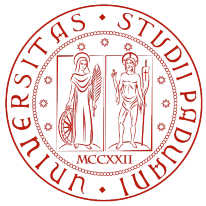

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SCUOLA DI DOTTORATO DI RICERCA IN MEDICINA DELLO SVILUPPO E SCIENZE DELLA PROGRAMMAZIONE SANITARIA
INDIRIZZO MALATTIE RARE
XXVIII CICLO

L'INGEGNERIA TISSUTALE E L'USO DELLE MATRICI DECELLULARIZZATE NELLA TERAPIA SOSTITUTIVA.

OESOPHAGUS DECELLULARISATION AND STORAGE FOR TISSUE ENGINEERING PURPOSES.

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ABSTRACT

BACKGROUND: Oesophageal Atresia (OA) is a congenital defect that occurs during gestation and prevents the correct oesophagus development in 1 every 3000-4500 births. Tissue Engineering could represent a therapeutic alternative for the most severe cases, where oesophageal replacement becomes necessary. Decellularised matrices are the ideal option because they are derived from tissue-specific extracellular-matrix (ECM) after removing all the cells therefore avoiding the risk of incompatibility and rejection. However, appropriate preservation may significantly affect scaffold behaviour.

AIM: here we aim to create a decellularised oesophageal scaffold in a large animal and to establish an innovative method of scaffold storing.

METHODS: rabbit oesophagi were decellularised using a detergent-enzymatic treatment (DET) and evaluated at 2 and 4 weeks, 3 and 6 months of storage. Four storage methods were compared: **SCM** (slow cooling in medium with 10% DMSO at -1°C/min, then stored in liquid nitrogen), **SF** (snap-freezing in liquid nitrogen, then stored in -80°C), **FD** (freeze-drying, then stored in -20°C) and **4C** (phosphate-buffered saline solution at 4°C). Structural and functional analyses were performed prior to and after each storage condition.

RESULTS: efficient decellularisation with preservation of the ECM was achieved after 2 DET cycles as evidenced by histology and DNA quantification. Only the SCM method maintained the architecture and biomechanical properties of the scaffold up to 6 months. On the contrary, all other methodologies failed long-term preservation of the original structure. In particular, SF-oesophagi displayed irreversible tissue collapse, FD-samples were impossible to rehydrate 3 and 6 months post-storage and the 4C option led to a progressive distortion of the tissue architecture.

DISCUSSION: scaffolds for oesophageal tissue engineering can be obtained in a large animal using a combination of detergent and enzymatic agents. Efficient storage allows a timely use of decellularised oesophagi, essential for clinical translation. Here we describe for the first time that slow cooling in a DMSO/medium solution and liquid nitrogen leads to reliable long-term storage of decellularised scaffolds.

RIASSUNTO

INTRODUZIONE: vi sono patologie nelle quali è necessario procedere a sostituzione totale o parziale dell'esofago. Tra di esse vi è l'atresia esofagea, una malattia dello sviluppo esofageo che colpisce 1 ogni 3000-4500 neonati. Tra le terapie emergenti ed innovative per il trattamento delle forme più gravi si propone l'uso di matrici decellularizzate create mediante tecniche di Tissue Engineering. Queste strutture rappresenterebbero la scelta ideale, in quanto esse mantengono le caratteristiche della matrice extracellulare (ECM) dell'esofago nativo, ma sono private della componente cellulare abbattendo il rischio di incompatibilità e di rigetto. Tuttavia va identificata un'adeguata strategia di conservazione delle matrici prodotte in laboratorio per garantirne la preservazione in modo da renderle disponibili e utilizzabili nella pratica clinica.

SCOPI: identificare un metodo efficace di produzione e conservazione delle matrici esofagee decellularizzate in un modello di grande animale.

MATERIALI E METODI: esofagi di coniglio sono stati prelevati e decellularizzati utilizzando un metodo che combina l'uso di detergenti ed enzimi (DET). Le matrici sono state analizzare dopo 2-4 settimane e 3-6 mesi dopo averle conservate in 4 modi differenti: SCM (congelate lentamente in medium e conservate in azoto liquido); SF (snap-frozen in azoto liquido e conservate a -80°C), FD (freeze-dryed e poi conservate a -20°C); 4C (in PBS a 4°C). Analisi di struttura e meccanica sono state effettuate ad ognuno dei 4 time-points.

RISULTATI: abbiamo raggiunto una decellularizzazione efficace con preservazione dell'ECM dopo 2 cicli DET (come mostrato dalla quantificazione del DNA e dall'istologia). Solo il metodo SCM preserva le caratteristiche strutturali e meccaniche della matrice fino all'ultimo time point dei 6 mesi. Gli altri metodi non sono efficaci, in particolar modo SF porta a collasso della micro-architettura tissutale, i campioni in FD non possono essere reidratati dopo 3 o 6 mesi e la conservazione a 4°C porta a progressiva distorsione delle strutture.

DISCUSSIONE: è possibile creare matrici esofagee decellularizzate utilizzando la combinazione di detergenti ed agenti enzimatici. Un metodo di conservazione efficace permette di preservare la matrice esofagea decellularizzata rendendola uno strumento concretamente utilizzabile nella terapia sostitutiva dell'esofago. Abbiamo illustrato come il metodo SCM sia il migliore a tale scopo.

LIST OF ABBREVIATIONS

AB	alcian blue
BW	birth weight
DET	detergent-enzymatic treatment
DMSO	dimethyl sulphoxide
ECM	extra cellular matrix
EVG	elastin van gieson
FD	freeze-drying
GAG	glycosaminoglycan
H&E	hematoxylin and eosin
LGOA	long gap oesophageal atresia
MT	masson's trichrome
OA	oesophageal atresia
PBS	phosphate buffered saline
PDT	primitive digestive tube
PR	picrosirius red
SCM	slow cooling in medium
SDC	sodium deoxycholate
SDS	sodiumdodecyl sulphate
SEM	scanning electron microscopy
SF	snap-freezing
TE	tissue engineering
TOF	tracheo-oesophageal fistula
UTS	ultimate tensile strength
4C	4 degrees Celsius

INDEX

1.0 INTRODUCTION

1.1 OESOPHAGEAL ATRESIA: A BRIEF OVERVIEW

1.1.1 DEFINITION

Oesophageal atresia (OA) is a congenital defect that occurs during gestation and prevents the correct oesophagus development (Fig. 1).

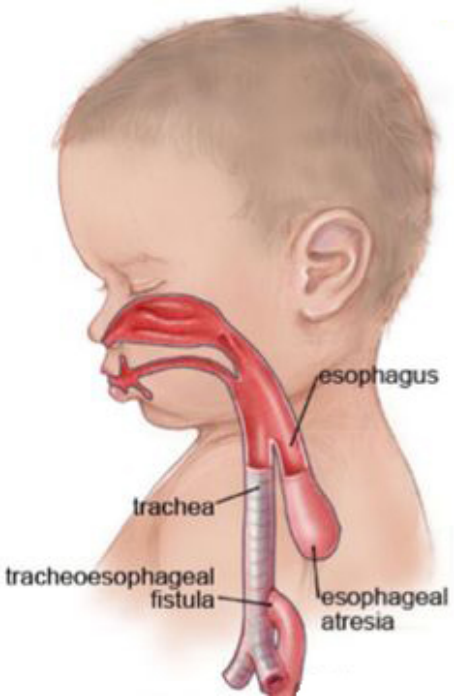

Figure 1. Oesophageal Atresia showing upper oesophageal pouch and a distal tract connected to trachea through a fistula.

The average incidence is 1 case in 3000-4500 births; the vast majority of the cases are sporadic even though a higher incidence can be detected in twins and a 2% risk of recurrence is present when a sibling is affected [1].

Despite numerous studies that investigated the embryological origin of the defect, the exact mechanisms behind OA development are still not fully understood. The primitive digestive tube (PDT) origins from the endoderm and gives rise to the first airway and digestive tracts.

A failure in PDT growth and septation could therefore lead to tracheo-oesophageal malformations. Moreover possible genetic basis are suggested by the higher incidence in twins and siblings and by the well-known association between OA and trisomies 21, 13, and 18. Many genetic pathways have been investigated in humans and animal models with no conclusive results.

From a structural point of view, OA can be classified based on the presence and position of a tracheo-oesophageal fistula (TOF) according to Gross classification [2] as shown in Fig. 2:

- type A: isolated oesophageal atresia: 8-9%
- type B proximal fistula with distal atresia: 1%
- type C: proximal atresia with distal fistula: 85%
- type D: double fistula with intervening atresia: 1-2%
- type E: isolated fistula (H-type): 4-6%

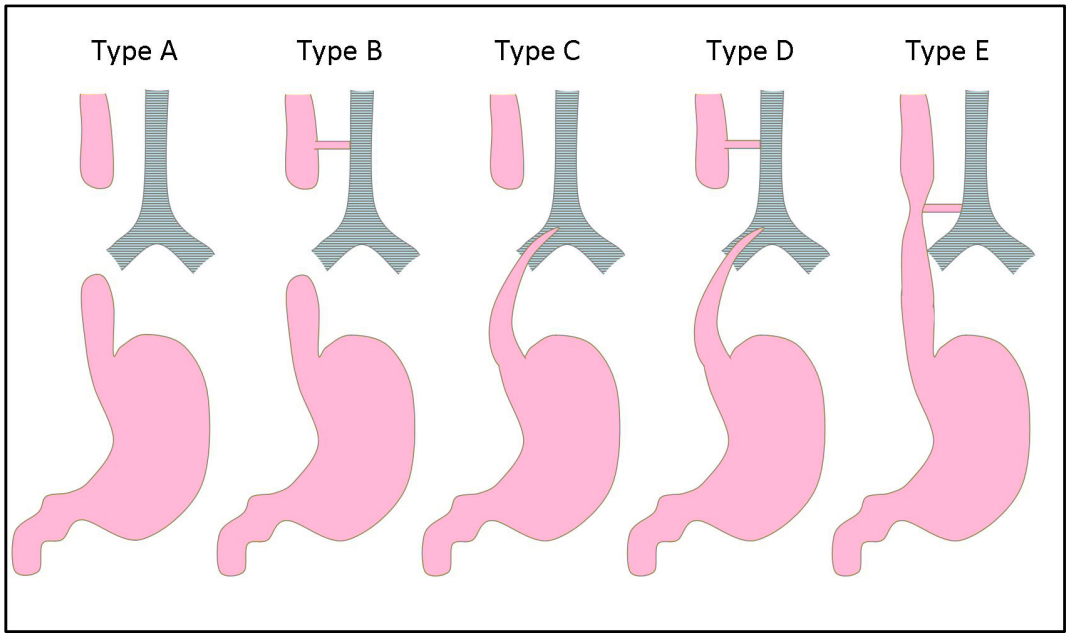

Figure 2. GROSS CLASSIFICATION OF OESOPHAGEAL ATRESIA. Among the different atresia types, the most common is associated with a distal fistula with the trachea (Type C).

1.1.2 DIAGNOSIS

Two signs that can suggest a prenatal diagnosis of OA are polyhydramnios and absent gastric bubble, both detectable using prenatal ultrasound scans. Even without fistula, these signs are non specific and their combination has a modest positive predictive value of roughly 50% [3]. Postnatal diagnosis is suspected in newborns that present with excessive drooling and abundant oral secretions, when episodes of coughing and

chocking manifest during feeding and when it is impossible to precede with a gastric tube. To confirm OA an X-Ray has to be performed showing the proximal oesophageal pouch where the gastric tube is curling, absence of the gastric bubble will exclude a distal tracheo-oesophageal fistula (Fig. 3). Tracheobronchoscopy can be useful to better define the presence and anatomy of the fistula and to identify other potential airways anomalies.

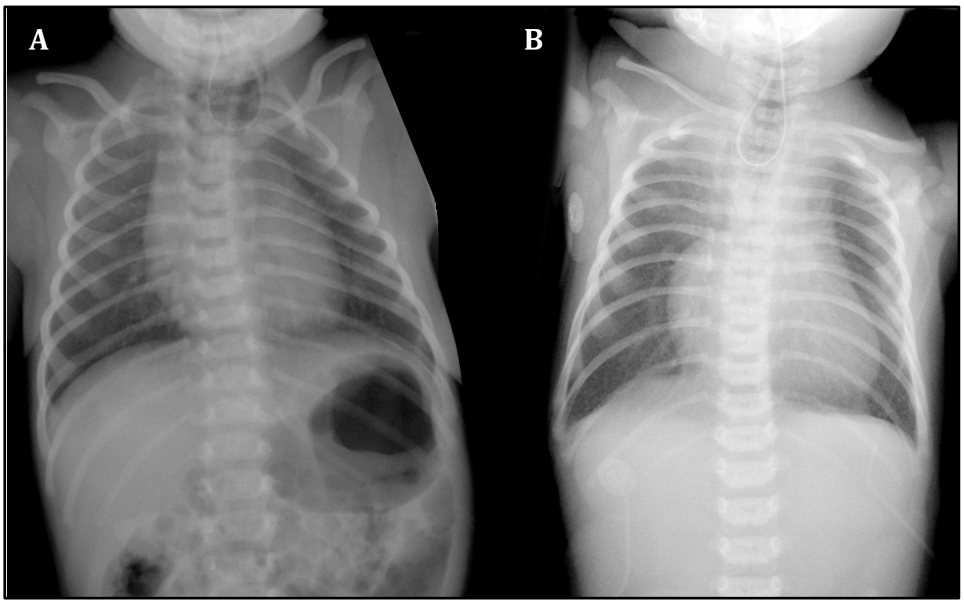

Figure 3. *X-RAY SHOWING OESOPHAGEAL ATRESIA. A nasogastric tube is coiling in the upper pouch of the oesophagus. Presence of a gastric bubble suggests a fistula connecting the trachea with the distal tract of the oesophagus.*

Further analysis are then necessary to investigate the presence of the multiple possible associated anomalies, which may involve 50% of the patients with an impact on their treatment and outcome. The most common anomalies include:

- cardiovascular defects (23%)
- musculoskeletal malformations (18%)
- anorectal and intestinal malformations (16%)
- genital-urinary malformations (15%)
- head and neck malformations (15%)
- mediastinal anomalies (8%)

- chromosomal anomalies (5.5%)
- complex syndromes like VACTERL (vertebral, anal, cardiac, tracheal, renal, limbs malformations) and CHARGE (coloboma, heart defects, choanal atresia, retarded growth, genital hypoplasia, ear deformities)

1.1.3 OUTCOME

A detailed pre-operatory evaluation will allow determining the risk classification and the survival rate of the patients. Using the Spitz classification patients can be divided into 3 groups based on birth weight (BW) and presence of major cardiac anomalies (defined as cyanotic congenital heart diseases that require palliative or corrective surgery or non-cyanotic heart anomalies requiring medical or surgical treatment for cardiac failure) [4,5]:

- Group I, survival 98%: BW>1500 g without major cardiac anomaly.
- Group II, 82% survival: BW<1500 g or major cardiac anomaly.
- Group III, 50% survival: BW<1500 g with major cardiac anomaly.

1.1.4 THERAPEUTIC STRATEGIES

Medical Treatment: in the pre-operatory period it is mandatory to protect the airways from the oral secretions that could cause episodes of chocking, respiratory distress and pneumonia. A constant suction of the proximal pouch with a flexible orogastric tube is therefore necessary. In the preterm infant with respiratory distress there may be a need for endotracheal intubation and mechanical ventilation. The respiratory gases and the raised pulmonary resistance increase the risk of gastric overdistension and rupture, which could be minimized reducing the chosen respiratory pressure and placing the tip of the endotracheal tube below the estimated level of the tracheo-oesophageal fistula.

Surgical Treatment: from the early successful attempts done in 1939-1940, different surgical procedures have been optimized with the aim to regain continuity of the oesophageal tract and to close the fistula.
The timing and method of the surgical approach is based on OA type, clinical stability and on the associated malformations.

- Oesophagus primary repair and fistula ligation

  Most of the patients undergo surgical repair in early infancy. When the distal and proximal pouches are close enough to allow a tension-free anastomosis, this can be performed through a thoracotomy o thoracoscopy, according to patient clinical state and surgeon experience.

- Two steps repair: oesophageal-lengthening procedures

  Patients with OA and wide separation of the oesophageal ends continue to represent a major surgical challenge. The following procedures were designed to treat long gap oesophageal atresia (often Type A) aiming to induce an oesophageal elongation and to allow a subsequent anastomosis when the proximal and distal pouch would be closer enough to avoid an excessive tension. In more severe cases, anastomosis may be delayed, with placement of a cervical oesophagostomy, until sufficient oesophageal growth occurs to allow anastomosis.

  1. *Kimura procedure:* the protocol consists of initial cutaneous oesophagostomy of the proximal oesophagus followed by multistaged extrathoracic elongations of the proximal oesophagus prior to an end-to-end oesophageal anastomosis [6].

2. *Livaditis technique:* at time of anastomosis a circumferential oesophagomyotomy is used to elongate the upper pouch by about 1 cm for each myotomy [7]. In a modified version of this technique a preoperative bougienage step is used to further elongate the oesophagus [8].

3. *Scharli procedure:* incision of the lesser curvature of the stomach to allow a retrosternal transfer of the distal oesophagus and a tension-free oesophageal anastomosis [9].

4. *Foker technique:* it involves 2 thoracotomies. First, anchoring sutures are placed securely at the 2 ends of the oesophagus and are brought out diagonally to the chest wall. Over a period of days to weeks, the 2 ends are brought closer together by a series of daily lengthenings by traction on the exposed sutures. The closure of the gap is monitored radiologically. A second thoracotomy is then performed to effect a tension-free anastomosis [10].

- <u>Oesophagus replacement</u> [11]

  There are instances where replacement is necessary, particularly in cases where numerous recurring futile attempts to perform a primary repair have a negative impact on the child and family well-being. Examples are repeated failed procedures to achieve oesophageal continuity, the development of complications such as empyema from anastomotic leaks, refractory or extensive strictures, or multiple recurrences of fistulas. In these settings a gastric transposition, colonic interposition or jejunal interposition represents the best available options. Among them the gastric route involves transposing the whole stomach into the cervical region and

is preferred for the excellent blood supply of the stomach. Nevertheless, several complications can occur including: anastomotic leaks, strictures, reflux, dumping, poor gastric emptying and Barrett's esophagitis. Overall, all these procedures are associated with a higher prevalence of long-term respiratory and oesophageal complications.

1.1.5 POST-OPERATORY COMPLICATIONS

Anastomotic leakage (15-20%)

Major leaks occur in the early postoperative period (<48 hours) and present with life threatening tension pneumothorax. Minor leaks may be detected on a contrast study, these will all seal spontaneously but there is an increased incidence of later stricture formation.

Anastomotic stricture (30–40%)

Risk factors implicated in stricture formation include anastomotic tension, anastomotic leakage and gastro-oesophageal reflux. Most of which will respond to one or two dilatations carried out introducing a balloon under fluoroscopy to carefully dilate the oesophagus until the "waisting" at the stricture is abolished.

Recurrent tracheo-oesophageal fistula (5-14%)

Infants with a recurrent fistula manifest respiratory symptoms: coughing during feeds, apnoeic or cyanotic episodes, recurrent respiratory infections. Whilst a chest X-ray may show an air oesophagogram, a contrast study is usually necessary to confirm the diagnosis: a water-soluble contrast is slowly instilled into the oesophagus through a nasogastric tube which is gradually withdrawn from the stomach to the level of the pharynx.

At surgery, bronchoscopic examination will reveal the recurrent fistula at the site of the original TOF and will allow the surgeon to insert stay sutures at both the oesophageal and tracheal ends of the fistula before it is divided.

Gastro-oesophageal reflux - GOR (40%)

Often, but not always, it follows a delayed primary repair or anastomosis under tension and can concur to the development of anastomotic strictures. Incompetence of the lower oesophageal sphincter mechanism may be due to a primary neurogenic defect, post-operatory shortening of the intraabdominal oesophagus or abolishment of the gastro-oesophageal angle of His. Symptoms of GOR include respiratory problems (as seen for a recurrent fistula), recurrent vomiting and strictures formation. The diagnostic workup includes contrast swallow, pH monitoring and endoscopy of the distal oesophagus. Medical treatment with gastric acid suppression is only successful in about half the cases and surgical approach is complicated by the distal oesophagus dysmotility with a high failure rate of fundoplication carried out in the first three months of life. Stricture formation often resolves spontaneously once the GOR is corrected.

Tracheomalacia (10%)

Tracheomalacia is a structural/functional weakness of the trachea that leads to airway collapses during expiration causing expiratory stridor, which varies in severity from a hoarse barking cough, to recurrent respiratory infections to acute life-threatening episodes of cyanosis or apnoea. The diagnosis is made at bronchoscopy or cine-bronchography and the area of collapse is usually restricted to the trachea at the level of the entry of the distal TOF. In severe cases the definitive treatment consists of aortopexy in which the ascending and arch of the aorta are

elevated anteriorly towards the sternum allowing space for the trachea to expand [12].

Dysmotility

Dysmotility of the distal oesophagus is related to an abnormal coordination of contractions and it is a major factor in the long-term swallowing problems encountered in these children. Oesophageal peristalsis, assessed by manometry, is abnormal in 75 to 100% of children and young adults with a history of OA, and in 100% of those with colonic interposition. In severe cases it can lead to failure to thrive and aspiration episodes. Many children with repaired OA/TOF need to eat slowly, and may need to avoid meats.

Respiratory function

Respiratory function tests performed after OA repair have detected increased airway resistance and abnormal air flow patterns [13]. Patients commonly develop wheezing and bronchial hyper-reactivity. Moreover during the first years of life, patients are exposed to an increased risk of re-hospitalization for respiratory infections. Infections can be related to oesophageal dysmotility, GOR or to a primary respiratory abnormality. The anti-infection mechanism can also be impaired by the presence of nonciliated squamous epithelium found in the trachea of 80% of infants with OA [14].

1.2 LONG GAP OESOPHAGEAL ATRESIA

Long-gap atresia (LGOA) is wildly recognized as the type of OA with a higher risk of surgical treatment failure and long-term complications. Its definition is nevertheless highly variable, being it described as a OA with a wide gap measured either in centimetres or vertebral bodies, defined pre-

operatively or intra-operatively (by distension or mobilization of the pouches), finally it can be defined as a OA where a primary anastomosis has been unsuccessful.

1.2.1 LONG GAP OESOPHAGEAL ATRESIA: THE CLINICAL PROBLEM

The optimal surgical technique for long-gap OA repair is still controversial. Ideally the best option would be the one that allows preserving the patient's own oesophagus. This is however not always possible due to the high risk of severe complications such as anastomotic leak, severe GOR or recurrent oesophageal strictures that would lead to an increased re-hospitalization rate and increased mortality. In these patients a replacement structure has to be insert in the chest to regain continuity of the gastro-oesophageal tract. As previously described the available options for oesophageal replacement (e.g. gastric pull-up or colon transposition) are all associated with a high short- and long-term complication rate. New approaches on LGOA are therefore needed to recreate a functional oesophagus even when the patient's own organ cannot be used.

1.2.2 TISSUE ENGINEERING AS AN ALTERNATIVE OPTION FOR LGOA

Novel therapies resulting from Regenerative Medicine and Tissue Engineering may offer new hope for patients with LGOA. These technologies can provide the surgeon of a new tool to replace the missing oesophagus using lab-made scaffolds that can restore not only oesophageal continuity but most importantly its function. These scaffolds, implanted in the affected baby, would reduce the risk of multiple stage surgery, high complications rate and frequent re-hospitalizations commonly associated with current surgical techniques and would offer the patient and the family a better quality of life.

1.3 TISSUE ENGINEERING

Tissue Engineering (TE) is defined as a multidisciplinary field, which applies the principles of engineering and life sciences towards the development of biological substitutes that aim to maintain, restore or improve tissue function. It integrates expertise of cellular biology, material engineering, physiology and gene-therapy to develop suitable replacements for tissues and organs. Moving forward toward the construction of engineered organs, it became necessary to integrate TE with the use of stem cells. The combination of the two approaches is now referred as Regenerative Medicine (Fig 4).

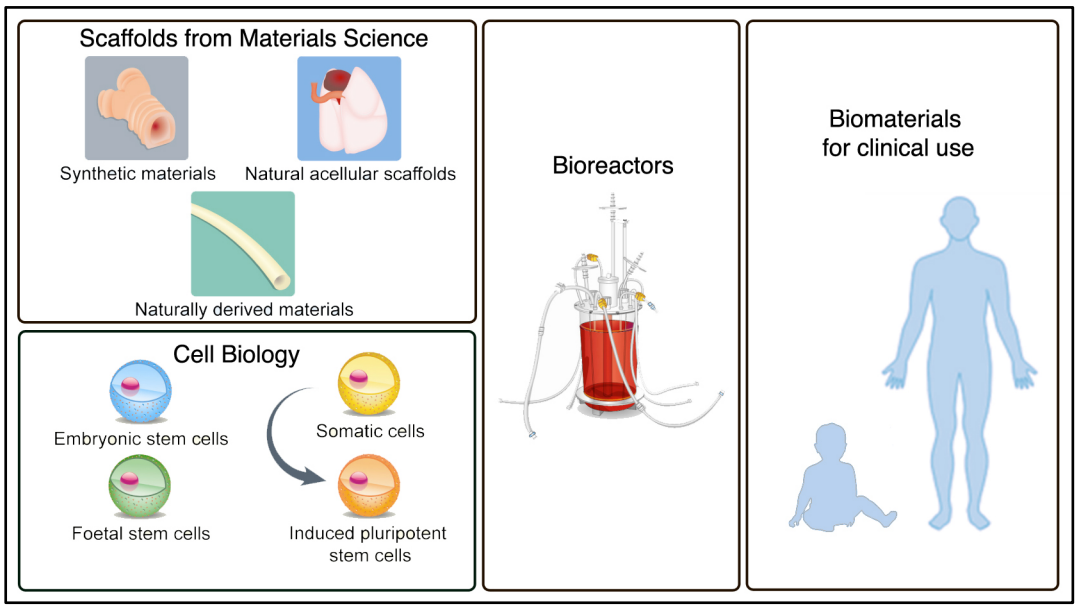

Figure 4. TISSUE ENGINEERING AND STEM CELLS IN REGENERATIVE MEDICINE. The combination of a synthetic or naturally derived scaffold and a pool of cells suitable for matrix repopulation represents a pivotal step in organs reconstruction for transplant purposes in humans. This approach is referred as Regenerative Medicine.

1.3.1 USE OF SCAFFOLDS IN TISSUE ENGINEERING

A major aim for TE is to develop functional organs to overcome the current limitations related to the shortage of donor organs, incompatibility problems, and the detrimental effects of long-term use of immunosuppressive drugs after transplantation. The process implies a

meticulous analysis and combination of different materials (synthetic or naturally derived), scaffolds production methods, pre implant treatment or cell repopulation.
Scaffold can be classified in synthetic and naturally derived matrices.

I) Synthetic scaffolds

The synthetic materials first suggested as suitable option for TE were the ones already in use as biomaterials in other areas of medicine. For example, materials belonging to the polyester family (PLA, PGA and PLGA) were already employed as sutures and orthopaedic fixatives such as pins, rods and screws. Polyesters were widely used; although some studies suggested that there could be problems due to their propensity to disintegrate into small particles or toxicity associated with acidic degradation. When designing a complex organ, the use of synthetic material become less efficient due to the complexity of the native organ and the need of an adequate vascular supply. Different materials have been manipulated in order to enrich them with specific properties that could resemble the native tissue characteristics. Scaffolds have been therefore modified to have to right porosity and allow better perfusion or to include cell adhesion molecules and growth factors. Despite all the efforts, synthetic scaffolds present evident limitations when the aim is a whole complex organ reconstruction.

II) Naturally derived matrices

Naturally derided macromolecules have been used to develop tissue-engineered scaffolds, among them the most commonly applied are the ones inspired by the components of the extracellular matrix (ECM) that represents the ultrastructure of the native tissue and characterize its structural and functional properties.

Matrices obtained from animal of human collagen have been used due to its high mechanical strength, good biocompatibility, low antigenicity and ability of crosslinking. Combinations of collagen and synthetic scaffolds or growth factors have also been tested with variable results. Fibronectin has been applied too, adding it to different biomaterials, to take advantage of its ability to enhance the availability of cell binding sites and increase cell adhesion. Fibrin has been used as cell delivery matrix for cartilage tissue engineering. It can be combined with other biodegradable substances, such as alginate or hyaluronic acid and it has also been used in the regeneration of the skin.

III) Decellularised matrices

Matrices obtained from decellularisation of native organs are increasingly used in regenerative medicine strategies for tissue and organ replacement. The idea is to remove all the cellular components from a native tissue, while maintaining its 3D structure, composition and biomechanical functions, which are retained in the ECM.

Absence of cellular components needs to be achieved to remove nucleic acids and major histocompatibility complex II and I in order to avoid any inflammatory responses and rejection processes after in vivo implantation of the scaffold. DNA removal has to be as complete as possible and previous analyses suggested that the decellularisation could be considered acceptable when the following criteria are satisfied: I) the amount of DNA is less than 50 ng per mg of dry tissue of ECM; II) the remaining DNA fragments have a length less than 200 bp; III) there is not visible nuclear material in tissue sections stained with DAPI or hematoxylin and eosin (H&E)[15].

The ECM is produced and modelled by the resident cells of each tissue and has been shown to play a key role in cell migration, proliferation and differentiation [16,17]. Moreover ECM influences cell mitogenesis and

chemiotaxis and induce host tissue remodelling responses. Recently it has been described the immunomodulatory effect of the ECM which could play a role in improving scaffolds quality [18]. Preservation of this complex structure is therefore pivotal in the decellularisation process [19,20].

As expected, several different decellularisation techniques have been developed, depending on the characteristics of the specific tissue, specifically its size, cellularity, density and thickness [15].

Several physical, or chemical/enzymatic treatments have been tested.

Examples of physical methods are:

- Freeze/thaw cycles: used to cause cell lysis with minor disruption of ECM ultrastructure;
- Pressure/force: mechanical abrasion and hydrostatic pressure can be applied, usually in combination with chemical agents;
- Electroporation: electrical pulses applied across the tissue alter the electrical potential of the cell membranes creating micropores and causing cell death;

For the chemical treatments the possibilities are again various:

- Acids and bases: can cause biomolecules removal by hydrolytic degradation (paracetic acid, acetic acid, alcium hydroxide, sodium sulphide, and sodium hydroxide are some examples). They are effective in decellularisation but are harsh enough to eliminate growth factors form ECM and alter its mechanical properties;
- Hypotonic/hypertonic solutions: cause cell lysis by osmotic effect or dissociate DNA from proteins;
- Detergents: solubilize cell membranes and dissociate DNA from proteins (Triton-X, sodium deoxycholate, Sodium dodecylsulfate, CHAPS). Prolonged exposure to detergent can alter ECM composition and leave cytotoxic residuals that could impair the results of scaffold implantation;

- Alcohols: isopropanol, ethanol and methanol are used to remove lipids, especially in valve leaflets to avoid post-implantation calcification. However they can precipitate proteins and damage ECM ultrastructure;
- Enzymes: usually employed in decellularisation include nucleases, trypsin, collagenase, lipase, dispase, thermolysin, and α-galactosidase. They can efficiently remove cells residual, but they need to be associated with other agents capable to disrupt cell membranes. Nucleases effectively cleave nucleic acids after cell lysis. Trypsin is commonly used, but has the drawback of being more disruptive to ECM components, specifically elastin;
- Chelating agents: ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) induce cell dissociation from ECM proteins;

The optimal application of the previously described decellularisation agents is dependent upon tissue characteristics such as thickness and density, the agents being used, and the intended clinical application of the decellularised tissue. The way in which these solutions have been applied can be different too, essentially two techniques have been used: whole organ perfusion or immersion with agitation. Examples of whole organ perfusion are heart [21], lungs [22,23] and liver [24], all organs characterized by a rich and easily accessible vascularization. This technique guarantees a complete DNA removal with optimal ECM preservation. Nevertheless, its application in small or less vascularized tissues is critical and in these cases the optimal choice is to immerge the tissue in decellularising solutions, usually in gentle agitation. Immersion/agitation has been applied in heart valves [25], blood vessels [26], skeletal muscle/tendon [27], peripheral nerves [28], trachea [29], esophagus [30], dermis [31] and urinary bladder [32].

1.3.2 SCAFFOLDS FOR TISSUE ENGINEERING OF THE OESOPHAGUS

When talking about an engineered oesophagus, we have to remember that the two main components that we aim to recreate are the appropriate cells and the scaffold they are seeded onto. All cells in our body depend on an anchorage and therefore need a scaffold support. In the native organ this scaffold is represented by the ECM, which mainly includes collagens, elastic fibres, glycosaminoglycans, proteoglycans, and adhesive glycoproteins. The ECM is organ specific and it provides many biologically important functions, including a three-dimensional environment for cell–cell and cell–matrix interaction, necessary for cell attachment, migration, and proliferation. This stable and yet biodegradable structure allows tissue remodelling in response to physiological and pathological needs, moreover it ensures exchange of nutrients and contains a range of growth factors for tissue development[33].

TE aims to recreate scaffolds similar to the native ECM, able to offer the same stability, as well as the same plasticity, to be biodegradable and tissue compatible. At present there is no consensus regarding the optimal technique for oesophagus engineering. Different approaches have been explored to create appropriate scaffolds able to substitute the native organ[34].

I) Synthetic oesophageal scaffolds

The first attempts were designed with the intent to treat and replace a damaged oesophageal tissue after an acquired injury. Despite the high incidence of leakages, stenosis and long-term complications these studies were successful in recognising the importance of the right stiffness of the scaffold: a rigid structure could be used only as a stent, while a more flexible and malleable scaffold was a too weak barrier exposed to high risk of leakages and high mortality.

More recent experiments on animals were designed to compare different

materials such polyethylene plastic [35,36,37] and silicon [38,39], all showing to be unsuccessful in stimulating cellular migration and restoring oesophagus function. Other known options are polyvinylidene fluoride (PVDF) and polyglastin-910 (Vicryl®) [40] or a combination of the latter and collagen with a lower mortality rate in dogs but still a high complication rate [41].

II) Naturally-derived oesophageal scaffolds

The possible use of collagen scaffolds was explored by a research group in Japan, in a series of experiments aimed to replace a short tubular oesophageal defect in dogs [42–44] . The scaffold was made of porcine dermal collagen and insert around a silicone tube used as a stent, which was removed at different time points. These studies showed that the stent allowed a reduction of strictures development. Increasing the stent duration also led to a better scaffold repopulation due to cellular migration. However when the defect was bigger or created in a less vascularized region of the oesophagus, cell migration was poor and muscle regeneration inadequate suggesting that there are limitations to the size and region of defect that may be replaced by this methodology.

III) Decellularised oesophageal scaffolds

In oesophageal TE an acellular matrix can be used as patches o tubular structures not only to fill the gap in the damaged oesophagus but also to promote cell migration and matrix repopulation. The ideal matrix should therefore resemble the 3D structure of the native organ, from the architectural and the functional point of view, ameliorating cell migration and proliferation, to ensure an efficient and durable integration with the host tissue. It should be a resistant and at the same time malleable structure, able to go through remodelling processes and to follow the needs of a growing organ resembling what happen in children and young

adults. Moreover it should be accepted by the host as a non-immunogenic structure, in order to avoid the need of a life-long immunosuppressive treatment and its well know side effects and complications. In recent years, lots has been done in research to improve the characteristics of decellularised oesophageal scaffolds and made them available for clinical use.

Significant heterogeneity exists among studies using different animal models, organs, decellularisation techniques, and repopulation methods.

- Decellularised Matrix Origin: scaffold used for oesophageal substitution have been derived from the oesophagus as well as from other tissues such as small intestine submucosa (SIS) or stomach with heterogeneous results [45]. In animal studies SIS patches were seen to be able to integrate in the host tissue end undergo physiological remodelling. On the contrary, use of SIS tubular structures was associated with higher complication rate (mainly strictures) and mortality. SIS has been used to repair oesophageal defects in patients with superficial cancers following endoscopic submucosal resection, with successful short and long term results despite a higher risk of stenosis. Urinary bladder submucosa and gastric acellular matrix are other examples of scaffolds used to repair an oesophageal defect in a canine and a rodent model respectively. AlloDerm® patches, a human decellularised skin usually employed for abdominal wound reconstruction, were used in a canine model demonstrating its potential to support tissue regeneration with a reduced risk of major complications (dysphagia, leakage, stricture and infection).

  However, it has been emphasized the importance of the matrix itself in retaining growth factors and other mediators to enhance cell repopulation and differentiation. These structural signals are

unlikely to occur with non-oesophageal scaffolds; therefore the optimal replacement scaffold should be derived by oesophageal tissue. Marzaro et al, e.i, used a patch of decellularised oesophageal tissue seeded with autologous smooth muscle cells to successfully repaired a 2 cm defect in a pig model showing promising results [46].

- Oesophagus decellularisation protocols: different physical, enzymatic and chemical treatments can be employed, even in combination, in order to remove cells and immunogenic material to create animal or human oesophageal scaffolds. It is however necessary to find the right balance between efficient cells removal and good preservation of ECM. The first decellularisation protocols included simple immersion and agitation of the oesophagus in a mix of detergent and enzymatic solutions[15]. The use of harsh chemicals to deeply wash the scaffold has led to poor ECM preservation. New techniques have been therefore developed, showing a better result with the use of a peristaltic pump that creates a continuous intraluminal flow to promote cell removal. It became then possible to avoid the use of harsh reagents such as sodium dodecyl sulphate (SDS) and Triton-X 100 (TX100). Also the use of EDTA to promote cell release has been proved to cause an uneven decellularisation, leading to a collapsed and disorganized ECM. The use of a peristaltic pump to deliver a less harsh detergent-enzymatic treatment (DET) has been proven in other organs to be efficient in removing the immunogenic material and preserving the ECM [47,48,49,50].

1.3.3 FROM BENCH TO BEDSIDE: OFF-THE-SHELF AVAILABILITY

The final challenge in TE is to effectively move from a bench-top concept to a feasible therapeutic tool for practical clinical use. The clinical request for oesophageal scaffolds cannot be met by making one construct at a time in

a research laboratory and immediately use it a patient. It becomes necessary to rethink about TE from a manufacturing point of view up-scaling the scaffolds production maintaining a clinically efficient and financially cost-effective approach. In order to reach an off-the-shelf availability it will be necessary to refine the entire process including: manufacturing, storage and shipping (Fig. 5).

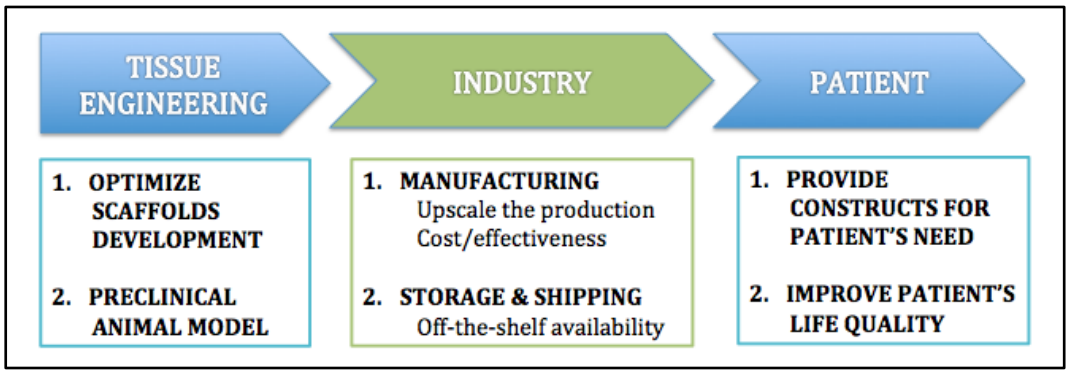

Figure 5. FROM BENCH TO BEDSIDE: necessary evolution of Tissue Engineering to reach a clinical application in humans.

For example Apligraf®, a skin cellularized matrix produced by Organogenesis, is kept fresh in a sealed shipping package at control room temperature (20-23°C) and has a 5 days shelf-life (http:// www.apligraf.com/professional/ what_is_apligraf). On the other hand, Dermagraft®, the dermal substitute developed by Advanced Tissue Science, is cryopreserved and shipped ensuring a 6 months shelf life, improving availability at the clinical side [51]. Although cryobiology is a relatively old field and most cell types can be cryopreserved, there is much that still needs to be learned to successfully preserve the structural and functional characteristics of 3-dimensional products.

At present the optimal storage method for oesophageal decellularised scaffolds has not been described.

2.0 AIM OF THE STUDY

2.1 CREATE A DECELLULARISED OESOPHAGEAL SCAFFOLD

In order to develop a decellularised scaffold from a large animal we used rabbit oesophagi and processed them using a detergent-enzymatic treatment that allowed cells removal and ECM preservation.

2.2 COMPARE DIFFERENT STORAGE PROTOCOLS

We identified 4 different storage methods for decellularised scaffolds:

- **SCM**: slow cooling in medium with 10% DMSO at -1°C/min, then stored in liquid nitrogen;
- **SF**: snap-freezing in liquid nitrogen, then stored in -80°C;
- **FD**: freeze-drying, then stored in -20°C;
- **4C**: phosphate-buffered saline solution at 4°C.

We then compared structural and functional characteristics of the stored matrices at the 4 time-points (2 weeks, 4 weeks, 3 months, 6 months) to identify the best storage protocol.

3.0 METHODS

3.1 SCAFFOLD DEVELOPMENT

3.1.1 HARVEST OF ORGANS

Oesophagi were obtained from adult rabbit. A midline incision was made to completely expose the abdominal thoracic cavities. The oesophagus was harvested from the cervical portion to the gastro-oesophageal junction and washed with PBS/AA.

3.1.2 DECELLULARISATION PROTOCOL

Decellularisation was achieved using a combination of a chemical detergent and enzymes, known as DET which has been previously optimized in our laboratory for tissue decellularisation [49,50].

The oesophageal lumen was perfused with continuous fluid delivery using a Masterflex L/S variable speed roller pump at 1 ml/min. Each DET cycle was composed of multiple steps during which the tissue was continuously perfused with different solutions. The cycle begins in deionised water (resistivity 18.2 MX/cm) at 4 °C for 24 h, to induce osmotic cell lysis. After that the oesophagus was perfused with 4% sodium deoxycholate (SDC - Sigma) at room temperature (RT) for 4 h, which acts as a detergent. Following SDC the samples were washed for 30 min with PBS, to remove the detergent that could inhibit DNase I and could be toxic for cells when the scaffold will be recellularized or implanted in vivo. The last step was a perfusion in 2000 kU DNase-I (Sigma) in 1 M NaCl (Sigma) at RT for 3 h to remove cleave nucleic acid sequences and aid in removal of nucleotides after cell lysis. The process was repeated for up to three cycles. Following treatment the constructs were stored according the four different storage protocols (Fig. 6).

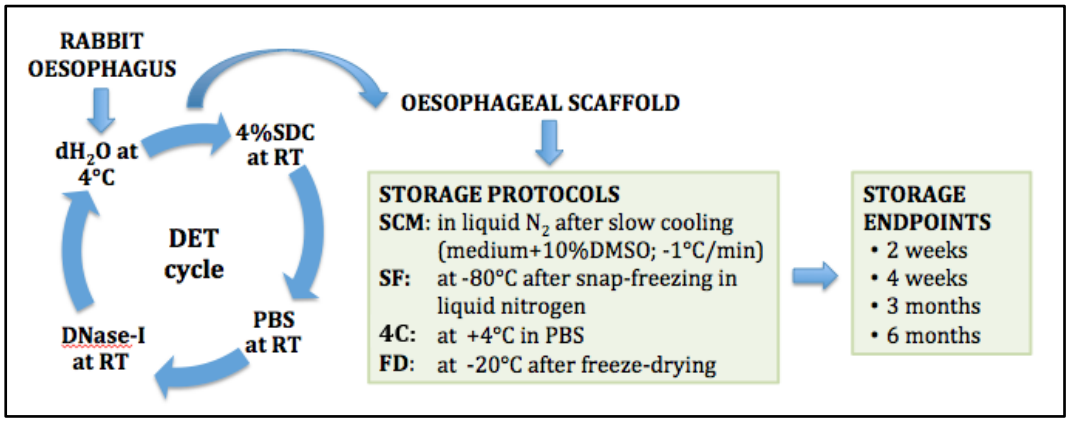

Figure 6. STUDY DESIGN: 30 oesophagi are decellularised according to DET protocol. Scaffold are stored according to the 4 different storage methods.

3.2 SCAFFOLDS STORAGE

As previously underlined, the optimal storage method for oesophageal decellularised scaffolds has not been defined yet. We therefore chose four different storage methods among the ones already described in literature and commonly used to preserve tissues and constructs for other purposes. We then compared them to identify the one that better preserve the structural characteristics of the decellularised scaffold.

3.2.1 SNAP-FREEZING (SF)

It is known that biological materials can be long-term preserved at low temperature, but they are also subject to irreversible damage in the process of cooling and recovering, which is known as cryoinjury. Whilst cryoinjury occurs mainly at low temperature from 0 to -80 ^{0}C, when the temperature decreases below -80 ^{0}C this damage is greatly reduced. Based on the principle that chemical, biological, and physical processes are effectively "suspended" at cryogenic temperature in liquid nitrogen (-196 ^{0}C), this method is considered a stable preservation method, which theoretically makes it possible to store a biosystem for extended period of

time [52]. Rapid freezing by immersion in N_2 (i.e. SF) causes tissue injury by intracellular ice formation and cellular osmotic rupture, a process that would not occur in decellularised scaffolds. Conventional storage in liquid nitrogen is therefore a common approach employed at cell and tissue banks [53].

In our study FD samples were rapidly frozen in liquid nitrogen vapors and then stored at -196 ^{0}C.

3.2.2 SLOW COOLING IN MEDIUM AND DMSO (SCM)

A well-known downside of freezing is that crystallization (ice formation) can occur during the process causing extensive tissue damage. Cryobiology was first developed in 1949 with the discovery by Polge of the cryoprotective properties of glycerol. Since then, other stabilising agents have been used including dimethyl sulphoxide (DMSO), a diffusible cell-permeating agent able to protect proteins from denaturation through electrostatic interactions, and to reduce the rates of ice nucleation and crystal growth increasing viscosity [54].

A combination of cryoprotective agents and slow cooling rate has been widely investigated, in order to reduce or control crystallization. If cells are cooled too quickly, intracellular ice crystals form, resulting in mechanical disruption of cells and their destruction. At slow rates of cooling, ice crystals form in the extracellular space, causing increased osmolality as free water is taken up. This causes cellular dehydration. Glycerol and DMSO prevent dehydration by inhibiting the increased concentration of sodium that can occur during ice formation and by decreasing the amount of water absorbed into ice crystals at any given temperature [55]. The current gold standard for preserving cells and allografts is controlled rate freezing with DMSO.

We thus used slow cooling (1 °C/min) of tissues immersed in 90% medium (RMPI - Gibco) and 10% DMSO. Slow cooling was achieved using

appropriate Nalgene freezing containers kept at -80 overnight, samples were then stored in liquid nitrogen.

3.2.3 FOUR CELSIUS DEGREES (4C)

Storage at 4°C in a solution of phosphate buffered saline supplemented with 1% antibiotic/antimycotic is a common storage protocol. It has been previously proved that the immunological and mechanical characteristics of decellularised pig matrices were unaffected by a 2-month storage in PBS at 4°C [56]. At a longer time point analysis, decellularised tracheas stored in 4°C for 1 year showed a general damage of the ECM architecture with a reduction of the collagen and elastin fibres. Tracheas were considered not usable for cell seeding and transplantation due to the dramatic changes in structure and mechanical properties [57]. Bonenfant et al evaluated 4°C storage for decellularised mouse lungs with 3 and 6 month time points [58]. This storage method led to a time dependent alteration of the lung structure, with atelectatic areas developing in the central region of the scaffold after 3 months, and involving also the peripheral structures after 6 months up to a general alteration of the entire lung.

3.2.4 FREEZE-DRYING (FD)

Freeze-drying is a dehydration process that has been widely used in a number of applications especially in the food and pharmaceutical industries.

Freeze-drying involves the removal of water or other solvent from a frozen product directly by sublimation so that the material does not go through the liquid phase. The process allows a preservation of the material characteristics, freeze dried products do not need refrigeration, and can be stored at ambient temperatures thus eliminating the costs for refrigeration. The methodology is based on a 2-step drying phase during which all the water in the tissue is removed by sublimation freezing the

sample and lowering the pressure (i.e. primary drying), followed by desorption of the residual moisture content (i.e. secondary drying). Following freeze-drying, materials present a long shelf-life that can be even prolonged when kept at low temperatures. Degradation of the product is in in fact related to the residual oxygen and moisture content, and therefore determined by the environment temperature and humidity.
Freeze-drying has been suggested as a methodology for long-term scaffold storage [59] as it safely preserves food and other products characteristics. Additionally, the scaffold could be restored to its previous state by water immersion.

3.3 SCAFFOLD ANALYSIS

Scaffold were analysed after decellularisation and compared to fresh tissue. Furthermore, scaffold analysis was performed after 2 weeks, 4 weeks, 3 months or 6 months storage at 4 different conditions: 4°C, snap-frozen, freeze-dryed, slow cooling in medium.

3.3.1 HISTOLOGY

Samples were fixed for 24 hours in 10% neutral buffered formalin solution in PBS (pH 7.4) at RT. Subsequently they were washed in distilled water (dH_2O), dehydrated in graded alcohol, embedded in paraffin and sectioned at 5µm. Tissue slides were stained with haematoxylin and eosin (H&E) (Leica, Germany), Masson's Trichrome (MT), (Leica, Raymond A Lamb, BDH Chemicals Ltd), Picrosirius Red (PR) (Polysciences Inc., Germany), Elastin Van Gieson (EVG) (VWR, Leica, Raymond A Lamb), and Alcian Blue (AB) (BDH Chemicals Ltd, Cellpath Ltd) stains. All images were captured on a Zeiss Axioplan 2 Imaging microscope (Germany) with Improvision Openlab software (Perkin-Elmer, USA, 5.5.1).

3.3.2 DNA QUANTIFICATION

DNA was isolated using a tissue DNA isolation kit (PureLink Genomic DNA MiniKit, Invitrogen) following the manufacturer's instructions. Briefly, the samples were digested overnight using Proteinase K and a digestion buffer. DNA samples were purified using alcohol washes and measured spectrophotometrically (Nanodrop, Thermo Scientific, US). Optical densities at 260nm and 280 nm were used to estimate the purity and yield of nucleic acids.

3.3.3 ECM COMPONENTS QUANTIFICATION

Collagen content of native tissue and acellular matrices was quantified using the total collagen assay kit (QuickZyme Biosciences, The Netherlands). Briefly for collagen, the samples were hydrolysed in 6N HCl at 95 °C for 20 hour, the hydrolysate were mixed with a chromogen solution staining hydroxyproline residues and colour was developed at 60 °C for 1 hour. The absorbance for each sample was determined at 555 nm using a Tecan Infinity microplate reader and the collagen quantity was calculated from a standard curve from known concentrations of pure collagen hydrolysates.

Elastin content of native tissue and acellular matrices was quantified using the FASTIN elastin assay (Biocolor, UK) according to the manufacturer's instructions. Briefly, the samples were homogenized, and elastin was solubilized in 0.25 M oxalic acid. Two consecutive incubations were performed at 95 °C to ensure complete extraction of elastin. Extracts were incubated with 5,10,15,20-tetraphenyl-21H,23H-porphine tetrasulfonate (TPPS) dye, and absorbance was determined at 555 nm spectrophotometrically (Tecan Infinity). Elastin concentrations from a standard curve were used to calculate the elastin content of the tissue.

Glycosaminoglycan (GAG) content of native tissue and acellular matrices was quantified using the Blyscan GAG Assay Kit (Biocolor, UK). Briefly, the tissues were digested with papain at 65 °C for 18 hours and aliquots of each sample were mixed with 1,9-dimethyl-methylene blue dye and reagents from the GAG assay kit. The absorbance at 656 nm was measured spectrophotometrically (Tecan Infinity) and compared to standards made from bovine tracheal chondroitin-4-sulfate to determine the GAG content.

3.3.4 SCANNING ELECTRON MICROSCOPY

Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer and left for 24 hours at 4°C. Following washing with 0.1 M phosphate buffer, they were cut into segments of approximately 1 cm length and cryoprotected in 25% sucrose, 10% glycerol in 0.05 M PBS (pH 7.4) for 2 hours, then fast frozen in Nitrogen slush and fractured at approximately -160°C. The samples were then placed back into the cryoprotectant at room temperature and allowed to thaw. After washing in 0.1 M phosphate buffer (pH 7.4), the material was fixed in 1% OsO_4 / 0.1 M phosphate buffer (pH 7.3) at 3°C for 1½ hours and washed again in 0.1 M phosphate buffer (pH 7.4). After rinsing with dH_2O, specimens were dehydrated in a graded ethanol-water series to 100% ethanol, critical point dried using CO_2 and finally mounted on aluminium stubs using sticky carbon taps. The material was mounted to present the fractured surfaces across the parenchyma to the beam and coated with a thin layer of Au/Pd (approximately 2nm thick) using a Gatan ion beam coater. Images were recorded with a Jeol 7401 FEG scanning electron microscope.

3.3.5 SYNCHROTRON-BASED X-RAY PHASE CONTRAST IMAGING

Measurements were performed at the biomedical beamline (I17) of the European Synchrotron Radiation Facility in Grenoble, France. The samples were placed approximately 150 m from the source (a 21-pole, 15 cm

period wiggler), to ensure spatially coherent illumination. The beam was monochromatized by a fixed-exit Laue/Laue silicon double crystal to 26 keV ($\Delta E/E \sim 0.02\%$) and filtered using 0.8 mm of copper and 3 mm of aluminium. The sample had been placed on a PI miCos rotation stage (PI mi- Cos GmbH, Eschbach, Germany) to enable tomographic acquisitions. The detector was placed at 3.45 m from the sample, a propagation distance sufficient to generate intense phase effects. Images were recorded by a FReLoN CCD camera coupled to a 47 µm thick $Gd_3Ga_5O_{12}$ scintillator. The effective pixel size was 3.5 x 3.5 micron^2. 2000 projections over 360^0 were acquired, with an angular step of 0.18^0. Images were phase-retrieved using the "single-distance" method developed by Paganin et al, and 3D reconstructions were performed using standard filtered back-projections [60].

3.3.6 CHICKEN CHORIOALLANTOIC MEMBRANE ASSAY

To evaluate the angiogenic properties of the decellularised materials *in vivo* the chicken chorioallantoic membrane assay (CAM) assay was used as previously described [29]. Fertilized chicken eggs were incubated at 37°C and constant humidity. At 3 days of incubation an oval window of approximately 3 cm in diameter was cut into the shell with small dissecting scissors to reveal the embryo and CAM vessels. The window was sealed with tape and the eggs were returned to the incubator for a further 5 days. At day 8 of incubation, 2 mm diameter acellular matrices were placed on the CAM between branches of the blood vessels. Polyester sections soaked overnight either in a PBS solution or in PBS with 200ng/ml vascular endothelial growth factor (VEGF) were used as negative and positive controls respectively. Samples were examined daily until 10 days after placement wherein they were photographed *in ovo* with a stereomicroscope equipped with a Camera System to quantify the blood vessels surrounding the matrices. The number of blood vessels less than

10 μm in diameter converging towards the placed tissues was counted blindly by assessors, with the mean of the counts being considered.

3.3.7 BIOMECHANICAL TESTING

Each material presents a characteristic relationship between the amount of deformation (strain) expressed at different levels of tensile loading (stress). This relationship reveals many of the properties of a material and it's therefore a way to describe the biomechanical features of a sample. To evaluate the biomechanical properties of the decellularised oesophagi, specimens were tested and subjected to uniaxial longitudinal tension until failure. This test records the tensile strength "σ" (Stress) versus strain "ε"; the highest point of the stress-strain curve is the Ultimate Tensile Strength (UTS) defined as maximum stress that a material could withstand until it breaks. The ratio of stress to strain is the Young's modulus, which is a measure of the stiffness of an elastic material and graphically corresponds to the slope of the stress/strain curve. Mechanical tests were performed with the application of uniaxial tension in an Instron 5565 at room temperature (20±1 °C). Specimens in the form of flat dumbbells with a 20 mm long working part were loaded at a constant tension rate of 100 mm/min. The thickness of the samples was measured using a digital electronic micrometer (RS components) at three places of the dumbbell and averaged. Stress–strain relationships, UTS and YM were obtained for samples and graphs plotted. Four samples were considered for each evaluated tissue.

4.0 RESULTS

4.1 DECELLULARISED OESOPHAGEAL SCAFFOLDS

Rabbit oesophagi have been decellularised following a DET protocol. This method, previously used in our laboratory to achieve a rat oesophagus decellularisation, was now scaled up to a large animal model in rabbit. The DET protocol was effective in inducing a macroscopic decellularisation of the oesophagi leading to tissues that appeared colourless and transparent after the first cycle (Fig. 7). Microscopic analysis was then performed showing a gradual loss of cells with each cycle of decellularisation as seen in H&E stained samples. After 2 DET cycles there were no nuclei in the scaffold (Fig. 8).

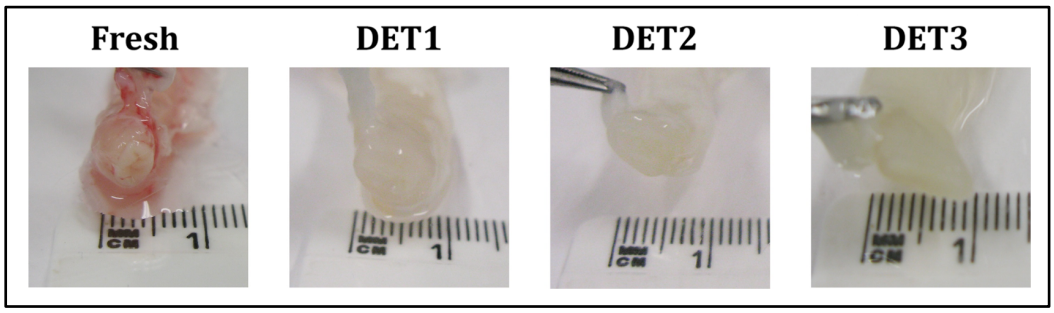

Figure 7. MACROSCOPIC APPEARANCE OF FRESH AND DECELLULARISED SCAFFOLDS.

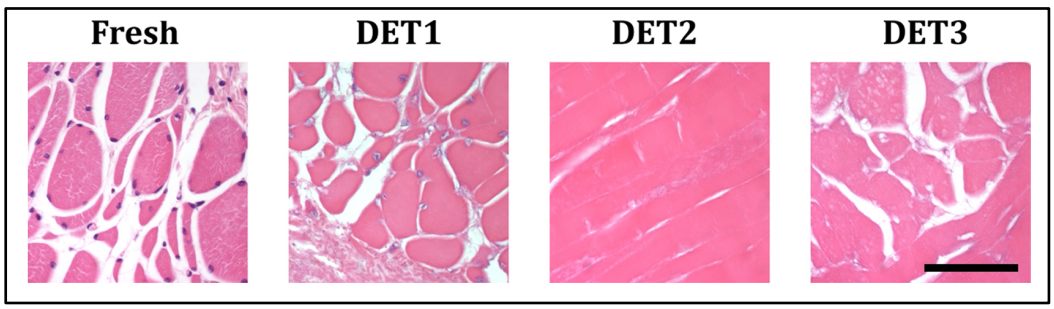

Figure 8. H&E STAINING OF FRESH AND DECELLULARISED SCAFFOLDS. Efficient cells removal was achieved after 2 DET cycles (bar=100 μm) .

Decellularisation efficiency was examined by DNA quantification after each DET cycle (Fig. 9). After cycles 2 and 3 there was significantly lower

DNA concentration compared to fresh or DET 1 oesophagi samples ($P < 0.01$ for both). The samples after DET cycle 2 and cycle 3 had no significant difference in DNA concentration.

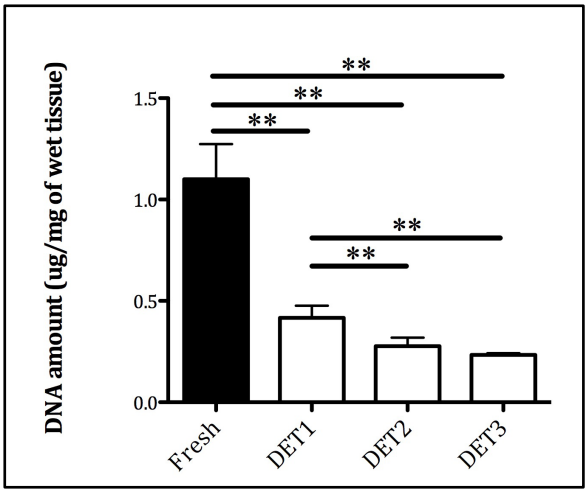

*Figure 9. DNA QUANTIFICATION. Efficient DNA removal was detected after DET treatment. **= p<0.01.*

As previously described, an efficient balance between cells removal and ECM preservation must be achieved in order to obtain a functional matrix. Therefore, to better analyse scaffolds microstructure and ECM preservation, 4 different histological staining have been used, as well as ECM components quantification.

Masson's Trichrome (MT) staining confirmed nuclei absence, as seen in H&E slides, and a preserved ECM structure with conservation of collagen (blue) in lamina propria, submucosa and intramuscular septae. Picrosirius red (PR) stains collagen in red and muscle fibres in yellow, showing preservation of collagen in submucosa and around the fibres (inlet) (Fig. 10). Elastin Van Gieson staining showed the presence of elastin (marked in black) in the submucosa as circular strands, around blood vessels present within the submucosa and surrounding muscle fascicles. Elastin Van Gieson staining showed the presence of elastin (marked in black) in the submucosa as circular strands, around blood vessels present within the submucosa and surrounding muscle fascicles. The submucosal and

vascular elastin was well preserved with muscular elastin fading following 3 cycles of decellularisation (Fig. 10). Alcian Blue staining, which stains the GAG blue, supported GAG preservation (Fig. 10).

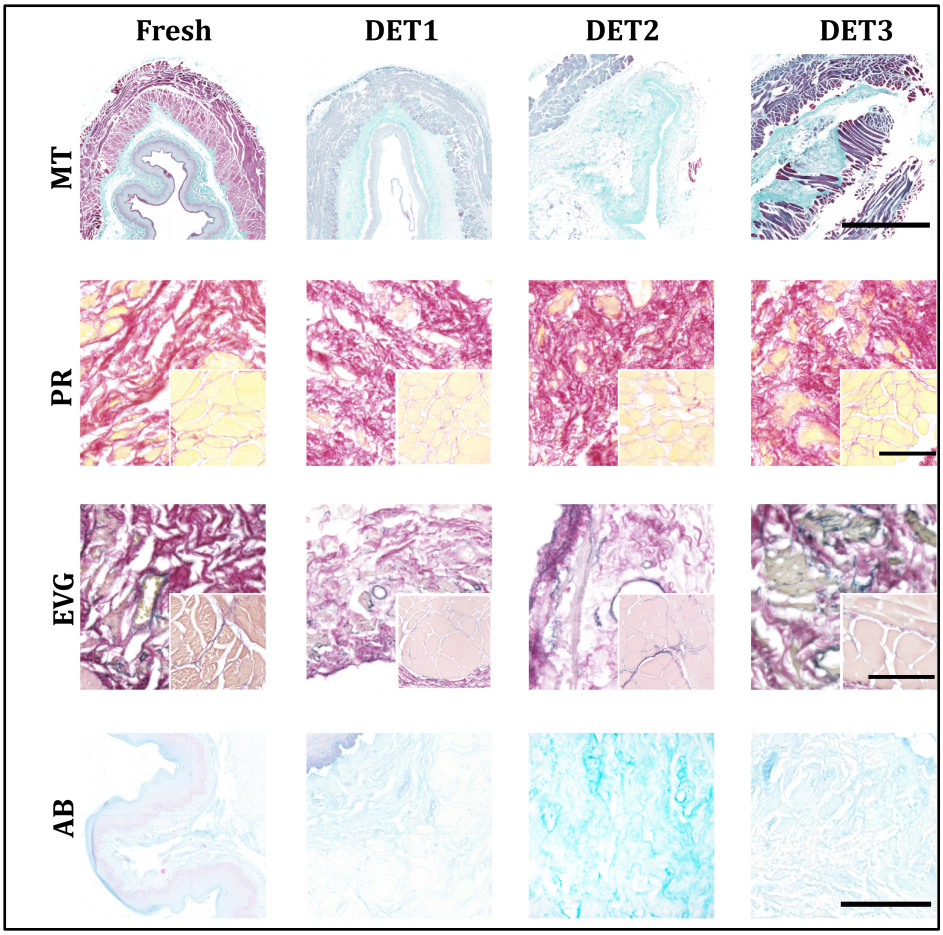

Figure 10. IMMUNOHISTOCHEMISTRY FOR ECM IN DECELLULARISED SCAFFOLDS. MT and PR staining demonstrated collagen preservation in both submucosa and among muscle fibers (inset). EVG staining showed elastic fibers in the submucosa, around blood vessels and surrounding muscle fascicles both in the fresh and DET tissues. Muscular elastin staining was reduced after 3 DET cycles (inset). AB staining indicated GAG preservation (bar=100 μm).
MT= Masson's Trichrome, PR=Picrosirius Red , EVG=elastin Van Gieson, AB=alcian blue, DET=detergent-enzymatic treatment.

Collagen quantification showed a significant reduction of collagen content after the first and the second DET cycle ($P<0.01$ and $P<0.05$ respectively). Elastin quantification showed that whilst there was no significant loss of elastin after the first and second DET cycle, 3 cycles of decellularisation

significantly decreased the amount of elastin in the oesophagi samples compared to both the fresh samples (P<0.01) and DET cycle 2 samples (P<0.05). Decellularisation also leads to lower levels of glycosaminoglycan (GAG) after DET cycles (P < 0.01), while there were no further changes between DET cycle 1 and the following cycles (Fig. 11 and Tab. 1).

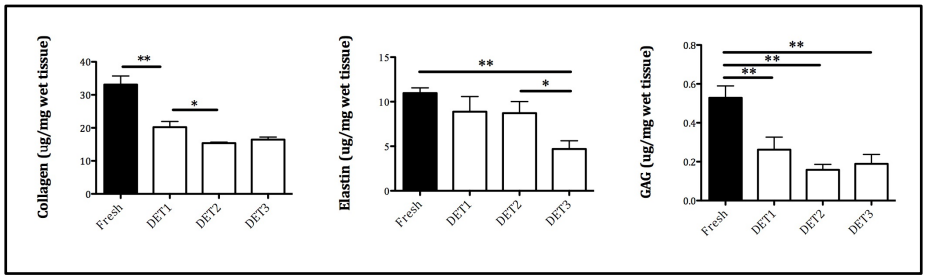

Figure 11. DECELLULARISATION EFFICIENCY AND SCAFFOLDS CHARACTERIZATION. Extracellular components showed a gradual decrease in collagen after the first and second DET cycle. Elastin dropped after 3 DET cycles. GAG were partially reduced by the first DET cycle.

	FRESH	DET1	DET2	DET3
COLLAGEN μg/mg	33.10 ± 9.47	20.21 ± 4.23	15.40 ± 0.28	16.45 ± 0.75
ELASTIN μg/mg	10.97 ± 1.44	8.89 ± 2.93	8.71 ± 2.92	4.69 ± 1.30
GAG μg/mg	0.53 ± 0.24	0.26±0.19	0.16 ± 0.05	0.19 ± 0.13

Table 1. ECM COMPONENTS ANALYSIS IN DECELLULARISED SCAFFOLDS. Quantification of collagen, elastin and glycosaminoglycans in decellularised scaffolds stored according to SCM protocol. Average ± standard deviation.

Having identified DET2 as the optimal cycle for efficient decellularisation and ECM preservation, further structural and functional analyses were performed on DET2 samples. Synchrotron imaging allowed a deeper investigation of preservation of the microarchitecture across a large scaffold segment. The analysis of the decellularised scaffold after 2 DET cycles confirmed ECM preservation for both lamina propria and submucosa (Fig. 12A). The muscularis mucosae showed maintained morphology and organization across the whole thickness of the tissue (Fig.

12B). Furthermore, an intact basement membrane was also evident in the cross section image (Fig. 12C).

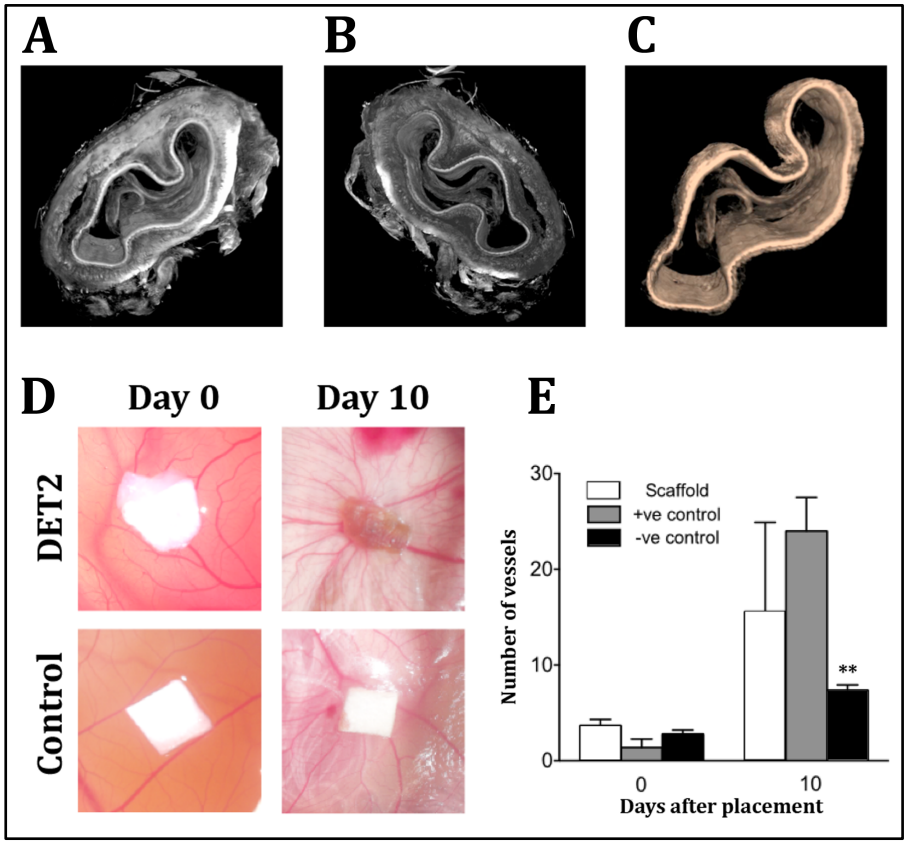

Figure 12. FUNCTIONAL ANALYSIS OF DECELLULARISED SCAFFOLDS. ***A,B****: synchrotron analysis after 2 DET cycles confirmed ECM preservation in the lamina propria, submucosa and muscularis.* ***C****: an intact basement membrane was detected across the whole scaffold segment.* ***D****: blood vessels were seen converging towards the scaffold in a spoked wheel manner after 10 days from placement on the CAM as confirmed in* ***E*** *by blinded quantification of the vessels compared to the negative control (** p< 0.01)*

After placement on the CAM numerous blood vessels were seen converging towards the scaffold in a spoke wheel manner while less vessels were detected in the negative control sample (Fig 12D). Blinded quantification of the converging blood vessels indicated that there was a significant difference in the number of vessels attracted by the scaffold at day 10 compared to day 0 after placement ($P < 0.01$) and to the negative control at day 10 ($P < 0.01$). There were no significant differences between the scaffold and the positive control loaded with VEGF (Fig. 12E).

Biomechanical tests of decellularised scaffolds were performed to quantify stiffness and elasticity of the construct using Stress/Strain curve, UTS and YM. Decellularised scaffolds were analysed for stiffness and elasticity using the Stress/Strain curve, Ultimate Tensile Stress (UTS) and Young's Module. There was no significant difference in the UTS among fresh and decellularised samples after 1 or 2 cycles; hence the maximum strength of the acellular scaffold was preserved (Fig. 13 A). Characteristic stress-strain curves showed that with increasing number of DET cycles the curve became steeper and more S-shaped, while the tensile stress at which the samples break remained the same (Fig. 13 B).

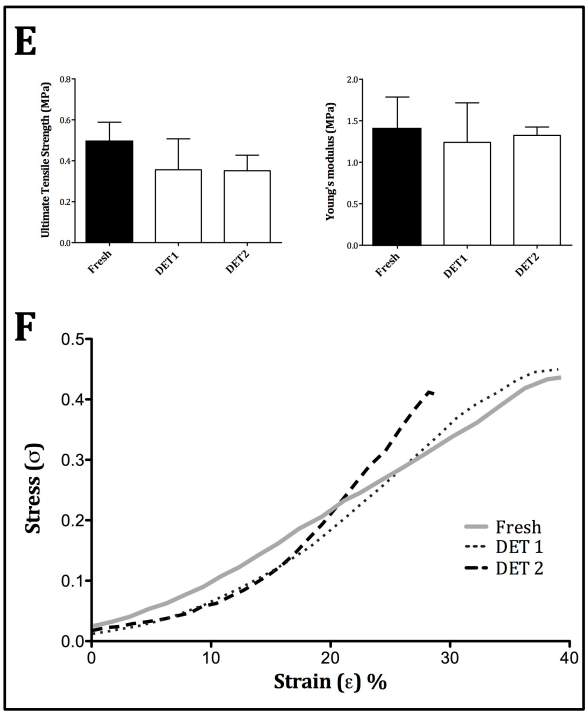

Figure 13. BIOMECHANICAL TESTING IN ACELLULAR SCAFFOLDS. ***A:*** *the maximum tensile stress at which the samples broke and the elasticity modulus remained comparable to fresh after decellularisation.* ***B:*** *characteristic stress-strain curves showing that increasing the number of DET cycles the tensile stress at which the samples break remains the same.*

To prove the idea that a scaffold, without any cellular component, could be successfully transplanted in a recipient animal, we performed a single transplant experiment. In a recipient rabbit, a surgical cervical incision

was performed to expose trachea and oesophagus. The latter was then isolated and a gap was created to mimic a 1,5 cm OA defect. An acellular scaffold was used to fill the gap and restore oesophageal integrity (Fig. 14). The animal withstood surgery with no surgical complications.

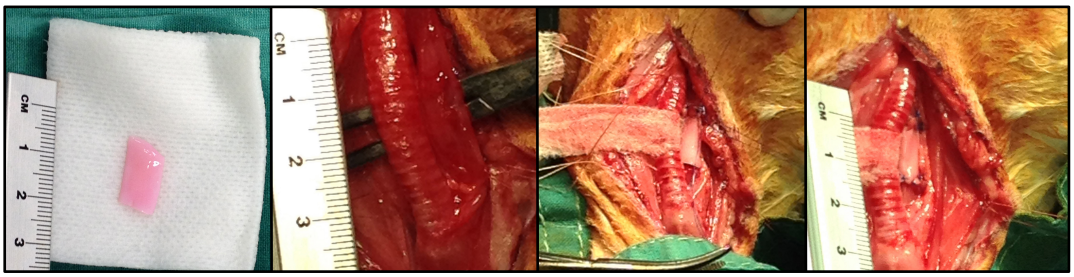

Figure 14. Oesophageal scaffold preparation and transplant in recipient rabbit.

4.2 STORAGE PROTOCOLS COMPARISON

After bringing back the stored scaffolds to room temperature, oesophageal matrices showed different appearances among the four storing protocols and after each time point. From a preliminary macroscopic analysis, SCM scaffolds appeared pink/orange due to the immersion in medium containing phenol red during storage, even after repeated washes with PBS. Except from the colour, SCM samples were comparable with a freshly decellularised. Following thawing of SF acellular oesophagi, clear tears could be seen in about 50% of the scaffolds, running in the longitudinal direction. Whilst 4C samples stored for 2 and 4 weeks were macroscopically comparable to freshly decellularised oesophagi, after storing for longer periods (3 and 6 months) the matrix showed degradation and loss of consistency and structural organization. This discrepancy between short and long time point storage was more remarked in FD samples, where scaffolds freeze-dried for up to 1 month could be rehydrated within 1 hour following PBS immersion, while oesophagi stored for 3 and 6 months could not be successfully rehydrated even after prolonged PBS immersion (Fig 15 A).

SEM analysis was performed to compare ultrastructure of the scaffolds. Here we present the comparison among the 4 storage methods at the last time point (6 months), with a sub-analysis of the different structural elements: lumen, submucosa and muscolaris (Fig. 15 B). Cross-sections of SCM- and SF-stored oesophagi show a similar preservation of the strata with detectable intact blood vessels in the submucosa of the SCM oesophagus. 4C-stored oesophagi demonstrate detachment of the mucosa and layers of the muscularis. The ECM is destroyed with collagen and elastin fibres forming spherical bundles. The FD oesophagus shows poor rehydration. All the layers are thin, condensed and stacked.

1. Lumen: examination of the oesophageal lumen at higher magnification shows no substantial differences between protocols. The ridges of the (now acellular) stratified epithelium are preserved except for in the FD-sample, where the surface is compact with longitudinal creases that run across it.
2. Submucosa: clear differences between storage methodologies were seen, that mirrored the histology results. The SCM-stored sample had a compact submucosa with the collagen fibres still present in bundles, whereas in the SF-stored sample the submucosa had frozen in dense layers that were separated by empty spaces created during the freezing process. The submucosa in the 4C-stored sample was completely disorganised with the collagen bundles having disintegrated to single fibres. In the FD-stored sample the ECM of the submucosa is hardly recognizable with bundles being reduced to sheets of dense matrix.
3. Muscularis: the stratum is well preserved in SCM-stored oesophagi with clear demarcation between the inner circular and outer longitudinal layers. In the SF-treated samples while the muscle layer is preserved, the muscle bundles are compact and separated from each other. Similarly, to the submucosa the muscle freezes one layer

at a time, pulling the tissue apart. The muscularis in the 4C is relatively well preserved with the bundles slightly coming apart. In the FD-treated oesophagus the muscle layer is compact and dense.

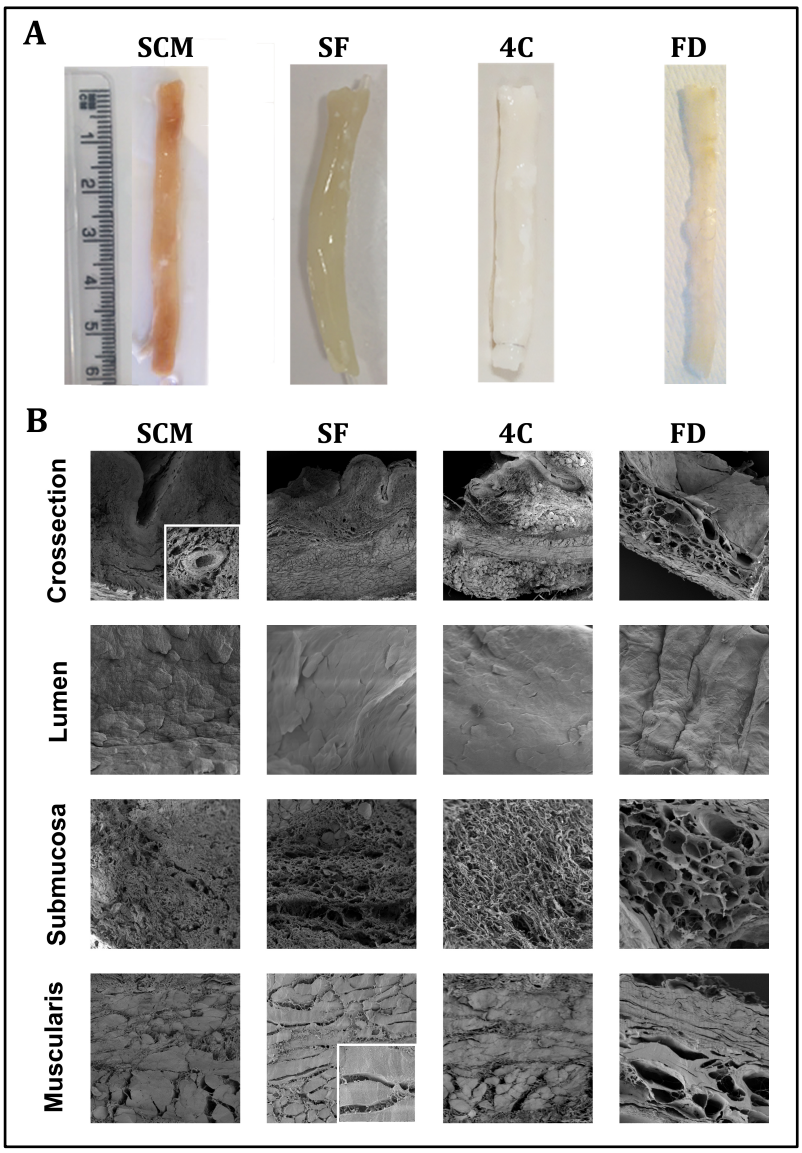

Figure 15. MACRO AND MICROSCOPIC APPEARANCE OF STORED DECELLULARISED SCAFFOLDS AT 6 MONTHS. ***A****: Macroscopic appearances varied across protocols with fractures present in the SF samples and heterogeneous rehydration in the FD scaffolds.*
B*: SEM analysis. SCM scaffolds demonstrated a good preservation of all oesophageal layers. SF scaffolds showed a stepwise freezing pattern that leads to areas of condensed and sparse ECM. In 4C samples ECM was falling apart with signs of degradation. FD scaffolds were completely collapsed, with the layers of the initial scaffold barely recognizable.*
SCM=slow cooling in medium, SF=snap-freezing, 4C=4^{0}C in PBS, FD=freeze-dried.

Histological analysis showed further differences among the storage methods.

- Masson's Trichrome (MT) staining for collagen (blue) showed a preserved structure and orientation of the connective tissue in SCM-stored oesophagi throughout all the time points. Even after 6 months, mucosa and muscularis were intact with the submucosa that connects the two being well maintained. SF stored oesophagi showed a variable degree of cryopreservation, as previously noted. Samples free of clear tears were analysed showing microscopic transverse fractures in the muscularis layers after 2 and 4 weeks, that extended to the mucosal layer after longer storage period. Moreover, the submucosal layer showed variable degrees of detachment from the muscle layer across all the timepoints. Masson's Trichrome (MT) in 4C stored oesophagi showed good preservation of the collagen up to 4 weeks, however at longer time points the connective tissue demonstrates a progressive deterioration with the muscle bundles increasingly thinning until they start detaching in layers. The mucosa breaks off completely at 3 months and couldn't be detected at 6 months. In FD-stored oesophagi the muscle, submucosal and mucosal layers were dense and clearly separated from each other in the first two time points. As expected from macroscopic analysis, storage for more than 3 months showed the irreversible collapse and breaking up of the tissue (Fig 16 A).
- Picrosirius Red (PR) staining was used to further understand changes in the ECM of the stored oesophagi: muscle fibres stain yellow while collagen type I/III stains red. The staining confirmed muscularis, mucosa and submucosa preservation in SCM samples, as well as no deterioration in SF-stored oesophagi (a part from the fractures caused by the freezing step). Progressive loss of muscle fibres was emphasized by picrosirius red staining in 4C samples, with gradual degradation of the muscle layer and collagens after 3 and 6 months.

Muscle fibre staining was rather not recognizable in FD samples too, supporting the profound changes in the ECM seen with MT staining (Fig 16 B).

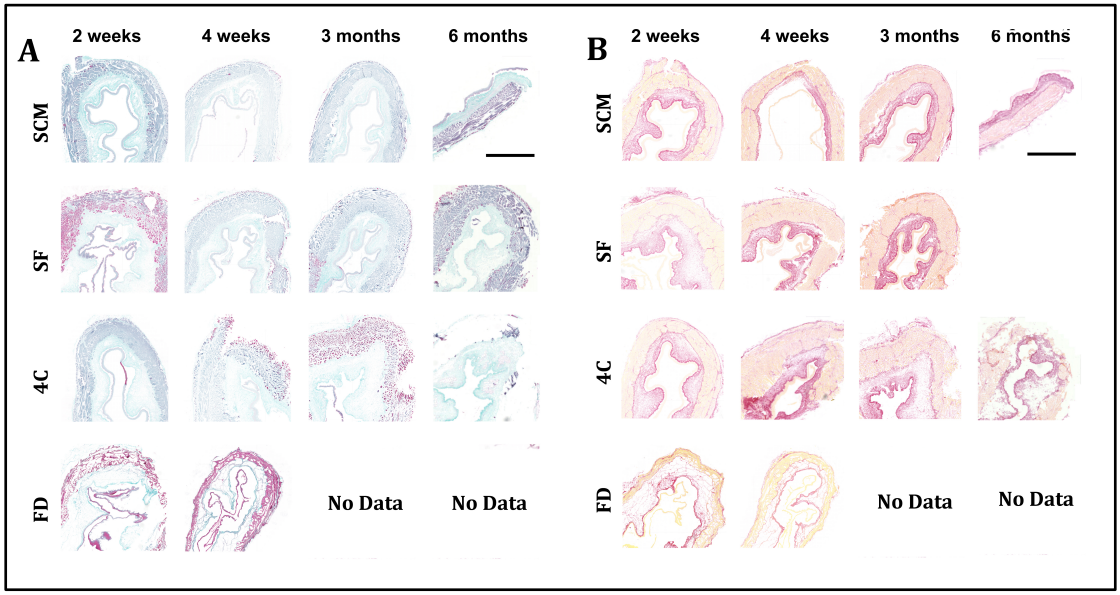

*Figure 16. COMPOSITION OF DECELLULARISED SCAFFOLDS AFTER STORAGE. Masson's Trichrome **(A)** and Picrosirius Red **(B)** staining demonstrated microfractures in SF-treated, a progressive loss of architecture in 4C-treated, and a markedflattening in FD-treated scaffolds. No data are available for FD samples at 3 and 6 months because the oesophagi could not be rehydrated.*
SCM=slow cooling in medium, SF=snap-freezing, 4C=4^{0}C in PBS, FD=freeze-dried.

- Elastin Van Gieson (EVG) staining highlighted changes in the elastin fibres present in the ECM, stained in black, distinguishable from collagen fibres in red. SCM-stored oesophagi demonstrated a well-maintained presence of vascular and submucosal elastin. Conservation of the perimuscular elastin is similar across samples and time points if the muscular layer is preserved. SF-stored oesophagi up to 3 months showed a good preservation of the vascular elastin, though the submucosal elastic fibres were hypercontracted. After 6 months the submucosa was broken up and showed weak staining. 4C-stored samples demonstrated a good preservation of vascular and

submucosal elastin only at the first timepoint, after that the submucosa was increasingly broken and elastin staining was poor and scattered. In FD-samples the elastin was preserved but compressed after 2 and 4 weeks. However, elastin was undetectable after 3 and 6 storage months (Fig 17 A).

- Alcian Blue (AB) staining in the stored oesophagi showed preservation of GAG in the submucosa across storage protocols and timepoints (Fig 17 B).

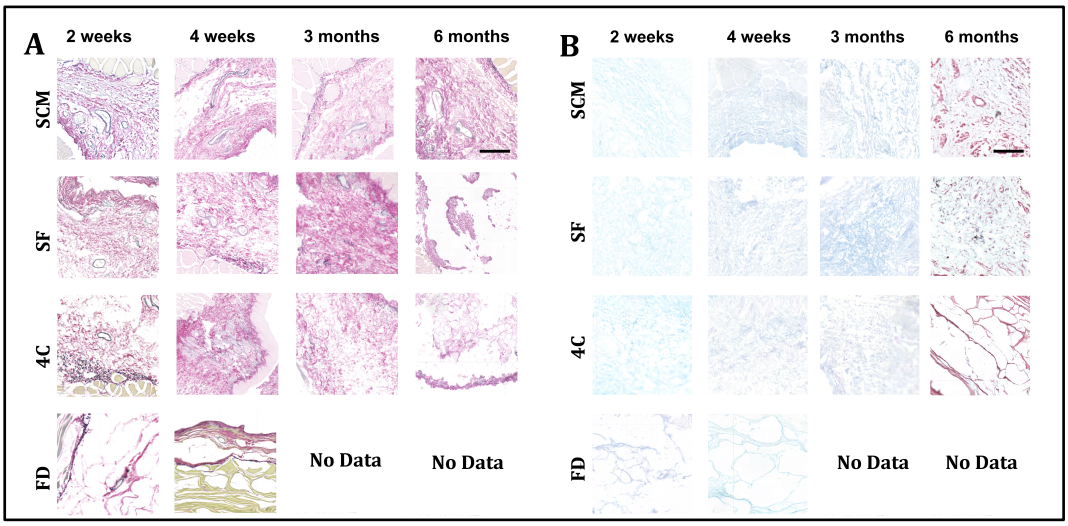

Figure 17. COMPOSITION OF DECELLULARISED SCAFFOLDS AFTER STORAGE.
***A:** elastin staining (EVG) showed maintenance of this protein in SCM scaffolds, with contractures in SF samples. Elastin was progressively lost in 4C scaffolds. **B:** AB staining showed GAG maintenance across storage. No data are available for FD samples at 3 and 6 months because the oesophagi could not be rehydrated.*
SCM=slow cooling in medium, SF=snap-freezing, 4C=4^{0}C in PBS, FD=freeze-dried.

Quantification of ECM components was performed to analyse presence and preservation of collagen, elastin and glycosaminoglycan after storage protocol (Fig 18 and Tab. 2). Having determined a better preservation of ECM architecture after 2 DET cycles and after SCM storage, this analysis is presented only for this storage method across the 4 time points and compared with fresh and decellularised samples.

After 2 weeks storage, no differences in collagen or elastin was seen compared to decellularised samples. A relative increase of GAG composition was detected after 2 weeks storage. No significative changes in collagen, elastin and GAG composition was detectable at the following time points.

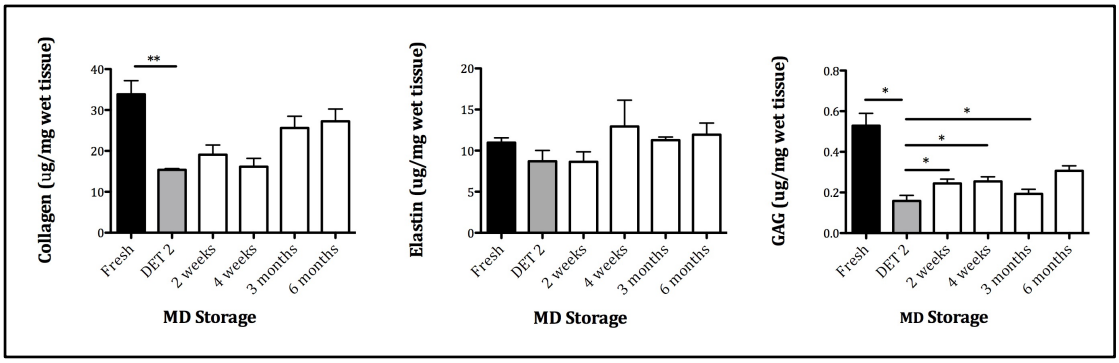

*Figure 18. ECM component quantification in fresh, decellularised (2 DET cycles) and SCM stored samples. *=p<0.05 and **=p<0.01.*

	2weeks	4weeks	3months	6months
COLLAGEN µg/mg	19.09 ± 3.32	16.14 ± 2.04	25.62 ± 2.86	27.27 ± 7.87
ELASTIN µg/mg	8.64 ± 3.66	12.94 ± 4.54	11.27 ± 0.65	11.92 ± 2.01
GAG µg/mg	0.24 ± 0.06	0.25 ± 0.06	0.29 ± 0.06	0.30 ± 0.03

Table 2. ECM COMPONENTS ANALYSIS. Quantification of collagen, elastin and glycosaminoglycans in decellularised scaffolds stored according to SCM protocol. Average ± standard deviation.

Biomechanical properties were analysed, as seen for fresh and decellularised samples, to describe stress/strain curve, UTS and Young's module after storage at the different time points. Four samples were considered for each evaluated tissue.

Comparison of Young's modulus and UTS at the first and last time points (2 weeks and 6 months) shows that samples stored in SCM maintain the same UTS after long term storage despite a decreased Young's module. The scaffold is therefore less stiff, but this change has no impact on the maximum stress that the material can withstand until it breaks, making it

more elastic and less rigid. On the other hand, 4C samples show UTS and Young's module values similar to SCM group at 2 weeks. However, prolonged 4C storage has a profound impact on the scaffold with a reduction of both values. The material becomes less resistant and cannot withstand the applied stress, with lower UTS compared both to the previous time point and to SCM group. Whilst SF samples did not present major changes over time, UTS is significatively lower compared to SCM at 6 months. FD results are presented only for the first time point because after 6 months it was impossible to rehydrate and test the samples (Fig 19).

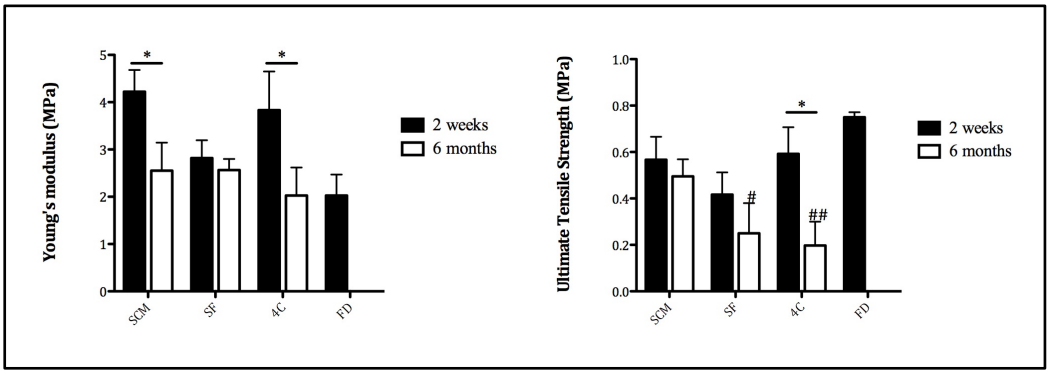

Figure 19. MECHANICAL PROPERTIES OF DECELLULARISED SCAFFOLDS AFTER STORAGE. Samples stored for 6 months with SCM maintained comparable ultimate tensile stress with 2 weeks stored scaffolds despite a decreased Young's module with no impact on the maximum stress that the material could withstand. While 4C samples at 2 weeks showed similar values to SCM, prolonged 4C storage had a profound impact on the scaffold with a reduction of both values. No data are available for FD samples at 3 and 6 months because the oesophagi could not be rehydrated.
*SCM=slow cooling in medium, SF=snap-freezing, 4C=4^{0}C in PBS, FD=freeze-dried. *p<0.05; #p<0.05 and ##p<0.01 vs SCM.*

5.0 DISCUSSION

Tissue engineering and regenerative medicine are becoming effective and applicable alternatives to conventional treatments in medicine. Different areas of medical interest could take advantage of bio-structures (scaffolds) created in laboratory and made available to restore a damaged tissue or to increase organ availability for transplant use. The scaffolds need to mimic the native structure, to be non-immunogenic - in order to avoid rejection risk and the need of a life-long immunosuppressive treatment - and to facilitate cells repopulation. With this view in mind, naturally derived decellularised scaffolds appear to be the optimal bio-engineered response to medical needs.

A large number of TE approaches involve the use of decellularised scaffolds that preserve features of the original ECM [24,50,61–64]. The rapid evolution of the field is bringing these decellularised scaffolds into pre-clinical and clinical use [65–67], highlighting limitations that have not been fully addressed yet. In particular, the lack of an established methodology for accurate long-term scaffold storage could limit clinical application, since this is an essential criterion to develop an off-the-shelf product, which could be used when clinically required. For a consistent and controlled use of scaffolds that will enable proof-of-principle studies to evolve into clinical practice, matrices need to be efficiently stored and made readily available for surgical implantation.

Therefore, we have studied the potential of decellularised scaffolds application in a congenital disease such as oesophageal atresia in a large animal model (rabbit), showing the feasibility of the process itself: from oesophageal isolation, decellularisation and long-term preservation.

In this study we have proven that the DET protocols, similarly to what established for other organs [24,29,49,50,56,61–63,67,68], is effective in removing

cell components from the rabbit oesophagus without damaging the ECM structure. The treatment is based on a combination of detergent (sodium deoxycholate) and enzymatic (DNase-I) solutions for tissue perfusion through the lumen. The number of cycles needed for an efficient decellularisation is dependent on tissue composition and therefore the method needed to be titrated on the rabbit oesophagus. DNA content analysis and H&E staining showed effective DNA removal after 2 DET cycles with no differences in DNA content found after further cycles. Based on these results we could have chosen to use alternatively 2 or 3 cycles, but further analysis had to be performed in order to verify if the ECM structure was properly preserved. The integrity of ECM structural and functional characteristics is in fact mandatory to allow for cell repopulation, implantation and favourable host interaction. Whilst multiple staining assays (MT, PR and AB) showed a good preservation of collagen fibres and GAG after decellularisation, staining for elastin (EVG) proved a clear decline of elastin presence after the 3rd DET cycle. Quantification analysis of matrix composition confirmed no differences in collagen and GAG content between cycle 2 and 3, but a clear drop in elastin content. Finally synchrotron images confirmed the preservation of the different strata. These findings suggested that 2 DET cycles were efficient in removing the cellular component while preserving ECM integrity. To confirm these data, biomechanical tests have been performed analysing the ability of the scaffolds to withstand a longitudinal force applied to the extremities. Each material has in fact a characteristic relationship between the amount of deformation (strain) expressed at different levels of tensile loading (stress). This relationship reveals many of the properties of a material and it is therefore a way to describe the biomechanical features of a sample. In our study there were no changes in UTS prior and after decellularisation, even after 3 cycles. This meant that the maximum stress that could be applied to the oesophagus before it broke did not change.

However, Young's modulus significantly increased after the last DET cycle proving that a 3rd cycle alters the scaffold characteristics making it stiffer and less malleable. It was therefore again proved that the best DET protocol for rabbit oesophagus decellularisation was the one based on 2 treatment cycles. Moreover the scaffold elicited a pro-angiogenic effect as seen at the CAM assay, which would facilitate its integration within the host tissue. These results are in line with our previous studies on DET application for tissue decellularisation. DET was first described in pig trachea[56] and proved capable of removing cellular content while preserving ECM components and features. Besides the successful clinical application of DET-decellularised trachea in a child [68], this simple treatment was more recently explored in more complex organs such as the lung and small bowel [48,50,69]. The same protocol has been previously successfully applied for the decellularisation of porcine oesophagi, allowing the development of a scaffold which preserved biomechanical characteristics of the original tissue [49]. In this study we confirm the versatility and reliability of the DET treatment in achieving a good decellularisation in another large animal model. As proof of surgical feasibility, a single acellular scaffold has been transplanted in a recipient rabbit. The animal survived the procedure without surgical complications.

In order to move this concept from the laboratory to its clinical application it was then necessary to identify a storage modality that could allow anticipatory preparation of the scaffold (prior to neonate birth) and its shipping to the receiving facility/surgical centre. It was therefore pivotal to analyse different storage conditions and to verify which of them could be effectively applied. The compared protocols have been chosen among the ones commonly used for tissue or cells storage.

We used rapid freezing by immersion in N_2 (SF), Storage at 4°C in PBS (4C), low temperature dehydration (FD) and slow cooling (1 °C /min) of

tissues immersed in 90% medium and 10% DMSO followed by storage in liquid nitrogen vapour (SCM). Stored scaffolds were analysed after short and long term time points to mimic the clinical need of an off-the-shelf availability of the product.

Comparison among the scaffolds has been performed from the structural and functional point of view through different methods and different time points. Composition and biomechanical properties are strictly connected and are extremely important especially in hollow organs like the oesophagus. Under the macroscopic point of view, no major differences have been noticed after short time points (2-4 weeks), apart from a pink nuance of the SCM scaffold due to the medium used, that could be easily avoid using another buffer or medium without phenol red in future applications. On the contrary, long term storage (3-6 months) induced major changes in SF (presence of clear longitudinal cracks), 4C (loss of consistency) and FD samples (unsuccessful rehydration even after prolonged immersion in PBS).

From an ultrastructure point of view, SEM analysis confirmed the superiority of SCM storage protocol in preserving the architectural characteristic of the scaffold strata: lumen, submucosa and muscolaris. Only SF protocol was able to achieve a similar result even for long term time points, but only for those scaffolds that did not developed tears.

For detailed structural composition analysis, ECM composition was studied by histology and by collagen/elastin/GAG quantification.

Staining for collagen showed fibres preservation after 2 and 4 weeks in SCM and 4C samples, microscopic tears were detectable after SF storage and a clear separation of the condensed strata was detectable in FD decellularised oesophagi. Analysis performed after 3 and 6 months showed collagen preservation only in SCM samples. These results, together with other founding from our study, confirmed the notion that the 4C methodology is efficient only for short term storage of natural

derived scaffolds. No major changes were detectable in GAG disposition among protocols, while elastin fibres were clearly well represented at 3 and 6 months only in SCM samples. To confirm this peculiar ability of SCM protocol to preserve the ECM composition after both short and long term time points, ECM components quantification has been performed. We confirmed that, despite a known reduction in collagen and GAG presence in decellularised scaffolds compared to fresh tissue, there were no further changes in collagen/elastin/GAG composition during SCM storage period, from 2 weeks up to 6 months.

Since SCM storage seemed to be the best protocol from the architectural point of view, we performed biomechanical test to verify if the structural preservation allowed a functional advantage over the other protocols. Despite a decreased Young's module in SCM samples, UTS did not differ over time showing that scaffolds became less stiff, but did not loose the ability to respond to a longitudinal tress and withstand the applied force. SF samples did not change over time, but UTS at 6 months was significantly lower compared to SCM samples. 4C scaffolds showed a decrease in Young's module values as seen for SCM samples, but it was associated with a concomitant reduction in UTS proving that the scaffold was loosing consistency and resistance. Regarding FD samples, it was impossible to perform these tests after 6 months because rehydration of the matrices was unsuccessful as previously described.

All together these data proved the superiority of SCM protocol for long term storage, suggesting its use to reach the off-the-shelf availability that is required for clinical use. While this technique has widely been tested for cells freezing and banking, its use in decellularised scaffold storage represents an innovative application.

It is known that different decellularisation methods can affect composition, structure and biomechanical properties of the resulting ECM. Nevertheless, even though we compared the efficacy of the 4 storage

techniques only on DET derived decellularised scaffolds, we believe that the SCM protocol, thanks to its cryobiology origin, will allow optimal preservation of other decellularised scaffolds independently from their size and the method used for their decellularisation.

In conclusion, TE is becoming a valid alternative for organ replacement owing to the improvements in natural scaffold production, in particular with decellularisation techniques. However, when moving from bench to a clinical scenario TE needs to evolve and develop new strategies for scaffold storage and shipping. In this study we successfully decellularised rabbit oesophagi using the described DET method. Most importantly we identified the best storage methodology among different protocols using slow cooling medium and liquid nitrogen, for the long term preservation of the structural and mechanical properties of the decellularised scaffold.

REFERENCES

1. Orford J, Glassom M, Beasley S, Shi E, Myers N, Cass D. Oesophageal atresia in twins. *Pediatr Surg Int*. 2000;16(8):541-545.
2. Gross RE. *The Surgery of Infancy and Childhood.* Philadelphia: WB Saunders; 1953.
3. Houben H, Curry JI. Current status of prenatal diagnosis, operative management and outcome of esophageal atresia/tracheo-esophageal fistula. *Prenat Diagn*. 2008;28(7):667-675.
4. Spitz L, Kiely EM, Morecroft J, Drake DP. Oesophageal atresia: at-risk groups for the 1990s. *Pediatr Surg*. 1994;29(6):723-725.
5. Lopez PJ, Keys C, Pierro A, et al. Oesophageal Atresia: improved otcome in high-risk groups? *Pediatr Surg*. 2006;41(2):331-334.
6. Kimura K, Soper TR. Multistaged extrathoracic esophageal elongation for long gap esophageal atresia. *J Pediatr Surg*. 1994;29(4):566-568.
7. Livaditis A, Radberg L, Odensjo G. Esophageal end-to-end anastomosis. Reduction of anastomotic tension by circular myotomy. *Scand J Thorac Cardiovasc Surg*. 1972;6(2):206-214.
8. De Lorimer A, Harrison M. Long gap esophageal atresia: primary anastomosis after esophageal elongation by bougienage and esophagomyotomy. *J Thorac Cardiovasc Surg*. 1980;79(1):138-141.
9. Scharli A. Esophageal reconstruction by elongation of the lesser gastric curvature. *Pediatr Surg Int*. 1996;11(4):214-217.
10. Foker JE, Linden BC, Boyle EM, Marquardt C. Development of a true primary repair for the full spectrum of esophageal atresia. *Ann Surg*. 1997;226(4):533-541; discussion 541-543. doi:10.1097/00000658-199710000-00014.
11. Spitz L. Esophageal replacement: Overcoming the need. *J Pediatr Surg*. 2014;49(6):849-852. doi:10.1016/j.jpedsurg.2014.01.011.
12. Torre M, Carlucci M, Speggiorin S, Elliott MJ. Aortopexy for the treatment of tracheomalacia in children: review of the literature. *Ital J Pediatr*. 2012;38(1):62. doi:10.1186/1824-7288-38-62.
13. Kovesi T, Rubin S. Long-term Complications of Congenital Esophageal Atresia and / or Tracheoesophageal Fistula*. *Chest*. 2004;126:915-925. doi:10.1378/chest.126.3.915.
14. Fragoso AC, Tovar J a. The multifactorial origin of respiratory morbidity in patients surviving neonatal repair of esophageal atresia. *Front Pediatr*. 2014;2(May):6-10. doi:10.3389/fped.2014.00039.

15. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials*. 2011;32(12):3233-3243. doi:10.1016/j.biomaterials.2011.01.057.

16. Vorotnikova E, McIntosh D, Dewilde A, et al. Extracellular matrix-derived products modulate endothelial and progenitor cell migration and proliferation in vitro and stimulate regenerative healing in vivo. *Matrix Biol*. 2010;29(8):690-700. doi:10.1016/j.matbio.2010.08.007.

17. Bornstein P, Sage EH. Matricellular proteins: extracellular modulators of cell function. *Curr Opin Cell Biol*. 2002;14(5):608-616. doi:10.1016/S0955-0674(02)00361-7.

18. Fishman JM, Lowdell MW, Urbani L, et al. Immunomodulatory effect of a decellularized skeletal muscle scaffold in a discordant xenotransplantation model. *Proc Natl Acad Sci U S A*. 2013;110(35):14360-14365. doi:10.1073/pnas.1213228110.

19. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomater*. 2009;5(1):1-13. doi:10.1016/j.actbio.2008.09.013.

20. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. *Biomaterials*. 2006;27(19):3675-3683. doi:10.1016/j.biomaterials.2006.02.014.

21. Ott HC, Matthiesen TS, Goh S-K, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008;14(2):213-221. doi:10.1038/nm1684.

22. Petersen TH, Calle E a., Colehour MB, Niklason LE. Bioreactor for the long-term culture of lung tissue. *Cell Transplant*. 2011;20(7):1117-1126. doi:10.3727/096368910X544933.

23. Price AP, England K a, Matson AM, Blazar BR, Panoskaltsis-Mortari A. Development of a decellularized lung bioreactor system for bioengineering the lung: the matrix reloaded. *Tissue Eng Part A*. 2010;16(8):2581-2591. doi:10.1089/ten.tea.2009.0659.

24. Shupe T, Williams M, Brown A, Willenberg B, Petersen BE. Method for the decellularization of intact rat liver. *Organogenesis*. 2010;6(2):134-136. doi:10.4161/org.6.2.11546.

25. Meyer SR, Chiu B, Churchill TA, Zhu L, Lakey JRT, Ross DB. Comparison of aortic valve allograft decellularization techniques in the rat. *J Biomed Mater Res A*. 2006;79(2):254-262. doi:10.1002/jbm.a.30777.

26. Montoya CV, McFetridge PS. Preparation of Ex Vivo–Based Biomaterials Using Convective Flow Decellularization. *Tissue Eng Part C Methods*. 2009;15(2):191-200.

doi:10.1089/ten.tec.2008.0372.

27. Deeken CR, White AK, Bachman SL, et al. Method of preparing a decellularized porcine tendon using tributyl phosphate. *J Biomed Mater Res Part B*. 2011;96B(2):199-206.

28. Karabekmez FE, Duymaz A, Moran SL. Early Clinical Outcomes with the Use of Decellularized Nerve Allograft for Repair of Sensory Defects Within the Hand. *Hand (N Y)*. 2009;4(3):245-249. doi:10.1007/s11552-009-9195-6.

29. Baiguera S, Jungebluth P, Burns A, et al. Tissue engineered human tracheas for in vivo implantation. *Biomaterials*. 2010;31(34):8931-8938. doi:10.1016/j.biomaterials.2010.08.005.

30. Ozeki M, Narita Y, Kagami H, et al. Evaluation of decellularized esophagus as a scaffold for cultured esophageal epithelial cells. *J Biomed Mater Res A*. 2006;79(4):771-778. doi:10.1002/jbm.a.30885.

31. Reing JE, Brown BN, Daly KA, et al. The Effects of Processing Methods upon Mechanical and Biologic Properties of Porcine Dermal Extracellular Matrix Scaffolds. 2010;31(33):8626-8633. doi:10.1016/j.biomaterials.2010.07.083.The.

32. Yang B, Zhang Y, Zhou L, et al. Development of a porcine bladder acellular matrix with well-preserved extracellular bioactive factors for tissue engineering. *Tissue Eng Part C Methods*. 2010;16(5):1201-1211. doi:10.1089/ten.TEC.2009.0311.

33. Hynes RO. Extracellular matrix: not just pretty fibrils. *Science (80-)*. 2013;326(5957):1216-1219. doi:10.1126/science.1176009.Extracellular.

34. Totonelli G, Maghsoudlou P, Fishman JM, et al. Esophageal tissue engineering: A new approach for esophageal replacement. *World J Gastroenterol*. 2012;18(47):6900-6907. doi:10.3748/wjg.v18.i47.6900.

35. Berman EF. The experimental replacement of portions of the esophagus by a plastic tube. *Ann Surg*. 1952;135(3):337-343.

36. Freud E, Efrati I, Kidron D, Finally R, Mares AJ. Comparative experimental study of esophageal wall regeneration after prosthetic replacement. *J Biomed Mater Res*. 1999;45(2):84-91.

37. Lister J, Altman RP, Allison WA. Prosthetic Substitution of Thoracic Esophagus in Puppies: Use of Marlex Mesh with Collagen or Anterior Rectus Sheath. *Ann Surg*. 1965;162(5):812-824.

38. Fryfogle JD, Cyrowski GA, Rothwell D, Rheault G, Clark T. Replacement of the middle third of the esophagus with a silicone rubber prosthesis. An experiment and clinical study. *Dis Chest*.

1963;43:464-475.

39. Watanabe K, Mark JB. Segmental replacement of the thoracic esophagus with a Silastic prosthesis. *Am J Surg*. 1971;121(3):238-240.

40. Lynen Jansen P, Klinge U, Anurov M, Titkova S, Mertens PR, Jansen M. Surgical mesh as a scaffold for tissue regeneration in the esophagus. *Eur Surg Res*. 2004;36(2):104-111. doi:10.1159/000076650.

41. Shinhar D, Finaly R, Niska A, Mares AJ. The use of collagen-coated vicryl mesh for reconstruction of the canine cervical esophagus. *Pediatr Surg Int*. 1998;13(2-3):84-87.

42. Yamamoto Y, Nakamura T, Shimizu Y, et al. Intrathoracic esophageal replacement with a collagen sponge--silicone double layer tube: evaluation of omental-pedicle wrapping and prolonged placement of an inner stent. *ASAIO J*. 2000;46(6):734-739.

43. Natsume T, Ike O, Okada T, Takimoto N, Shimizu Y, Ikada Y. Porous collagen sponge for esophageal replacement. *J Biomed Mater Res*. 1993;27(7):867-875. doi:10.1002/jbm.820270705.

44. Takimoto Y, Okumura N, Nakamura T, Natsume T, Shimizu Y. Long-term follow-up of the experimental replacement of the esophagus with a collagen-silicone composite tube. *ASAIO J*. 1993;39(3):M736-M739.

45. Baiguera S, Urbani L, Gaudio C Del. Tissue Engineered Scaffolds for an Effective Healing and Regeneration : Reviewing Orthotopic Studies. *Bio Med Res Int*. 2014.

46. Marzaro M, Vigolo S, Oselladore B, et al. In vitro and in vivo proposal of an artificial esophagus. *J Biomed Mater Res A*. 2006;77(4):795-801. doi:10.1002/jbm.a.30666.

47. Sjöqvist S, Jungebluth P, Lim ML, et al. Experimental orthotopic transplantation of a tissue-engineered oesophagus in rats. *Nat Commun*. 2014;5:3562. doi:10.1038/ncomms4562.

48. Maghsoudlou P, Georgiades F, Tyraskis A, et al. Preservation of micro-architecture and angiogenic potential in a pulmonary acellular matrix obtained using intermittent intra-tracheal flow of detergent enzymatic treatment. *Biomaterials*. 2013;34(28):6638-6648. doi:10.1016/j.biomaterials.2013.05.015.

49. Totonelli G, Maghsoudlou P, Georgiades F, et al. Detergent enzymatic treatment for the development of a natural acellular matrix for oesophageal regeneration. *Pediatr Surg Int*. 2013;29(1):87-95.

50. Totonelli G, Maghsoudlou P, Garriboli M, et al. A rat decellularized small bowel scaffold that preserves villus-crypt architecture for

intestinal regeneration. *Biomaterials*. 2012;33(12):3401-3410. doi:10.1016/j.biomaterials.2012.01.012.

51. Mansbridge J. Commercial considerations in tissue engineering. *J Anat*. 2006;209(4):527-532. doi:10.1111/j.1469-7580.2006.00631.x.

52. Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol*. 1984;247(3 Pt 1):C125-C142.

53. Wusteman MC, Pegg DE, Warwick RM. The banking of arterial allografts in the United kingdom. A technical and clinical review. *Cell Tissue Bank*. 2000;1(4):295-301. doi:10.1023/A:1010167604609.

54. Bakhach J. The cryopreservation of composite tissues: Principles and recent advancement on cryopreservation of different type of tissues. *Organogenesis*. 2009;5(3):119-126.

55. Pamphilon D, Mijovic A. Storage of hemopoietic stem cells. *Asian J Transfus Sci*. 2007;1(2):71-76. doi:10.4103/0973-6247.33848.

56. Jungebluth P, Go T, Asnaghi A, et al. Structural and morphologic evaluation of a novel detergent–enzymatic tissue-engineered tracheal tubular matrix. *J Thorac Cardiovasc Surg*. 2009;138(3):586-593. doi:10.1016/j.jtcvs.2008.09.085.

57. Baiguera S, Del Gaudio C, Jaus MO, et al. Long-term changes to in vitro preserved bioengineered human trachea and their implications for decellularized tissues. *Biomaterials*. 2012;33(14):3662-3672. doi:10.1016/j.biomaterials.2012.01.064.

58. Bonenfant NR, Sokocevic D, Wagner DE, et al. The effects of storage and sterilization on de-cellularized and re-cellularized whole lung. *Biomaterials*. 2013;34(13):3231-3245. doi:10.1016/j.biomaterials.2013.01.031.

59. Wang S, Goecke T, Meixner C, Haverich A, Hilfiker A, Wolkers WF. Freeze-Dried Heart Valve Scaffolds. *Tissue Eng Part C Methods*. 2012;18(7):517-525. doi:10.1089/ten.tec.2011.0398.

60. Paganin D, Mayo SC, Gureyev TE, Miller PR, Wilkins SW. Simultaneous phase and amplitude extraction from a single defocused image of a homogeneous object. *J Microsc*. 2002;206(1):33-40. doi:10.1046/j.1365-2818.2002.01010.x.

61. Nakayama KH, Batchelder CA, Lee CI, Tarantal AF. Decellularized Rhesus Monkey Kidney as a Three-Dimensional Scaffold for Renal Tissue Engineering. *Tissue Eng Part A*. 2010;16(7):2207-2216. doi:10.1089/ten.tea.2009.0602.

62. Ross EA, Abrahamson DR, John PLS, et al. Mouse stem cells seeded into decellularized rat kidney scaffolds endothelialize and remodel basement membranes. 2012;(June):49-55.

63. Barakat O, Abbasi S, Rodriguez G, et al. Use of decellularized porcine liver for engineering humanized liver organ. *J Surg Res*. 2012;173(1):e11-e25. doi:10.1016/j.jss.2011.09.033.

64. Ott HC, Clippinger B, Conrad C, et al. Regeneration and orthotopic transplantation of a bioartificial lung. *Nat Med*. 2010;16(8):927-933. doi:10.1038/nm.2193.

65. Raya-Rivera A, Esquiliano DR, Yoo JJ, Lopez-Bayghen E, Soker S, Atala A. Tissue-engineered autologous urethras for patients who need reconstruction: an observational study. *Lancet (London, England)*. 2011;377(9772):1175-1182. doi:10.1016/S0140-6736(10)62354-9.

66. Hamilton NJ, Kanani M, Roebuck DJ, et al. Tissue-Engineered Tracheal Replacement in a Child: A 4-Year Follow-Up Study. *Am J Transplant*. 2015;15(10):2750-2757. doi:10.1111/ajt.13318.

67. Macchiarini P, Jungebluth P, Go T, et al. Clinical transplantation of a tissue-engineered airway. *Lancet*. 2008;372(9655):2023-2030. doi:10.1016/S0140-6736(08)61598-6.

68. Elliott MJ, De Coppi P, Speggiorin S, et al. Stem-cell-based, tissue engineered tracheal replacement in a child: A 2-year follow-up study. *Lancet*. 2012;380(9846):994-1000. doi:10.1016/S0140-6736(12)60737-5.

69. Maghsoudlou P, Totonelli G, Loukogeorgakis SP, Eaton S, De Coppi P. A decellularization methodology for the production of a natural acellular intestinal matrix. *J Vis Exp*. 2013;(80). doi:10.3791/50658.

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