and carotid arteries.
Harvested vessels were maintained for 72 hours in an antibiotic mixture (mefoxin 240mg/ml, lincomycin 120mg/ml, colimycin 100mg/ml, vancomycin50 mg/ml); after decontamination were taken to the lab for cleaning, or the removal of all the connective tissue using a scalpel, razor blade, and scissors.
Crypreserved vessels were first thawed in a 37°C water bath until the structure was malleable; both vessells were washed in physiologic solution (0,9 % sodium chloride) to eliminate DMSO in cryopreserved ones and antibiotic solution from the fresh ones. After all the procedures the vessels were ready to be used for any experimentation.

The preliminary decellularization parameters were based on existing protocols in literature, including the duration of decellularization, detergents and temperatures.

Protocol 1

Short time protocol but too aggressive for the ECM. This protocol requires two incubations in two different sterile buffers.

Buffer I: 0,1% Trypsin 0,02 ethylenediaminetetraacetic acid (EDTA) in PBS without Mg^{++} Ca^{++}, for 36 hours at 37°C (all Sigma, St. Louis, Mo., USA). The trypsin/EDTAsolution was changed every 12 hours.

Buffer II: 20 μg/ml RNase; 200 μg/ml DNase (Boehringer, Mannheim, Germany), for 4 hours at 37°C.

All incubations were conducted under continuous shaking.
After the decellularization process the scaffolds were washed several times for removal

of residual substances with PBS and stored in sterile PBS at 4°C.

Protocol 2

Short time protocol but not very efficient.

Buffer I: 0.1% SDS in distilled water, for 16 hours at 37°C

Buffer II: 0.9% NaCl 3 passages for 3 hours each.

All incubations were conducted under continuous shaking. After the decellularization process scaffolds were left in NaCl for 24 hours and after that washed in sterile PBS.

Protocol 3

A nonenzymatic protocol that associate hypotonic shock and low-concentration ionic detergent.

Buffer I: Tris 10 mmol/L; 0.1%; ethylendiamine tetraacetic acid (EDTA) in distilled water, pH 8 for 14 hours

Buffer II: 0.1% Sodium dodecylsulfate (SDS) for 24 hours

Buffer III: Tris 50 mmol/L; NaCl, 0.15 mol/L; 0.1%ethylendiamine tetraacetic acid (EDTA) in distilled water, pH 8 for 24 hours.

All incubations were conducted at 37°C under continuous shaking.

5.2 Evaluation of decellularization process.

Histology was used to evaluate the decellularization process

5.2.1 Hematoxilin Eosin

Samples were fixed in 10% formaldehyde solution, embedded in paraffin to obtain 3μm-thick vessel sections for the histological staining. For conventional histopathological analysis sections were stained with hematoxylin & eosin (H&E)

according to the histological procedures and observed with LM. The staining method involves application of the basic dye hematoxylin which colours basophilic structures with blue-purple hue and the alcohol-based acidic eosin Y that colours eosinophilic structures in bright pink. Images were digitalized through a video camera (JVC 3CCD video camera, KY-F55B, Jokohama, Japan) connected with a Leitz Diaplan LM (Wetzlar, Germany); original images were taken at magnificatio 10X-20X and analyzed using the Image-Pro Plus® 6 software (Media Cybernetics, Inc., Bethesda, MD, USA).

5.3 Histological evaluation of Extracellular Matrix

5.3.1 Trichrome

Trichrome Picro-Mallory (Bio Optica Milano SPA) stains some components of connective tissue according to the different degree of affinity between dyes and tissue macromolecules. In particular, connective tissue is markedly acidophil because of the presence of many basic groups. 3µm-thick paraffin embedded sections were brought to distilled water and processed for the trichrome staining following the Picro-Mallory Trichrome manufacturer's instruction.

5.3.2 Weigert Assay

Weigert Van Gieson (Bio Optica Milano SPA) is a combination of stains used in histology to identify elastic fibres. The method is based on the affinity towards elastic fibers displayed by resorcin fuchsin, a precipitate resulting from a reaction between resorcin and basic fuchsin and ferric chloride. 3µm-thick paraffin embedded sections were brought to distilled water and processed for the weigert staining following the Weigert Van Gieson manufacturer's instruction.

5.4 Ultrastructural evaluation of Extracellular Matrix

5.4.1 Transmission Electron Microscopy

To evaluate the integrity of ECM samples were washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde and processed for transmission electron microscopy (TEM). Vascular tissue were rinsed in phosphate buffer and post-fixed in 1% buffered Osmium Tetroxide for 1 hour at room temperature (rt) to preserve the lipid structures and cell membranes. After fixation samples were washed, dehydrated in a graded series of ethanol from 70% to 100% (3x10 minutes each at rt), dipped in propylene oxide (2x15 minutes at rt) and then embedded in epoxy resin (50% Araldite CY12 + 50% Araldite HY + 1,5% Benzyldimetylammina (BDMA) as accelerator; all purchased from TAAB, England). The semi-thin sections obtained with ultramicrotome were stained with Toluidine blue. Ultrathin sections were cut with diamond knives using an ultramicrotome, transferred to specimen support grids, counterstained with uranyl acetate and lead citrate and observed in a Philips 400T trasmission electron microscope.

6. RESULTS

6.1 Haematoxylin Eosin

In Figure 9, two images from each protocol are compiled together. All sections were stained with H&E and imaged at 5X and 10x magnification.

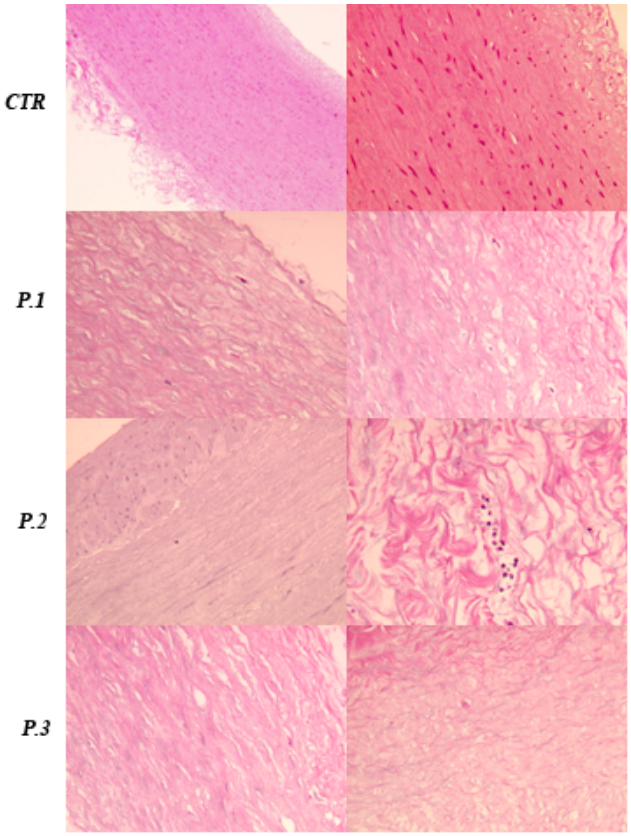

FIG.9 Haematoxylin/eosin images of the different decellularization protocols

Protocol 3 (P3) was considered to be the optimal for the decellularization process. Hematoxylin Eosin stain showed that the decellularization solution P3 is optimal for the elimination of cells or cellular component, apparently without degrading the vessel wall.

6.2 Thricrome Picro-Mallory and Weigert stain

In figures 10 and 11 are shown samples stained with Trichrome Picro-Mallory and Weigert. Here a comparison between control and cell-free samples, using the optimized decellularization protocol (P3).

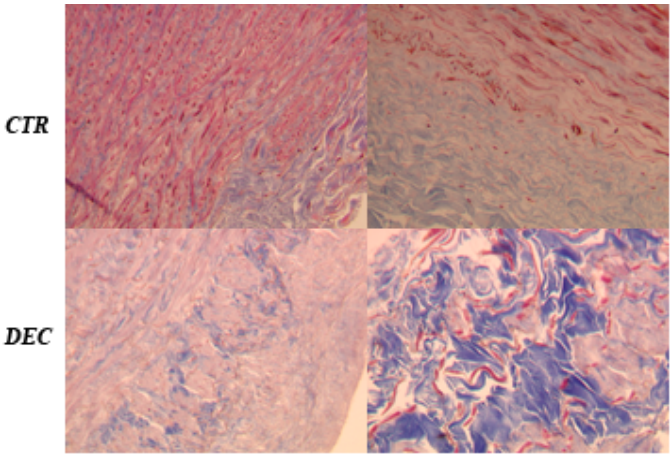

FIG.10 Trichrome Picro-Mallory Stain. A comparison between positive control (Ctr) and decellularized sections. In blue the collagen fibres.

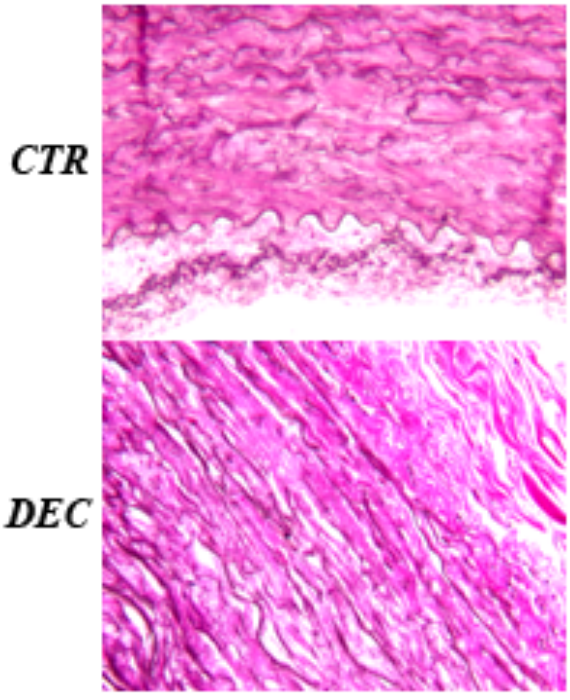

FIG.11 Weigert stain, A comparison between positive control (Ctr) and decellularized sections. In black the elastic fibres.

Trichrome Picro-Mallory and Weigert stains showed that the decellularization protocol did not damaged the collagen and elastic fibres of the extracellular matrix (ECM).

6.3 Transmission electron microscopy

With transmission electron microscopy (TEM) was evaluated the ultrastructure of the collagen and elastic fibres to better investigate the integrity of the ECM (FIG.12).

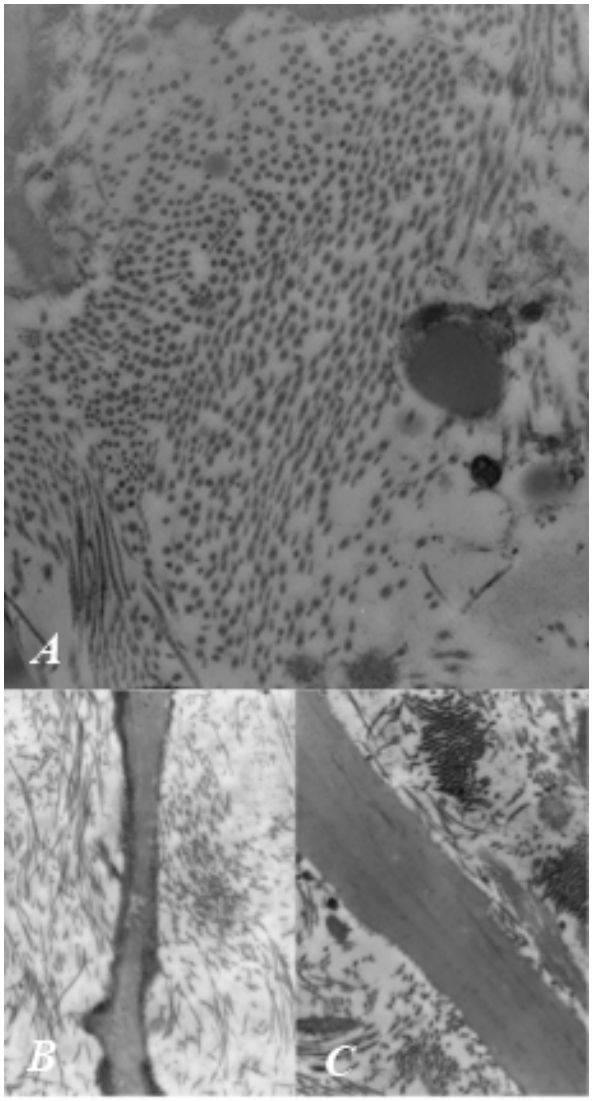

FIG.12 Collagen (A) and elastic (B,C) fibres in the ECM of a cell-free vessel

From the ultrastructural analysis emerged that collagen fibres are not damaged by

decellularization solutions but it seems they were subjected to a change in their normal arrangement, maybe caused by the empty spaces left from the eliminated cells.
Not any significant change was observed in the elastic fibres.

7. ISOLATION OF MSCs

7.1 Isolation of vascular wall mesenchymal stem cell from huma thoracic aorta

Cells were obtained from thoracic aorta segments harvested from heart beating (HB) and non-hearth beating (NHB) donors. Several pieces of thoracic aorta tissue were washed with sterile Hepes, mechanically minced and enzymatically digested with 0.1% collagenase type II (Sigma,Milan, Italy) in Hepes at 37°C for 40 min. The homogenate was subsequently pelleted by centrifugation at 1600 rpm and washed twice in PBS; the cells were then counted and plated at $1x10^6/cm^2$ in collagen biocoated culture flasks (BD Labware, Franklin Lakes, NJ) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% heat inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. After one week the non-adherent cells were removed by replacing the medium supplemented with 10% FBS. When the cultures were near confluence (approximately 1-2 weeks) the cells were detached by treatment with 0.25% trypsin-EDTA (Sigma) and were expanded up to passages 12- 15.

7.2 Isolation of myofibroblast from human umbilical cord

Human umbilical cord cells were harvested from fresh umbilical cords of healthy individuals after the obtainment of informed consents; human umbilical cord tissue was excised by biopsy from Wharton's jelly, washed with PBS and minced into pieces of approximately 8 mm^3. The tissue was then placed into petri-dishes and cultured in a humidified incubator at 37°C with 5% CO_2 in advanced DMEM medium (Gibco, Invitrogen) after few days supplemented with 10% fetal bovine serum (PAN Biotech,

Germany), 2mM Glutamax (Gibco) and Gentamycin (50μg/ml, PAN). After one week the non-adherent cells were removed by replacing the medium supplemented with 10% FBS. Umbilical cord myofibroblast cells (ucMFBs) were expanded up to passage 11.

7.3 Cells Characterization

7.3.1 Cytofluorimetry

Flow cytometer is able to analyze several thousand of cells every second and actively separate and isolate cells with specified phenotype. To detect surface antigen, cells taken at P3-P5 were washed twice with PBS containing 2% FBS and incubated for 20 minutes at room temperature using the following extensive conjugated moAbs panel: anti-CD29-fluorescein isothiocyanate (FITC), anti-CD31-phycoerythrin (PE), anti-CD34-PE, anti-CD146-PE, anti-CD44-FITC, anti-CD45-allophycocyanin (APC), anti-CD73-FITC, anti-CD90-phycoerythrin-cyanine 5 (PC5), anti-CD105-PE, anti-CD117-FITC (all from Beckman Coulter), anti-CD133-PE (Milteny Biotech) von Willebrand Factor (vWF, clone F8/86, Dako Cytomation), anti-KDR (Sigma), anti-NG2 (R&D System, Inc., MN, USA), anti-STRO-1 (clone STRO1, R&D System), anti-Notch-1 (clone C-20, Santa Cruz Biotecnology), anti-Oct-4 (clone C-10, Santa Cruz Biotecnology) and anti-Sca-1 (clone CT-6A/6E, Cederlane Laboratories). Secondary moAbs were used after cell staining with unlabeled primary moAbs: anti-mouse IgG-APC (Beckman-Coulter), anti-rabbit IgG-FITC (Dako Cytomation). For vWF and Oct-4 revelation cells were fixed and permeabilized with the IntraPep Kit (Beckman-Coulter) and then incubated with anti-mouse IgG-FITC (Dako Cytomation). Negative controls were performed using appropriate conjugated non-relevant antibodies. Samples were analyzed using a Cytomics FC500 flow cytometer equipped with two lasers for data acquisition (Beckman Coulter). Results were analyzed using the CXP Software (Beckman Coulter).

7.3.2 Immunofluorescence

Cells were plated at $1x10^3$ in 24 well culture dishes (Cellstar), cultured until near confluence, and fixed in 4% paraformaldehyde in PBS for 10 min. at room temperature. For intracytoplasmic protein staining, cells were permeabilizated with 0.2% Tryton X-100 in PBS. Unspecific staining was blocked by incubating cells with 5% Goat Serum in 1% Bovine Serum Albumine (BSA) in PBS for 30 min. at room temperature. The samples were then incubated sequentially with MoAbs for 60 min. at room temperature; MoAbs included the following: CD44 (1:50, Santa Cruz), CD90 (1:100 Biolegend), CD166 (1:50, Biolegend), Oct4 (1:20), CD146 (1:500), CD31 (1:10, Biolegend) and CD45 (1:200, Biolegend). Samples were rinsed three times with PBS and then incubated for 45 min at room temperature with the following secondary MoAbs: Cy2-G-a-M (1:100, Jackson ImmunoResearch) and Alexa Fluor 546-Phalloidin (1:50, Invitrogen); Nuclei counterstaining was performed with DAPI (1:200, Sigma-Aldrich). Negative controls were performed omitting the primary antibodies.

After three washes samples were mounted with the anti-fading reagent Lisbeth's embedding medium (0.1 M Tris-HCl pH 9.5; Glycerol containing 50mg/ml n-propyl gallate). Samples were observed under a Leica fully automated inverted fluorescence microscope DMI6000 B using a DAPI filter (Leica Microsystems, Milan, Italy).

7.4 Molecular analysis

7.4.1 RNA extraction and RT-PCR

Total RNA was isolated from human umbilical cord myofibroblast-derived cells using RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's instruction, and reverse-transcribed into cDNA (ThermoScript RT-PCR System, Invitrogen, Carlsbad, CA USA), which was used for PCR amplification with Taq polymerase (Bio Therm DNA Polymerase, GeneCraft GmbH, Ludinghausen, Germany).

The primers sequences for Oct4 were: forward 5'cgaccatctgccgctttgag 3' and reverse 5' ccccctgtcccccattccta 3' (577pb). For NANOG forward 5' cagaaggcctcagcacctac 3' and

reverse 5’ ctgttccaggccaggcctgattgtt 3’(214bp). For SOX2 forward 5’ cccccggcggcaatagca 3’ and reverse 5’ tcggcgccggggagatacat 3’(448bp).

Amplification of GAPDH (235pb; forward 5’ gaaggtgaaggtcggagt 3’ and reverse 5’ gaagatggtgatgggatttc 3’) was used as a positive control.

The amplified fragments were analyzed by 2% agarose gel electrophoresis, GelRed stained.

8. RESULTS

8.1 Cytofluorimetry

Umbilicard cord myofibroblast isolated were analyzed for the single expression of characteristic markers generally used to identify the MSCs and stem cells using a flow cytometric analysis.

Fibroblast-like cells were evaluated at their surface with a panel of antigens found in MSCs (CD90, CD44, CD105, CD29, CD73); hematopoietic lineage (CD45), hematopoietic progenitor (CD117, CD34, CD133), endothelial cells (CD31, vWF, CD146, KDR) and stemness markers (c-Kit, Notch-1, Oct-4, Sca-1, Stro-1) and perycite cells (NG2). As other mesenchymal stem cells, ucMFBs expressed typical mesenchymal antigens such as CD90, CD44, CD105, CD29, CD73 and NG2. On the contrary, they were negative for the early and mature hematopoietic markers CD34 and CD133 and for mature hematopoietic markers CD45. ucMFB were also negative for KDR, CD146, CD31 and vWF which are markers of differentiated endothelium whilst c-Kit was not detected. (FIG.13 A)

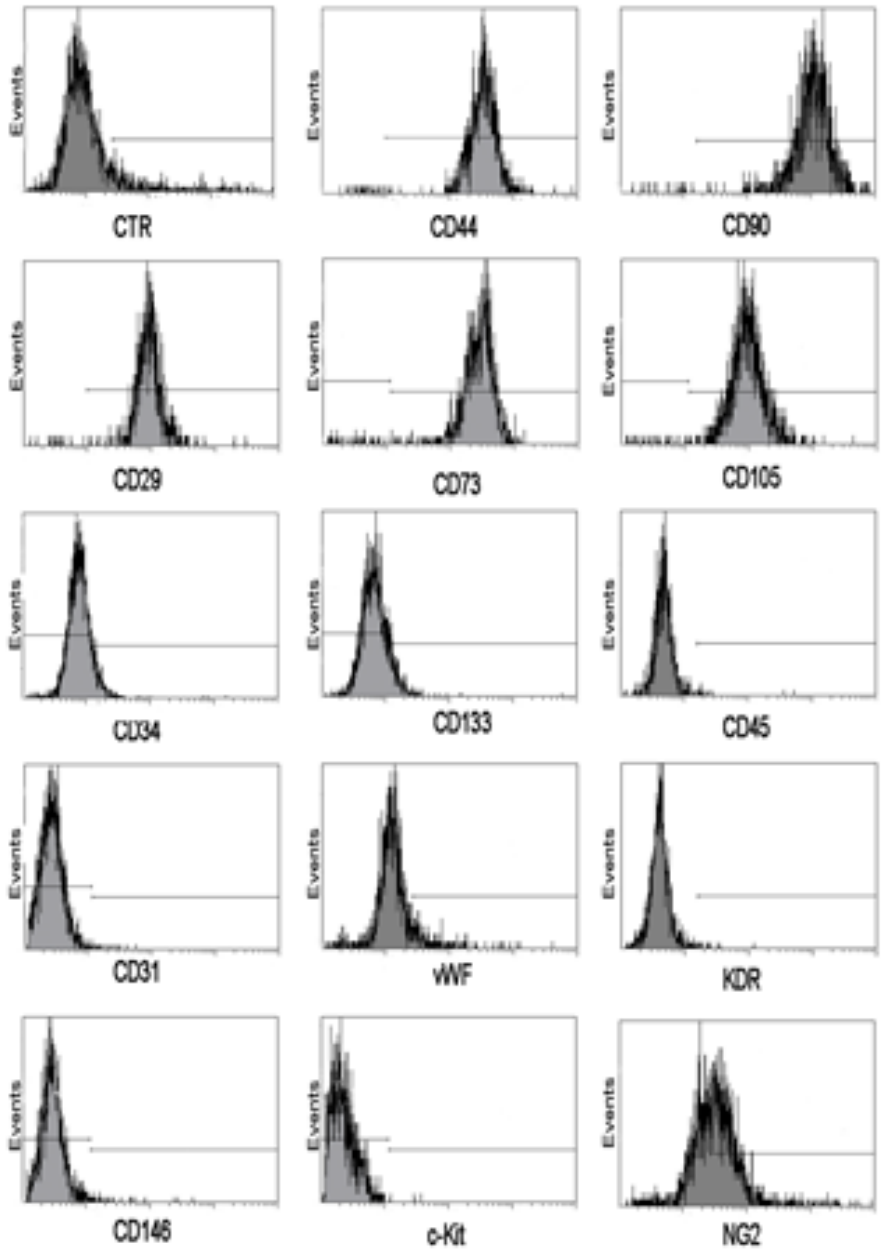

FIG.13 (A) Flow cytometry analysis of typical CD markers present on MSCs and typical hematopoietic and endothelial markers which are not expressed by MSCs

Cells expressed staminality markers such as Oct-4 and Notch-1; Sca-1and Stro-1 were also expressed although at lower level**.** (FIG.13 B)

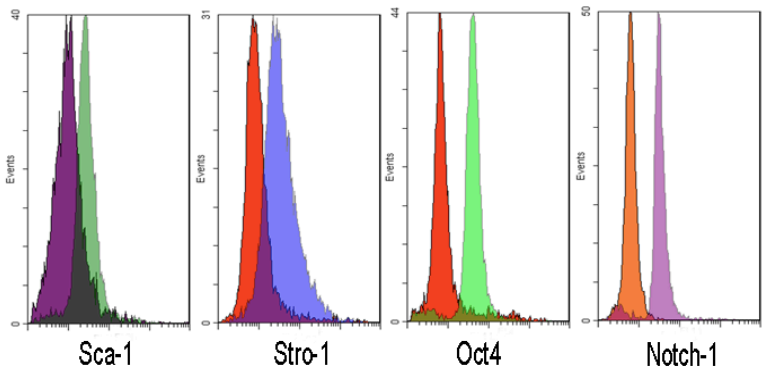

FIG.13 (B) Immunophenotype of staminality markers.

8.2 Immunofluorescence

In addition to flow cytometry analysis, a single immunofluorescence staining was performed to investigate the smooth muscle phenotype of ucMFB isolated. Cells were stained with intracytoplasmatic antigens against to smooth muscle markers (α-SMA, Calponin, h-Caldesmon, Vimentin, Desmin, and PDGF-rβ) and Ki-67 a proliferation‟s marker. The fibroblast-like cells showed a mesenchymal phenotype resulted positive for Vimentin and Calponin while, surprisingly, we observed rare cells positive for the α-SMA staining and the absence of other smooth muscle markers like h-Caldesmon, Desmin and PDGF-rβ (FIG.14).

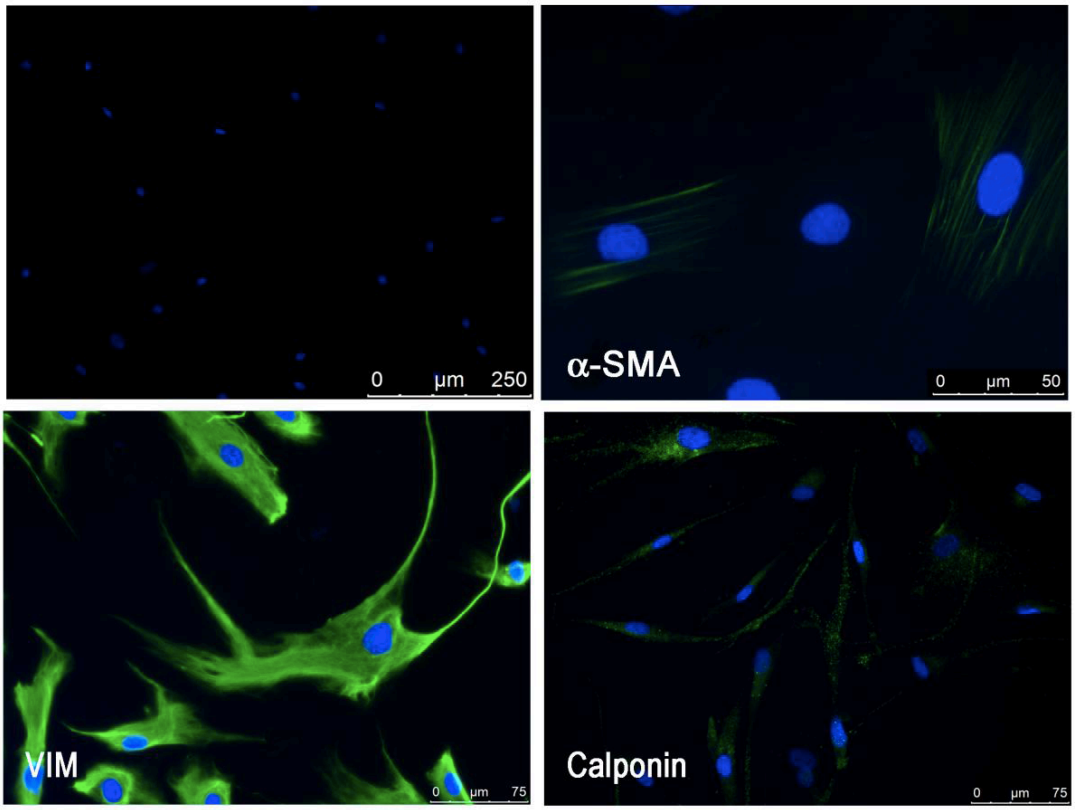

FIG.14 Immunofluorescence characterization of H-MSCs cultured on slide chambers. Cells were stained with moAbs directed against to smooth muscle markers. Negative control (A), α-SMA (B), Vimentin (C) and Calponin (D). Nuclei in blue and cell positive in green

8.3 RT-PCR

Gene expression of ucMFBs was evaluated by RT-PCR. This semiquantitative analysis confirmed that these cells expressed transcripts associated with embryonic stem cell marker, like Oct4 and NANOG.

Both Oct4 and NANOG are transcriptor factors involved in the self-renewal of undifferentiated embryonic stem cells. Nanog, is also known as a key factor in maintaining the pluripotency. The presence of SOX2 expression was not detected in umbilical cord myofibroblast cells.

The positive control, BM-MSCs, expressed all the molecules of undifferentiated embryonic stem cells, Oct-4, NANOG, SOX2. GAPDH was used as housekeeping gene.

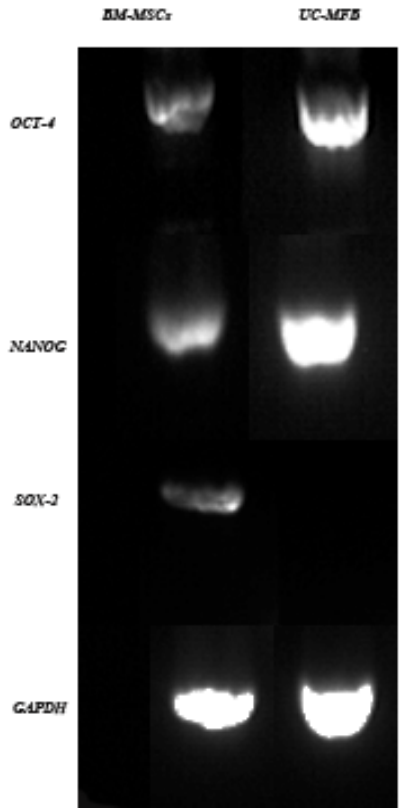

FIG.15 Stem cell genes expression. On the left it is shown the transcript expression of Bone Marrow-derived cells; on the right it is shown the transcript expression of ucMFB

9. DEVELOPMENT OF RE-CELLULARIZATION PROTOCOL

The aim of recellularization is to produce small diameter tissue engineered blood vessel (TEBV). For this purpose two different cell types were seeded on the cell-free blood vessels. vwMSCs and ucMFB were tested in their ability to adhere, penetrate and proliferate into the tissue.

9.1 Static cellularization

Decellularized vessels were cut into eight pieces of about $1cm^2$ each and placed in 12-well dishes (FIG.16). Four of these segments were seeded with $1.2x10^6/cm^2$ vwMSCs at the eleventh culture passage, covered with DMEM medium and placed in a humidified atmosphere incubator at 37°C with 5% CO_2. The other four segments were instead seeded with $1.2x10^6/cm^2$ ucMFB at the twelfth culture passage, covered with DMEM medium and placed in a humidified atmosphere incubator at 37°C with 5% CO_2.
Medium was replaced every 2/3 days, in both the experiments.

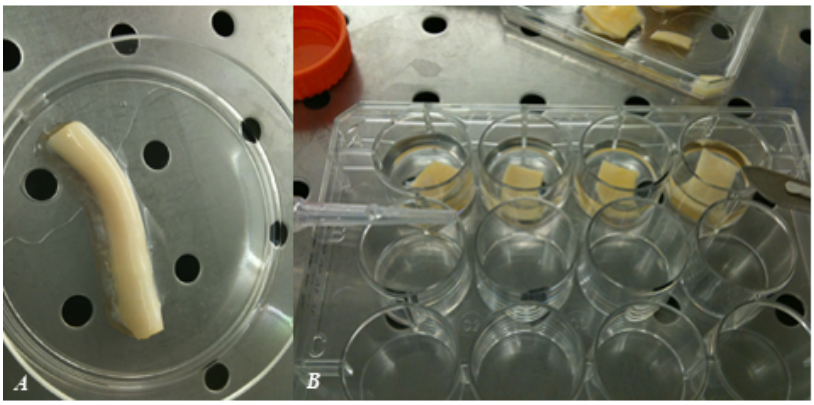

FIG.16 Carotid artery after decellularization (A); $1cm^2$ segments

Segments were taken at days 7, 14, 21, and processed for histological analysis.
From the analysis emerged that just few cells remained or penetrate into the tissue, instead most of the cells migrated outside the tissue preferring the plastic of the well dish, where they adhere.
Even though the amount of cell on the surface of the tissue was low umbilical cord

myofibroblast appeared more prone to interact with the tissue.

A second experiment was made seeding $1.5x10^6$ cells/cm^2 scaffold, using fibrin (Sigma-Aldrich, St. Louis, USA) as a carrier. The concentration of Fibrinogen (10 mg/mL) and Thrombin (10 IU/mL) was in accordance with the standard concentrations used in cardiovascular tissue engineering. After static incubation of seeded constructs in advanced DMEM supplemented with 10% FBS for seven days, they were processed for a preliminary histological analysis, that showed that cells thanks to the fibrin-thrombin compound stays on the surface of the decellularized scaffold.

9.2 Dynamic cellularizzation

Mammary artery of about 15 cm of length was decellularized with the optimized decellularization protocol, maintaining the natural tubular structure, $1.5x10^6$ cells/cm^2 scaffold were seeded inside the tubular scaffold using the Fibrinogen-Thrombin compound as carrier and after seeding the construct was placed in humidified atmosphere incubator at 37°C with 5% CO_2. After four days of static incubation in advanced DMEM, the construct was placed into a bioreactor flow system mimicking a pulsatile pressure cardiovascular flow environment. The bioreactor consisted in a silicone tube with a diameter of 1cm inside which is fixed the tubular graft, this silicone tube permit the flow of medium in both internal and external surface (FIG.17).

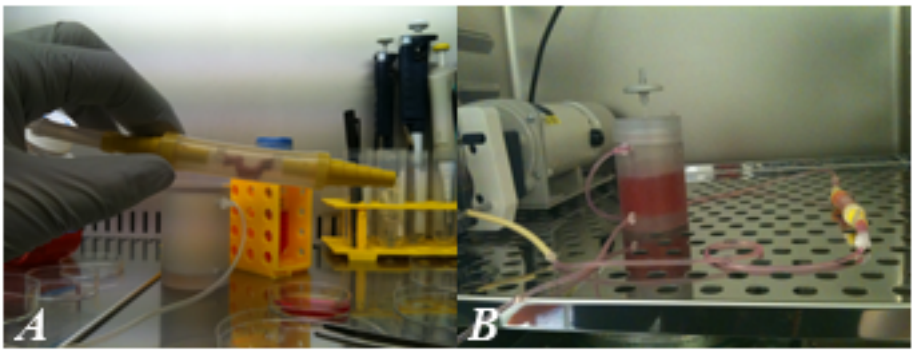

FIG.17 The tubular vascular graft seeded with uvMFB placed into the silicone tube (A); whole system into the incubator (B).

The whole system was placed in a regular humidified incubator at 37°C and 5% CO2 and air driven by a simple dual-phase control ventilator, which was placed outside the incubator and connected to the air chamber of the bioreactor by a single silicone tube.
The pulsatile flow was slightly increased over the study period and DMEM medium was changed every 3/4 days. Construct was harvested after 14 days of biomimetic conditioning accordingly (Weber B. et al, 2011).

10. EVALUATION OF RE-CELLULARIZATION AND MATRICE FORMATION

10.1 Histology

The tubular graft after fourteen days of dynamic incubation was harvested and processed for the paraffin embedding, as previous described. 3µm-thick sections were stained with haematoxylin & eosin (H&E) for the conventional histopathological analysis, according to the histological procedures and observed with LM.

10.2 Sirius Red Stain

Collagen is the major insoluble insoluble fibrous protein in the extracellular matrix. There are several methods for evaluating tissue fibrosis/collagen deposition, one of this is the Sirius Red stain. Sirius Red is a dye that binds to the [Gly-x-y] triple-helix structure found in all collagen fibers. (Corinne R. et al, 2011). 3µm-thick paraffin embedded sections previously stained with haematoxylin & eosin were brought to distilled water and processed for the Sirius Red staining following the manufacturer's instruction. Briefly, after de-waxing and rehydration sections were stained in 0,2% picro-sirius red for one hour at rt, washed in water and counterstained with hemalum. Sections were washed in water and dehydrated in three changes of 100% ethanol, cleared in xylene and mounted in a resinous medium.

Sirius Red is studied with a polarization microscopy, it presents different colours in regions where collagens I, II and III have been described. Collagen type I appears yellow, orange or red while collagen type III appears green.

10.3 Pop-off

The Pop-off method allows a precise area in a paraffin-embedded section to be re-embedded in resin. The area of interest in the stained paraffin-embedded section mounted on a glass slide is lightly circled with a diamond pencil on the surface of the slide before the coverslip is removed, the circled area should be of the same size of a BEEM capsule. The slide is then immersed in xylene to remove the coverslip and then rinsed with fresh xylene to ensure removal of the mounting medium.
When the slide is clean it is immersed in a solution containing xylene and propylene oxid in equal part for about two minutes at rt. This immersion is followed by another immersion in pure propylene oxid (2min at rt). The slide is then placed in a 2:1 mixture of propylene oxid and resin (2min at rt), followed by the placement in a 1:1 mixture of propylene oxid and resin (2min at rt) and then transferred in a 1:2 mixture of propylene oxid and resin for 10 minutes at rt. A labelled BEEM capsule is placed in a BEEM capsule holder (FIG.18). The BEEM capsule is completely filled with resin and placed in the holder. The section adhering to the glass slide is inverted over the top of the resin-filled capsule. The BEEM capsule holder and the slide are placed in a oven at 76°C for about 72 hours to carry out the polymerization process. After polymerization and cooling at room temperature the glass slide-BEEM unit is removed by pushing the capsule up from the underside of the holder. The glass slide is then placed on a 100°C hot plate for 15 seconds and after the capsule is cut off. The area of interest remains in the resin block and it is processed for the usual TEM analysis.

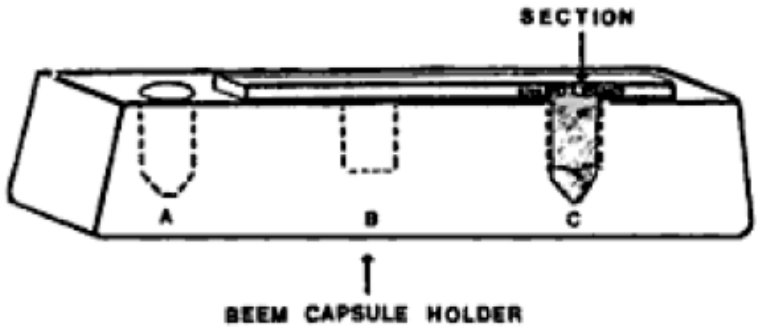

FIG.18 BEEM capsule holder for paraffin section reembedding. Standard capsule well (A); bottom of well cutaway (B); resin-filled capsule in cutaway well. The section is inverted over the capsule (Bretschneider et al, 1981)

11. RESULTS

11.1 Haematoxylin Eosin

In Fig.19, H/E stained sections of a seeded a-cellular scaffold. Samples were harvested after 14 days of dynamic culture in a flow-bioreactor. Seeded cells showed a little and almost undetectable tissue formation. Cells on the intimal surface appear healthy and in specific areas they start migrating into the tissue.

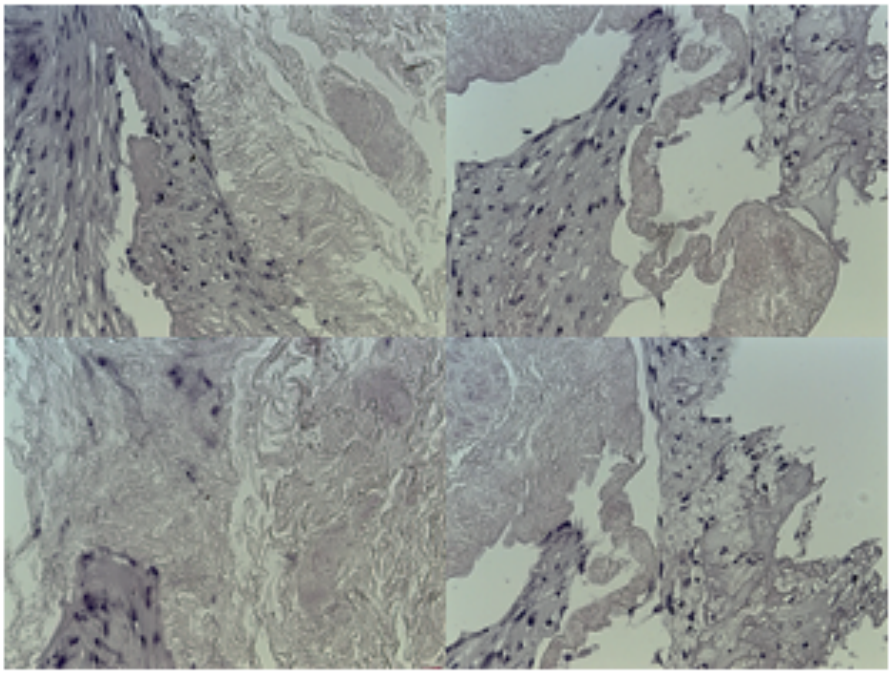

FIG. 19 10X magnification HE stained sections. Presence of cells on the intimal surface and their progressive migration into the tissue

11.2 Sirius Red

Sirius Red is a dye that binds to the [Gly-x-y] triple-helix structure found in all collagen fibers. It presents different colours in regions where collagens I, II and III have been described. Collagen type I appears yellow, orange or red while collagen type III appears green. In figure 20, Sirius Red staining showed layers of immature collagen type III,

presumably synthesized from the seeded ucMFBs lying on the intimal surface.

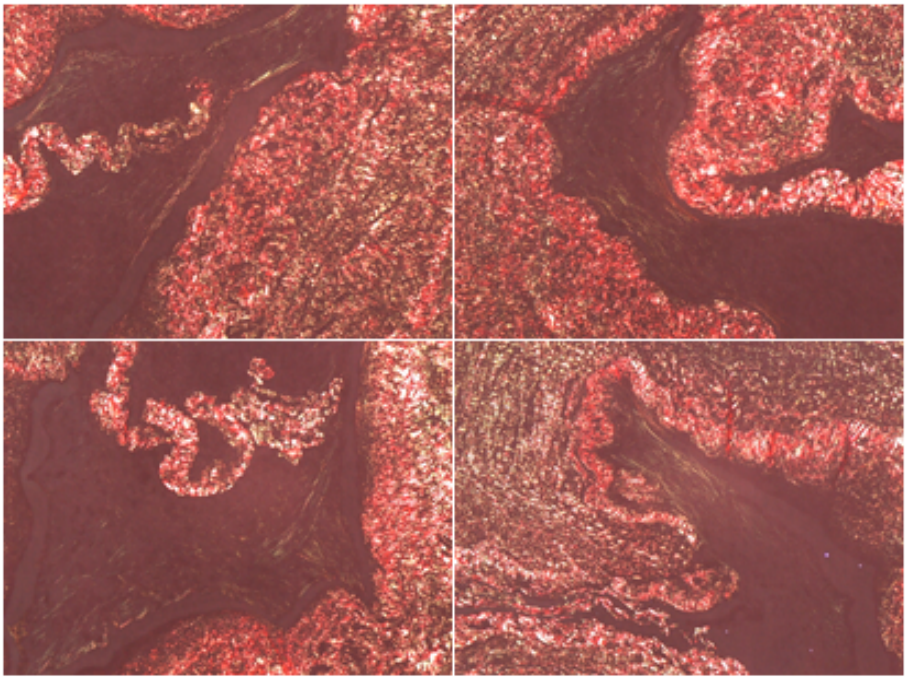

FIG.20 Sirius Red Staining; In green fibres of collagen type III close to the intimal surface

11.3 Pop-Off

Pop-off is method that allows a precise area in a paraffin-embedded section to be re-embedded in resin. Haematoxylin eosin stained sections of cell-free scaffold seeded with ucMFBs after the 14th day of dynamic culture in the flow-bioreactor, were processed with the Pop-off for the transmission electron microscopy (TEM) anlysis. Ultrathin sections were cut with diamond knives using an ultramicrotome, transferred to specimen support grids, counterstained with uranyl acetate and lead citrate and observed in a Philips 400T trasmission electron microscope.

In figure 21, ultrastructural analysis revealed 30 nm thick collagen fibres, presumably corresponding to the immature collagen.

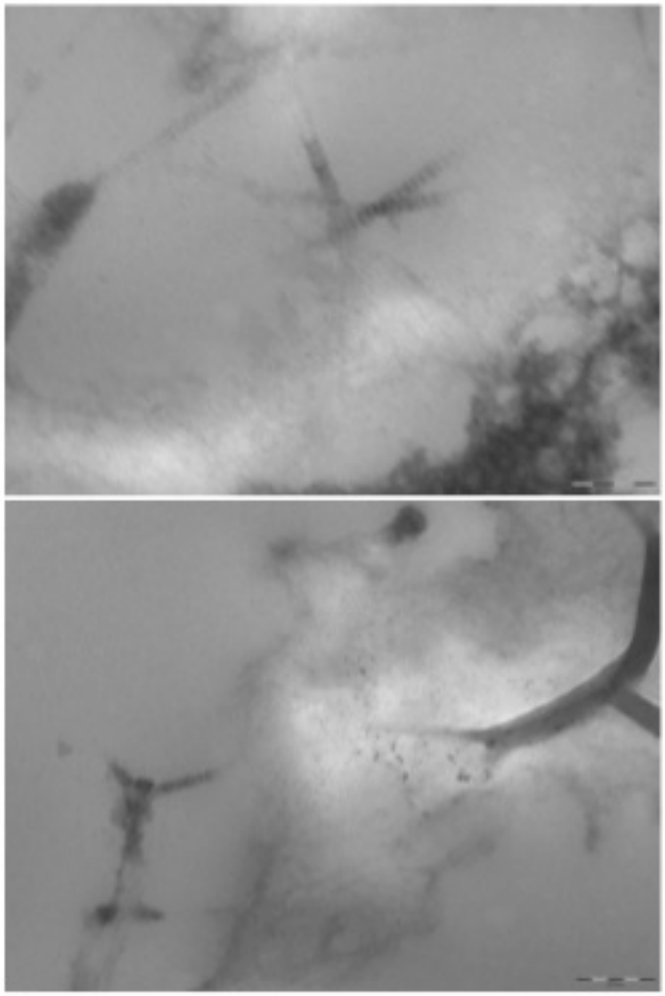

FIG.21 Ultrastructure of 30 nm thick collagen fibres

12. DISCUSSION

Cardiovascular disease, including coronary artery and peripheral vascular disease, is the leading cause of morbidity and mortality in word population, as reported by the World Health Organization (WHO). Peripheral vascular disease refers to a cluster of conditions in which atherosclerosis, or narrowing of blood vessels, occurs in the peripheral circulation, particularly in the legs. Even if, unlike myocardial infarction, has a relatively low risk of death PAD causes substantial disability as affected limbs are at higher risk of amputation and infection. These statistics demonstrate the growing need for the use and development of effective therapies to treat cardiovascular disease. The use of autologous vascular substitute like mammary or radial arteries seems to be a valid option in PAD treatment. However their use is limited by availability or quality, concomitant diseases, previous sampling, and moreover this approach require multiple surgical procedures. In the last years for these reasons synthetic xenograft made of poly(ethylene terphalates) [Dacron™] and expanded poly(tetrafluoroethylene) [ePTFE Teflon™] were successfully used. Good conduits in high-flow low-resistance conditions and clinically tested for large diameter (>6 mm) vessels, however almost all vascular conduits with a smaller diameter (<6mm) failed for several complications such as intimal hyperplasia, atherosclerotic degeneration or thrombosis. To summarize, the use of either a synthetic or natural graft has specific individual limitations resulting in a high demand for alternative treatments. Decellularization is a promising technique that can be utilized to remove cells from a tissue. Several groups have found a multitude of uses for the ECM from a decellularized tissue; a hypothetic idea is the in-vivo use of decellularized scaffold due to the lack of donors and/or viable tissues. New decellularization techniques will continue to develop to become a unique source of biologic materials for specific applications. The aim of this thesis is to examine the potential use of a decellularized artery as a more physiologically relevant scaffold type, when compared to current synthetic polymers. The use of decellularized scaffold provides several potential advantages including maintenance of structural integrity (mechanical strength and compliance) incorporation of natural binding sites for cells and reduction of immune response. The following summarizes the work performed in

this thesis to develop and implement the decellularized scaffold. After numerous trials, the final decellularization protocol included the successive immersion in 3 solution low-concentration ionic detergents containing Tris 10 mmol/L; 0.1%; ethylendiamine tetraacetic acid (EDTA); 0.1% SDS, Tris 50 mmol/L, NaCl, 0.15 mol/L, 0.1% EDTA, being continuously shaken at 37°C in a humidified atmosphere with 5% CO_2. Decellularization was histological evaluated with Haematoxylin and Eosin dyes to visualize eventual nuclei of resisted cells to the process and cellular material, and with Thricrome Picro-Mallory and Weigert staining to visualize the extracellular matrix. The histological evaluation showed the complete decellularization of artery resulting by the lack of hemotoxylin-stained nuclei, obtaining a potential biological-acellular scaffold type. Furthermore, histology and ultrastructural microscopy of collagen and elastic fibres showed that the ECM structure resisted to the harsh SDS washing remaining intact. The second aim of the work was the re-cellularization of cell-free scaffold. For this purpose was investigated their capacity to house cells. Myofibroblast-like cells isolated from human umbilical cord (ucMFB) and vascular wall-MSC cells (vwMSCs) were tested as potential cells source to re-cellularizate the scaffold. After a static culture seeding, ucMFBs showed a higher inclination to adhere, penetrate and populate the cell-free scaffold. To better mimic a native artery the seeded scaffold was placed in a bioreactor. The main advantage in the use of bioreactors in the production on vascular tissue engineered graft is that the perfusion system mimics the physiological flow of cardiovascular system. The use of bioreactors in tissue engineering field is widely increasing because it has been greatly described that they facilitate cell seeding procedures on both sides of a 3D tubular matrix; allow seeding and culturing of different cell types on either side of the tubular scaffold; enhance oxygenation of the culture medium and mass transport, between the medium and the adhering cells; and moreover stimulate the cells with hydrodynamic stimuli, favouring the metabolic activity and the differentiation process.

In conclusion, this research provided the documentation to support the potential for a decellularized natural human blood vessel to be utilized as a scaffold and it might provide a novel approach for generating small-diameter vascular grafts for vascular reconstruction surgery.

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