POLITECNICO DI MILANO

Scuola d'Ingegneria Industriale e dell'Informazione

Corso di Laurea Magistrale in Ingegneria Biomedica – Biomedical Engineering

Development of novel bioreactors for oesophageal and parenchymatous organs tissue engineering

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Academic year: 2019/2020

…A voi che mi avete sopportato durante questo percorso,

un sincero abbraccio

Abstract

Most congenital gastrointestinal anomalies result in some type of obstruction, frequently manifesting with feeding difficulties, abdominal distention, and emesis at birth or within the first days. Oesophageal atresia is the most common gastrointestinal atresia, with an estimated incidence of 1 in 3500 live births. Atresia is a congenital absence or closure of a normal body opening or tubular structure and is associated with disorders present in different parts of the body. Gastric pull-up, colon transposition and jejunum transposition represent the most endorsed treatments for long-gap oesophageal atresia. However, these procedures are afflicted by common severe complications that most often lead to later life further problems in operated children. Besides, a life-long immunosuppression is often needed. These problems leaded to the search for alternative therapeutic strategies, and tissue engineering comes within this context.

The main work presented in this thesis involves the development of a novel bioreactor used in the dynamic culture of a porcine-derived oesophagus. The bioreactor allows an adjustable set-up for the culture, basing on the organ's size. The dynamic culture included the harvesting of the oesophagus, the decellularization of the organ in order to remove the native cellular content, and successive re-population of the decellularized scaffold with human mesoangioblasts and human fibroblasts. The decellularization process of an organ discharges most of the original cellular material while aiming to retain the native organ architecture, obtaining a scaffold. The scaffold can be seeded with suitable cells and cultured *in vitro* to obtain a functional tissue suitable to transplantation. The bioreactor developed in this work was used for both decellularization and recellularization. The tissue analysed at the end of the dynamic culture showed clusters of cells re-populating areas of the scaffold where they started a local remodelling of the organ's internal structures. The realized bioreactor allows different size oesophagi to be cultured and its application could in the future be extended to all luminal organs.

In this thesis is also presented an additional work comprising the design of a bioreactor addressed to the dynamic culture of all parenchymatous organs. The work performed is relative to the design, construction and assessment of the sterility properties of the bioreactor. In a future perspective, the inclusion of this bioreactor in a dynamic culture of a

parenchymatous organ (e.g. liver or kidney), can bring further assessments on its suitability for tissue engineering purposes.

Sommario

La maggior parte delle anomalie congenite del tratto gastrointestinale sono caratterizzate da ostruzioni di vario tipo, che si manifestano frequentemente con difficoltà nell'alimentazione, distensione addominale ed emesi, alla nascita nei primi giorni di vita successivi. L'atresia esofagea è il tipo di atresia più comune del tratto gastrointestinale, con un'incidenza stimata di 1 su 3500 nativi vivi. L'atresia è un'assenza o una chiusura congenita di un orifizio o una struttura tubulare normalmente aperti, ed è associata con vari disordini in altre parti del corpo. Pull-up gastrico, trasposizione del colon e del digiuno rappresentano ad oggi i trattamenti più utilizzati nel caso di atresia esofagea di tipo long-gap. A questi interventi sono però associate severe complicazioni che possono successivamente portare ad ulteriori problemi nel corso della vita dei bambini operati. Inoltre, spesso è necessaria una terapia immunosoppressiva a vita. Queste controindicazioni hanno spinto nella direzione della ricerca di terapie alternative, ed è in questo ambito che l'ingegneria tissutale fa la sua comparsa.
Il lavoro principale che viene presentato in questa tesi è incentrato sullo sviluppo originale di un bioreattore utilizzato nella coltura dinamica di un esofago di maiale. Il bioreattore permette di essere regolato sulla base delle dimensioni dell'organo in coltura. La coltura dinamica prevedeva l'estrazione chirurgica dell'esofago, la relativa decellularizzazione al fine di rimuovere il contenuto cellulare nativo, e il successivo ripopolamento dello scaffold decellularizzato con mesoangioblasti e fibroblasti, entrambi di provenienza umana. Il processo di decellularizzazione di un organo mira a scartare la gran parte del materiale cellulare originale mantenendo però l'architettura nativa dell'organo, ottenendo uno scaffold. Lo scaffold può essere seminato con determinate cellule e coltivato *in vitro* per ottenere un tessuto funzionale adatto al trapianto. Il bioreattore sviluppato in questo lavoro è stato usato sia per la decellularizzazione che per la ricellularizzazione. Il tessuto analizzato alla fine della coltura ha mostrato la presenza di agglomerati di cellule in determinate zone, in corrispondenza delle quali era rilevabile un iniziale rimodellamento delle strutture interne native dell'organo. Il bioreattore realizzato permette la coltura di esofagi di differenti dimensioni e in future la sua applicazione potrebbe essere estesa all'intera classe degli organi luminali.

In questa tesi viene presentato anche un lavoro aggiuntivo che comprendeva il design di un bioreattore rivolto alla coltura dinamica di tutti gli organi parenchimatosi. Il lavoro eseguito è relative al design, alla costruzione e alla valutazione della sterilità di tale bioreattore. In una prospettiva futura, l'utilizzo di questo bioreattore nell'effettiva coltura dinamica di un organo parenchimatoso (e.g. fegato o reni) può condurre a ulteriori valutazioni sulla sua idoneità all'uso nell'ambito dell'ingegneria tissutale.

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Chapter 1

Introduction

1.1 Regenerative Medicine and Tissue Engineering

Organ and tissue loss through disease and injury promotes the development of regeneration-oriented therapies decreasing reliance on transplantations, considering the wide gap between demand and offer of organs [1]. The current therapy of transplantation of intact organs and tissues to treat failures and loss suffers from limited donor supply and often severe immune complications, but these obstacles may potentially be bypassed using regenerative medicine strategies [2].

The field of regenerative medicine encompasses numerous strategies, including the use of materials and de novo generated cells, as well as various combinations thereof, to take the place of missing tissue, effectively replacing it both structurally and functionally, or to contribute to tissue healing [3].

In this context, a broad range of strategies at both the preclinical and clinical stages of investigation are currently being explored; Such strategies have been broken up into three broad categories: (i) recapitulating organ and tissue structure via scaffold fabrication, 3D bioprinting, and self-assembly [4]; (ii) integrating grafts with the host via vascularization and innervation [5]; and (iii) altering the host environment to induce therapeutic responses, particularly through cell infusion and modulating the immune system [6].

Tissue engineering (TE) is a branch of regenerative medicine evolved from the field of biomaterials development, and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues.

The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs. [7]. TE employs different approaches:

- Direct cell implantation: individual cells, from the recipient or a donor, injected directly into the damaged tissue [8].

- In situ tissue regeneration: natural or synthetic scaffolds introduced to exploit the body's natural ability to regenerate [9].
- Implantation of tissues: engineered biological tissues cultured and implanted into defected tissue [10].

However, TE has currently significant limitations, such as:

- Size limit of tissues engineered in vitro: every cell need blood supply short distance (100-200 μm at most) over which oxygen can diffuse Therefore, it is fundamental to produce a vascularized construct, this is one of the most important challenges of TE for translation in clinic [11].
- Innervation: another key challenge in TE is the recreation of the nerves in the tissue [12].
- Cost-effectiveness of the approach [13].

1.2 Design of an engineered tissue

In general terms, the design process of an engineered tissue involves the following phases (Fig.1):

- Manufacturing of 3D supports (scaffolds).
- Culture and *in vitro* expansion of cells from a donor or recipient.
- Seeding cells on scaffolds.
- Culture under dynamic conditions: "conditioning" of the tissue with a bioreactor.
- Implantation of the construct in the model animal/patient.

Generally, a process of TE involves three components [14]:

- Cells.
- Biomaterial or scaffolds: they have the function of driving, cell growth and differentiation in a defined space.
- *Signals:* stimulating factors inducing cell responses such as proliferation and differentiation.

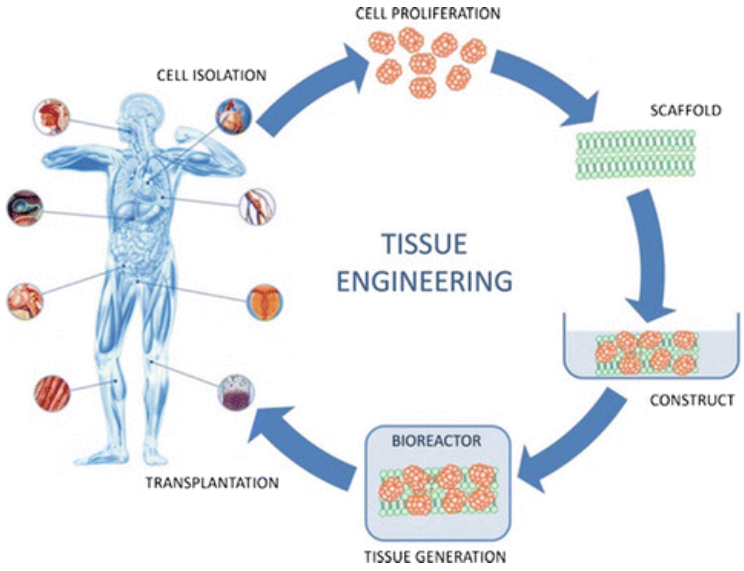


Figure 1 Schematic representation of a typical process followed in the development of an engineered tissue. The main components comprehend a cell culture, where cells are increased in number to be able to populate a scaffold which serves as the supporting structure of the tissue. The construct is the scaffold considering the cells engrafted in it, and it can be installed in a bioreactor. The bioreactor purpose is to better allow the growing of an organic tissue starting from the cells seeded in the scaffold, often mimicking the natural conditions to which the tissue is subjected in native environment. An appropriately developed tissue can be thus transplanted in the site of the body requiring it. Adapted from 'Tissue Engineering and Regenerative Medicine Research Perspectives for Pediatric Surgery' by A. Saxena, *Pediatric Surgery International* volume 26, pages557–573(2010).

1.2.1 Cells

We can classify the cell sources for regenerative therapies based on different criteria. The most important classification is related on the cell potency. Based on cell potency, cells can be distinguished for their ability of differentiate into any cell family (totipotent), or multiple cell families (pluripotent), or into a closely related family of cells (multipotent), or into a single cell type (unipotent) [15]. Stem cells are cells that replicate almost indefinitely and can differentiate into typical cells of the tissue in which they reside (if adult stem cells) or in various cell types (if embryonic). Their main limitation is availability. Recently induced pluripotent stem cells (iPSCs) have been inserted in this scenario. iPSCs are pluripotent stem cells derived from adult somatic cells appropriately" reprogrammed"; they have very similar characteristics to embryonic stem cells. Today iPSCs are a valid solution in regenerative medicine thanks to the possibility of using cells human somatic and to differentiate into

many cell types [16, 17].

Cells lines used for culture can be distinguished in primary and immortalized. Immortalized cells derive from tissues and subsequently modified. This modification is an alteration of the genetic code (immortalization), and this is possible with chemical transformation (via viral agents) or irradiation. They are able to grow rapidly and replicate indefinitely in vitro, but this is not a realistic and significant biological answer and they can give rise to tumours if implanted. Primary cells derive from biopsy directly from an organism (human or animal). The process to obtain these cells are:

- Dissection.
- Enzymatic digestion (e.g. collagenase, trypsin, etc.) and/or cell isolation.
- Cell characterization.

Primary cells do not replicate indefinitely, they can only be expanded in culture for a limited number of steps. These cells are used because they have an in vitro realistic response, they are also applicable in clinic. These two cell groups have different advantages and disadvantages; all different aspects are listed in Table 1.1. In the context of regenerative medicine, it is useful to classify different cell sources basing on immunogenicity, namely the property to provoke an immune response in the recipient. Cells can thus be divided in autologous, syngeneic, allogeneic and xenogeneic. When the donor of cells corresponds to the recipient we speak of autologous cells. When the donor and the recipient have identical genotypes, we refer to syngeneic cells. Autologous and syngeneic cells are generally not immunogenic. Allogeneic cells are involved if the donor and the recipient belong to the same species, while in the context of xenogeneic cells the donor and the recipient belong to different species. Allogeneic cells can cause immune rejection in the recipient, but if the donor of cells and the recipient are matched through their Human Leukocyte (HLA) group, the immune response can be controlled by immune-depressor drugs. Xenogeneic cells are highly immunogenic and their interaction with the recipient's immune system may cause a severe syndrome denominated "acute immune rejection. [18]. The comparison between the various types of cells mentioned is shown in Table 1.2.

Table 1.1: Differences between primary and immortalized cells. Adapted from 'Ask a scientist: what's the difference between primary and immortalized cells?' by Dr A. Lodge, *www.cellero.com*,2018

	Primary Cells	Immortalized Cells
Origin	Isolated directly from donor	Isolated from tumors or intentionally immortalized with viruses
Lifespan	Limited	Unlimited lifespan in culture
***In Vivo* Model**	Yes	No
Function	Closely resembles cell function	May lack functions or characteristics of normal cells
Maintenance	Not considered primary cells following passage or culture	Can be maintained to provide consistent experimental results
Donor Characteristics	Available	Not available
Use Cases	Immunology, inflammation, vaccination or other biological experiments requiring cells with a close match to *in vivo* function	Studying tumor cells; If the cell of interest is not available or it is impractical to use primary cells

Table 1.2: Comparison of different cell sources for regenerative medicine-therapies. Adapted from lesson slides of "Technologies for regenerative medicine" by Prof. M. T. Raimondi, Politecnico di Milano, AA 2017/2018

Cell source	Advantages	Criticalities
Autologous	Histo-compatible	Limited availability. Limited expandability. Risk of teratoma formation for induced-pluripotency stem (iPS) cells.
Syngeneic	Histo-compatible except for mitochondrial DNA	Risk of teratoma formation for embryonic cells. Ethical, technical and regulatory limitations for cloned cells.
Allogeneic	Available from deceased donors. Can be industrialized.	Risk of immune rejection. Risk of tumorigenesis for immortalized cell lines.
Xenogeneic	Largely available from animal livestock. Can be industrialized.	Risk of acute immune rejection. Risk of transmission of xeno-zoonoses from viruses (like the HIV infection from non-human primates and the EBOLA infection), endogenous retroviruses (like the PERV infection from pigs), or prions (like the BSE infection from cows).

Another criterion by which cells are classified, concerns the growth mechanism. There are

adhesive cells and suspension cells. The first ones grow adhering to the culture surface and require interaction between adhesion receptors of membrane and adhesive proteins adsorbed on the culture surface (e.g. fibronectin, laminin, poly-L-lysine, matrigel). The second ones normally grow in fluid medium without adhering to the culture surface.

1.2.2 Scaffold

Scaffolds are able to guide cell adhesion, proliferation, migration and differentiation (" contact-guidance theory"). They can also be a vehicle for the controlled release of bioactive factors (eg. peptides, growth factors, etc.) [19]. Based on their specific properties, scaffolds can induce the growth and development of different tissues. Scaffolds are classified based on composition:

- Natural polymers: scaffolds composed by natural proteins, like collagen, polysaccharide, or alginate (*HYAFF*®) used as a gel to carry cells. The rate of *in vivo* degradation can be regulated by varying the degree of inter-molecular cross-linking.
- Decellularized matrices: prepared by removing the cellular component from donor tissues. These preserve the natural composition and structure of the tissue of origin. Ultimately, they will gradually degrade in the post-implantation period, being replaced by a new Extra Cellular Matrix (ECM) (produced by implanted or resident cells).
- Synthetic polymers: they could be bio-stable or bio-degradable. A synthetic polymer is bio-stable if maintains the mechanical properties for all its life, such as SIBS, new generation bio-stable thermoplastic elastomers [20]. Scientific development is moving towards the use of biodegradable materials. Materials able to lose mechanical properties in a controlled manner in order to facilitate the correct regeneration of the tissue (eg. Poly-Lactic Acid (PLA) and Poly-Glycolic Acid (PGA)).

The most important properties required by a scaffold are:

- Biocompatibility: it is a measurement of how the material is compatible with a biological system. It is tested with *in vitro* cytotoxicity test (to evaluate the possible release of low molecular weight substances by the material in contact with the culture medium), *in vitro*

cytocompatibility test (to evaluate the cell viability in contact with the material), *in vitro* blood test (to evaluate absence of thrombogenic effects in contact with the material) and finally *in vivo* functional test to evaluate the *in vivo* biological interaction of the material.

- Porosity: scaffolds are 3D structures with different porosity, it is important for seeding and colonization by the cells. A structure with linked pores could help the regeneration of the tissue and are fundamental for cell adhesion and growth [21].
- Biodegradability/Biostability: generally, the scaffold is a temporary structure for cells with the purpose of maintaining the mechanical properties for a limited time. Once cells adhere, they usually secrete ECM. This process is usually accompanied by the degradation and remodelling of the scaffold. Sometimes non-biodegradable structures are preferred. For example, in the context of muscle tissue regeneration, a stable structure over time is required with the functionalized surface, in order to allow the regeneration and alignment of muscle fibres according to the correct orientation [22].
- Surface chemistry: aimed at promoting cell adhesion, proliferation and differentiation and incorporation of ECM elements (hydrophilicity, negative surface charge, etc.). The cells interact with the substrates through focal bonds of cytoskeletal adhesion proteins with those adsorbed on the surface of the material. The cells rearrange their cytoskeleton to better adapt to the surrounding substrate [23,24]. For example, it is known that rough surfaces, when compared with smooth ones, determine a more intense mechanical stress on osteoblasts, since they must be adapted to superficial irregularities [25].
- Mechanical properties: they should be similar to the tissue to be replaced or modelled, or they should be suitable for cell conditioning.

The main limitation of both synthetic and natural materials is the absence of vascularization of the engineered tissue. Through angiogenesis, the host environment induces vascularization of the tissue, but the capillarity has quickly to be sufficiently branched within the construct. Without this, the tissue could become necrotic [26]. Decellularized scaffolds are considered a better choice for TE, because it contains the vasculature structure of the natural organ.

1.2.3 Decellularization

Decellularization is a technique used to create a scaffold removing the cellular material from a tissue, maintaining the ECM. This is a very promising field because it can be applied to any type of tissue, both of animal and vegetal origin. This type of technology can be applied to entire organs and aims to obtain a product that preserves the geometry, the mechanical characteristics and the protein composition of the matrix, and that ultimately keeps the native vascular network intact [26]. The use of decellularized matrices has considerable advantages:

- Preservation of the ECM: the purpose of the decellularization is to efficiently remove all cellular and nuclear material. This process aims also at minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM.
- Immunogenicity: without the cellular part, the matrix induces only a limited immune response.
- Tissue-specific scaffold: the final acellular matrix possesses all the constitutive and mechanical characteristics of the organ of origin.

Various decellularization protocols have been evaluated; in general, they are based on three phases [26,27]:

1) Lithic process: it involves the breaking of cell membranes. Generally, it is done first with double filtrated water and then with a decellularizing agent. The cells die for osmotic shock and the residual cellular parts are washed away with the agent. Three types of decellularizing agents can be used:

    - Chemical agents: ionic and non-ionic solvent detergents.
    - Enzymatic treatments: enzymes and chelating agents.

- Physical agents: agitation or sonication, mechanical massage or pressure, or freezing and thawing cycles.

In (Table 1.2) are listed various kinds of decellularizing agents considering their main drawbacks.

2) Removal of cellular and nucleic components: the DNA is one of smaller part in the cell, it can get stuck in the ECM. These pieces of DNA can create an immune response. To avoid this, the organ is perfused with DNAse, an enzyme used to digest DNA.
3) Washing: it allows to clean the matrix from any residuals chemical or enzymatic agents used for the decellularization, as well as cellular debris.

The decellularization process also presents some disadvantages:

- Compatible dimensions: for every patient it is necessary to find a cadaveric donor organ with appropriate dimensions.
- Reproducibility: there are several facts that influence the outcome of the process, compromising the reproducibility of the process. Every time new solution is used to continue the process there is the risk of getting air into the organ. Furthermore, the final outcome is also determined by the quality and by the cannulation of the organ.
- Immunogenicity: in the context of clinical application, when using xenogeneic scaffold, genetic material (DNA, RNA) and antigens must be utterly eliminated to avoid a strong immune response after *in vivo* transplantation.

Every organ needs a different decellularizing protocol in terms of order of the chemicals, their type and dosage, based on organ's dimensions, anatomy, composition, density, structure and mechanical properties. Table 1.3 summarizes the principal agents used to decellularize a tissue or an organ.

Table 1.3: Listing of principal agents used for decellularization procedures, divided in physical, chemical and enzymatic agents, coupled with the harmful effects that they respectively have on native tissue. Adapted from 'Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications' by A. Gilpin and Y. Yang, *BioMed research international*, 2017, 9831534

Method	Mode of action	Possible negative effects
Physical		
Snap freezing	Intracellular ice crystals disrupt cell membrane	ECM can be disrupted or fractured during rapid freezing
Mechanical force	Pressure can burst cells and tissue removal eliminates cells	Mechanical force can cause damage to ECM
Mechanical agitation	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material remove	Aggressive agitation or sonication can disrupt ECM as the cellular material is removed
Chemical		
Alkaline; acid	Solubilizes cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs
Non-ionic detergents Triton X-100	Disrupts lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact	Mixed results; efficiency dependent on tissue, removes GAGs
Ionic detergents Sodium dodecyl sulfate (SDS)	Solubilize cytoplasmic and nuclear cellular membranes; tend to denature proteins	Removes nuclear remnants and cytoplasmic proteins; tends to disrupt native tissue structure, remove GAGs and damage collagen.
Sodium deoxycholate (SDC) Triton X-200		More disruptive to tissue structure than SDS Yielded efficient cell removal when used with zwitterionic detergents
Zwitterionic detergents-CHAPS	Exhibit properties of non-ionic and ionic detergents	Efficient cell removal with ECM disruption similar to that of Triton X-100
Sulfobetaine-10 and -16 (SB-10, SB-16)		Yielded cell removal and mild ECM disruption with Triton X-200
Tri(n-butyl)phosphate	Organic solvent that disrupts protein–protein interactions	Variable cell removal; loss of collagen content, although effect on mechanical properties was minimal
Hypotonic and hypertonic Solutions	Cell lysis by osmotic shock	Efficient for cell lysis, but does not effectively remove the cellular remnants
EDTA, EGTA	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g., trypsin)
Enzymatic		
Trypsin	Cleaves peptide bonds on the C-side of Arg and Lys	Prolonged exposure can disrupt ECM structure, removes laminin, fibronectin, elastin, and GAGs
Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chain	Difficult to remove from the tissue and could invoke an immune response
Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains	

All these methods disrupt the cell membrane, facilitate subsequent rinsing and removal of the cell contents from the ECM. Physical treatments are generally insufficient to achieve complete decellularization and must be combined with a chemical treatment

[28]. There are a great number of methods available to determine the efficiency of decellularization process. They can be divided in three classes:

1) Optical inspection: a preliminary and exclusively qualitative evaluation. This type of inspection is performed comparing the colour of the organ before and after the process. Generally, the native tissue has a red-orange or brown colour, while, if subjected to an efficient cell removal process, the organ tends to lose colour becoming increasingly white-transparent.

2) Histological inspection: consists in observing at optical and fluorescence microscope of specifically coloured tissue sections to identify the nuclear or the proteic component. Example of stainings are:

- DAPI; Hoechst: can serve as a first line of inspection to determine presence of cells nuclei.
- Haematoxylin & Eosin (H&E): staining used to evaluate the integrity of the tissue. It is a double staining, one for the nuclei (eosin) and one for the proteins (haematoxylin).
- Masson's Trichrome (MT) and Picrosirius Red (PR) can be used to examine tissues for the presence of various cytoplasmic and extracellular molecules.

3) Immunohistochemical methods can also be used to visualize specific proteins, such as actin collagens [58].

4) Vascular network imaging: carried out on scaffolds with perfusion in the vasculature of a dye to evaluate the integrity of the vascular tree [29].

5) Quantifications: quantitative analysis for the presence of proteins in the tissue, or for genetic material. A scaffold could be considered well decellularized if it respects these criteria [26]:

- less than 50 ng dsDNA per mg ECM dry weight.
- less than 200 bp DNA fragment length.

1.2.4 Signals

Cells in native tissues are under constant stimulation by mechanical forces [31]. For this reason, it is important to reproduce the same biological environment and conditions. Cells respond to them with chemical or physical stimulus transmitted to the nucleus to increase or decrease the expression of genes [31].
There are two kind of signals: chemical (e.g. soluble factors like grow factors (GFs), insoluble adhesion molecules, functionalised surfaces, etc.), physical (e.g. mechanical, electrical, etc.) or a combination of these two. In a TE setting, stimuli that mimic the biophysical environment are provided by the culture media and the bioreactor [31].

1.2.5 Bioreactors

The term "bioreactors" defines devices able to improve biochemical and biological processes under highly controlled environmental and operating conditions [32], to mimic in vivo microenvironment in the scaffold. The bioreactor consists of a culture chamber, control system, actuators and sensors, and eventually a software and hardware component. Bioreactors are commonly used as:

- Actuators: they improve the exchange of nutrients and oxygen, also providing physical stimulation (eg. mechanical guides, electrical, etc.). A stimulation of this type can help the differentiation and/or maintenance of the cell phenotype.
- Control system for monitoring parameters: systems that can control parameters and act in case of need, modifying the system so that everything falls into the desired condition (sensors for pH, glucose, pressure, etc.).
- In vitro model: the bioreactor is a technical tool to support and direct the in vitro development of living tissues.
- Cell seeding on 3D structures: the cells seeding must be carried out in the most efficient manner respecting the basic criteria of sterility. The bioreactors can be designed in such a way as to create a closed structure that maintains sterility, allows cell seeding and perfuse nutrients, to support cell growth and proliferation. A homogeneous distribution

of cell can be achieved thanks to the use of mixing bioreactor. Through a bioreactor is possible to set the right speed for an optimal seeding [33].

- Chemical-physical conditioning: bioreactors can improve the structural and functional properties of engineered tissues, thanks to the generation of a pattern of artificially stimuli, specific to the tissue.
- Medium monitoring/change media: bioreactors can allow an automatic semi-continuous supply of the medium, replacing it with fresh medium in order to re-establish the homeostasis around a predefined set-point.
- Stand-alone: the growing demand for automated bioreactor systems for clinical application has led to the development of stand-alone devices that integrate the environmental control functions performed by incubators.

The design of a tailored bioreactor is required, especially when mechanical stimulation is needed [34]. Bioreactors are an important device for successful clinical implementation of engineered tissue. They are excellent model systems and they helps to end the shortest way to a reliable, safe and economically sustainable clinical application. For the clinical use, bioreactors should be single use, closed and automated. That can permit the use of standardize processes and can minimize manual operations and reducing the risks of contamination [35,36]. The bioreactors in clinic have the task to make up for the lack of tissues to be transplanted and to solve the problems related to rejection. The bioreactors, in clinic, are able to reconstruct a functional tissue, starting from a limited number of cells. ARS (Aastrom Replicell System by Aastrom Bioscience Inc., Ann Arbor, MI, USA) [37] is an example of a system used for cell expansion in the clinic. It provides a concentrated cell suspension at the outlet, ready to be administered to the patient. ACTESTM (Advanced Clinical Tissue Engineering System, of Octane Biotech Inc., Ontario, Can) is designed for the automated generation of mature autologous constructs from single patient biopsies and appropriate scaffolds [38,39].

1.2.5.1 Design requirements for a bioreactor

Each project for scientific research is specific and difficult to be translated to other applications. The realization of a device starts from the specific requests of the project

and from an analysis of what already developed in the literature. At this point a prototype is created, then it is improved to obtain the final design.
Each bioreactor has to respect some fundamental needs, such as:

- Reliably and safety: the bioreactor must be able to work in a reliable and reproducible manner. Respect of Good Laboratory Practice (GLP) and ease of use in biological laboratories.
- Materials: they have to support the temperature and humidity conditions in which the bioreactor works; should be possible to be sterilized and maintain sterility during culture. Biocompatibility and no cytotoxicity of the materials in contact with biological tissue.
- Automation and stand-alone: real-time measurement systems and ability to maintain the conditions of temperature, humidity and pH without having to be placed inside of an incubator.
- Minimization of dimensions and optimization of geometries.
- Versatility to avoid cross-contamination with other samples cultured in parallel.
- Physical stimulation (eg. mechanical, chemical, electrical, magnetic, fluid-dynamics, etc.), to encourage the correct development of the desired tissue.

1.2.5.2 Bioreactors for continuous perfusion

Bioreactors designed for perfusion are devices where the culture medium flows in porosity scaffold, lumen or in an organ's vascular system. The fluid motion can be obtained through a hydraulic circuit equipped with a pumping system or by exploiting the relative movement between the scaffold and the culture medium. The simplest configuration consists in placing the chamber between two tanks and using two reciprocating pumps, to pass the fluid in two directions periodically [40] or moving the scaffold with a rotating chamber. Moreover, during the perfusion there is cellular sedimentation and the porosity decreases. Therefore it is good to change the flow accordingly. This aspect could be used to characterize the growth of a construct, using fluid dynamic parameters. For example, it is possible by evaluating the decrease in the porosity of the construct during tissue growth. The bioreactors are often associated with oxygen-permeable tubes (e.g. silicone), the length of the tubes is fundamental for a good oxygenation of the medium.

These bioreactors use fluid dynamic as a stimulus, reproducing the environment in vivo conditions. That supports the organism for the time required for tissue maturation and development. In general, the perfusion is done with media, to create the right condition for cell grown. Addition of growth factors may be necessary to support cell proliferation and differentiation. These bioreactors could:

- Improve efficiency of seeding process and uniformity of cell distribution.
- Improve efficiency of mass transport.
- Allow physical stimulation of cells.
- Improve traceability, reproducibility, efficiency and safety of the process.

Perfusion bioreactors have been used in the regeneration of various tissues, because static culture presents, very often, a central region of the scaffold with dead cells [41]. Perfusion allows a homogeneous arrangement of cells in the construct. It is demonstrated that the perfusion can increase cell metabolism, for example it implies an increase in calcified matrix deposition from bone marrow osteoblasts [42]. Viability, proliferative capacity and expression of specific markers are also improved [40], [43].

Another important feature of perfusion bioreactor is the ability to control mechanical stimulation through the application of fluid dynamic shear stress. Fluid dynamic stimulation, for example, imposes shear stresses that act directly on the cells [44] (e.g. in the case of cartilage, bone, cardiac tissue) creating a differential pressure [44]. An example of a perfusion bioreactor is OPB (Patent No. WO2008 / 098165). OPB is a seeding, culturing bioreactor using a confined perfusion with oxygenation. It is composed by an oscillating platform (to create a relative motion between the culture medium and the scaffold). This device has a sensing and a control system. This enables the management of oscillating patterns and the monitoring of critical culture parameters. The culture chambers are disposable (scaffold holder, tubes and fittings) in silicone (permeable to oxygen and carboxy dioxide).

The perfusion bioreactors are used for the regeneration of different organs, such as liver, heart and kidney. In Table 1.3 are descripted some bioreactors for different organs.

Table 1.4: Current state of perfusion bioreactor organ engineering. Adapted from 'Bioreactor design for perfusion-based, highly-vascularized organ regeneration' by B. M Bijonowski, W. M. Miller & J. A. Wertheim, *Current opinion in chemical engineering*, 2(1), 32–40, 2013.

Organ	Bioreactor Design	Implantation	State of development
Heart	Perfusion bioreactors for recellularization have been used for cardiac patches and whole organ recellularization. Most bioreactors incorporate electrical or mechanical stimulation to induce stretching.	Surgically created defects in the ventricle of rodent hearts have been repaired with tissue engineered myocardial patches in a rodent heterotopic heart transplant model. Cardiac patches have also been used to repair infarcted heart muscle in rats.	Mechanical and electrical stimulation in a bioreactor enhanced the contractile function of cardimyocytes almost to the level of native cells. At one month, cardiac patches showed seamless integration and vascularization with sur-rounding normal tissue. Patches placed on infarcted hearts showed decreased scarring, reduced dilation and improved ventricular function.
Lung	Both media infusion through the vasculature and gas distension of lung parenchyma in a perfusion bioreactor enhanced biomechani-cal properties of engineered lungs during recellularization.	Tissue engineered rat lungs were implanted into immune-compromized rodent recipients.	Rodents receiving a single tissue engineered lung transplant had superior oxygenation to pneumonectomy controls at day 7 while breathing 100% O_2.
Liver	Rodent livers have been decellularized and recellularized in bioreactors. These reactors provided inflow through either the portal vein or the inferior vena cava.	Recellularized liver grafts have been implanted in rats for 8 hours.	Hepatocyte function was modestly reduced in liver scaffolds compared to collagen sandwich cultures.
Kidney	Large perfusion bioreactors have been constructed for porcine kidney decellularization consisting of multiple perfusion circuits allowing for simultaneous decellularization of several kidneys. Organs are perfused through the renal artery and fluid exits through the renal vein.	Decellularized pig kidneys were implanted into the abdominal cavity of age matched pigs and sutured to the recipient aorta and vena cava.	The decellularized grafts maintained integrity, but were fully clotted upon retrieval. Decellularized grafts were perfused with increasing pressure in vitro to show that the scaffold could withstand physiological pressure.

1.2.6 3D seeding and culture

Before the seeding with cells, the decellularized organ and the chamber have to be sterile prior to reseeding. A common method of sterilization is gamma irradiation.
One report indicates that a dose of 25 kGy is necessary to achieve complete scaffold sterilization [46].
The injection of cells is done in two different ways: microinjected in a multifocal manner or directly injection of cells at high concentration into the vascular perfusion, the last

one tends to be more effective [47]. The directly injection is used for heart [48], lung [47] and liver [49 - 51]. For the seeding is important to check some parameters:

- Low flow rates are used to reduce shear stress on the cells (a decellularized organ has a decreased resistance to flow [52]).
- During the recellularization the cells reseeded fill the holes in the decellularized matrix and the pressure drop will increase as the porosity decreases [53]. For that it may be helpful to check the perfusion pressure before the inlet of the organ.
- Increasing the cell density, the possibility to have cell aggregates increases. This may cause hypoxia and the development of a necrotic core [53,54].

The seeding is done by bioreactors that maintains sterility and helps proliferation by providing the cells with nutrients, gas and fresh medium. In order to implant the organ, following its complete regeneration, it is necessary to restore some elements including the parenchyma, the vascular network and the supporting components. However, the study for the development of technologies concerning the regeneration of whole organs is not widespread. Because the recellularization is particularly difficult due to the structural complexity of the organs and cellular heterogeneity [55]. In literature there are many research groups working with this technology. Most of it are doing in vivo transplants (murine model) [56], and the major examples in clinical of decellularized matrices concern the heart valves coming from animal model [57].

1.3 Oesophagus

1.3.1 Anatomy

The oesophagus is a muscular tube that extends from the level of the 6th cervical to the 11th thoracic vertebra, spanning three anatomic regions [58]. The cervical oesophagus lies just left of the midline, posterior to the larynx and trachea and anterior to the prevertebral layer of the cervical fascia (Fig. 2). The upper portion of the thoracic oesophagus curves slightly to the right and passes behind the tracheal bifurcation and the left mainstem bronchus. The lower portion of the thoracic oesophagus runs behind the pericardium and the left atrium,

where it bends to the left and enters the abdomen through the oesophageal hiatus. The abdominal oesophagus, 2 to 4 cm long, ends at its junction with the stomach [59]. There are three areas of normal narrowing of the oesophageal lumen: at the cricoid cartilage; at the left main bronchus and the aortic arch, where it is compressed by these structures; and at the diaphragmatic hiatus [60].

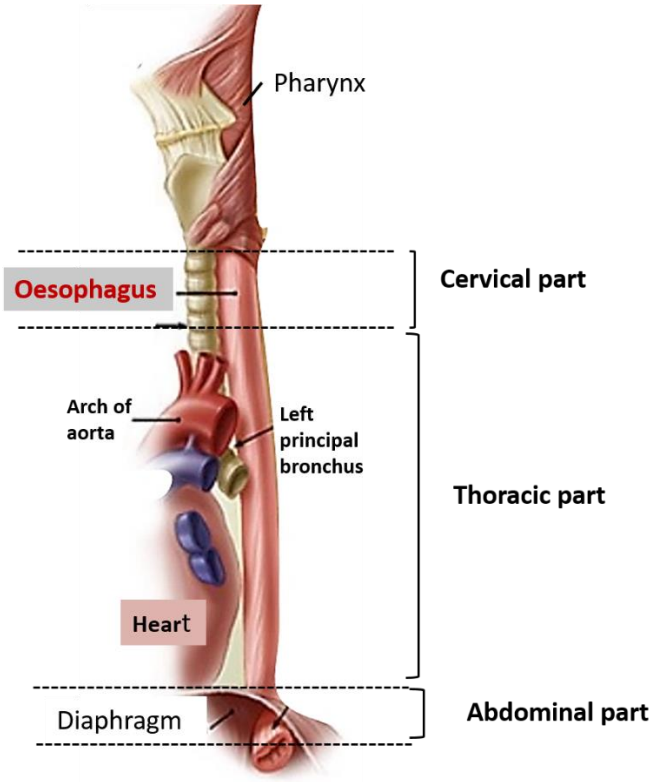


Figure 2: Oesophageal muscular tube, divided in cervical (4 cm), thoracic (20 cm) and abdominal part (1-2 cm). It begins as a continuation of pharynx in the neck at the lower border of the cricoid cartilage (at the level of C6 vertebra) to the cardiac end of the stomach in the abdomen (opposite T11 vertebra). Adapted by 'Anatomy QA>Blog>Anatomy>Oesophagus' by Anatomy QA blog, *www.anatomyqa.com/oesophagus-anatomy-exam-quiestions/*

Architecture of the oesophageal wall

The mucosa consists of squamous epithelium overlying a lamina propria and muscularis mucosa. The latter contains mainly longitudinal muscle fibers. The junctional columnar epithelium of the gastric cardia meets the squamous epithelium of the oesophagus in a sharp transition called the Z-line. The submucosa, which contains elastic and fibrous tissue, is the strongest layer of the oesophageal wall [61]. The oesophageal muscle is composed of an

inner circular and an outer longitudinal layer. The upper third of the oesophageal musculature consists of skeletal muscle, and the lower two thirds consist of smooth muscle. The upper oesophageal sphincter is formed by the cricopharyngeus muscle along with fibers from the oesophageal wall and the inferior constrictors of the pharynx. On gross inspection, the lower oesophageal sphincter is not a distinct anatomic structure. Some investigators have reported seeing a thickening of the circular oesophageal musculature at the location of the manometric high-pressure zone [62], but at operation a distinct sphincter is not evident [63]. Unlike most of the gastrointestinal tract, the oesophagus does not have a serosa [64].

Blood supply

The oesophagus receives its arterial blood supply from four sources.The cervical oesophagus is nourished by branches of the inferior thyroid arteries. The bronchial arterial system supplies the upper portion of the thoracic oesophagus, and the midthoracic oesophagus is supplied by vessels originating directly from the thoracic aorta. The lowermost thoracic and intra-abdominal oesophagus is supplied by tributaries of the left gastric and inferior phrenic arteries [65]. Complete oesophagel arterial supply is visible in Fig. 3.

An extensive submucosal venous plexus connects through the musculature with longitudinally oriented periesophageal veins In the neck these vessels drain into the inferior thyroid veins and in the thorax into the hemiazygos and azygos veins. Because the lowest portion of the venous drainage is into the left gastric vein, the caval and portal venous systems are connected through the submucosal plexus. Portal hypertension can transform these submucosal veins into large varices. Complete oesophageal venous system is visible in Fig. 4.

Innervation

The innervation of the striated muscle of the pharynx and upper oesophagus originates in the brain stem (nucleus ambiguous). The innervation of the distal oesophagus and lower oesophageal sphincter originates in the dorsal motor nucleus of the vagus and terminates in ganglia in the myenteric plexus. The myenteric plexus, which lies between the longitudinal

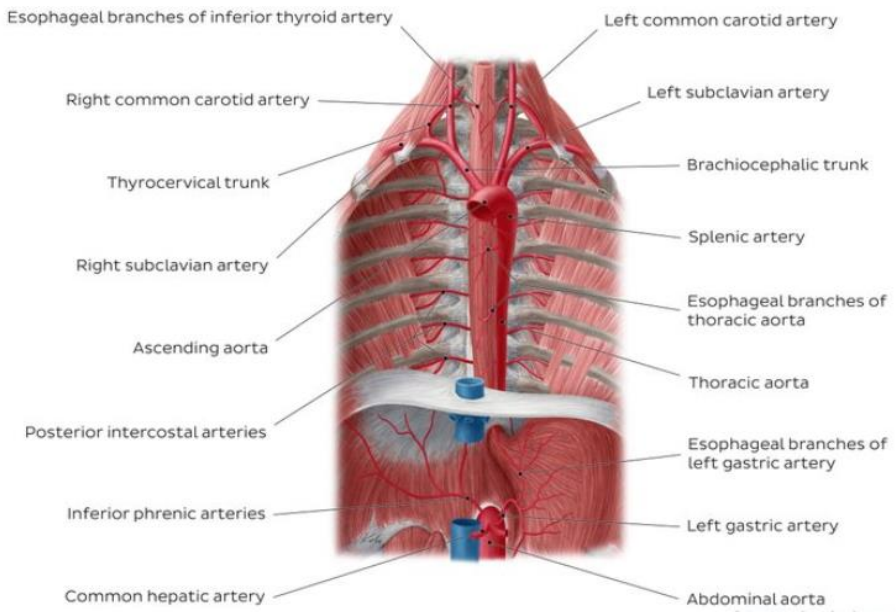


Figure 3: Arterial supply of the oesophagus. Adapted from 'Esophagus' by Adrian Rad BSc (Hons), Kenhub, www.kenhub.com/en/library/anatomy/esophagus

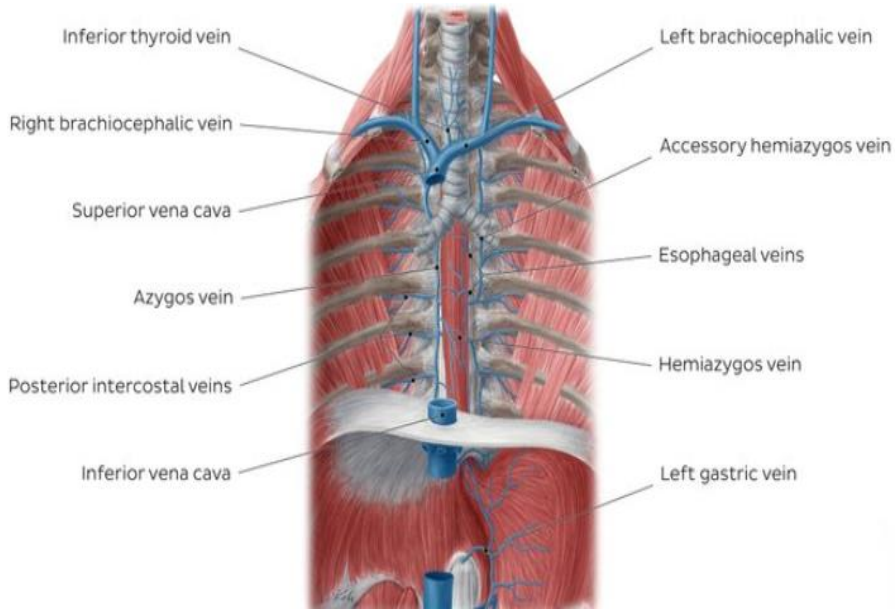


Figure 4: Venous supply of the oesophagus. Adapted from 'Esophagus' by Adrian Rad BSc (Hons), Kenhub, www.kenhub.com/en/library/anatomy/esophagus

and circular muscle layers, receives efferent impulses from the brain stem and afferent impulses from the oesophagus. Two main types of effector neurons are found within the oesophageal myenteric plexus: (1) Excitatory neurons mediate contraction of the muscle

layers via cholinergic receptors; and (2) inhibitory neurons affect the muscle layers via vasoactive intestinal polypeptide, a nonadrenergic, noncholinergic neurotransmitter.

The vagus nerves run along either side of the neck until they reach the thoracic oesophagus, where they form an extensive plexus. Above the diaphragm they coalesce once more into two trunks [64]. The left trunk courses more anterior and the right trunk posterior as they pass through the oesophageal hiatus. Each then divides. The anterior vagus gives off the hepatic branch and the anterior nerve of Latarjet. The posterior vagus divides into the celiac branch and the posterior nerve of Latarjet, which runs parallel but deeper to its anterior counterpart in the gastrohepatic ligament about 1 cm from the lesser curvature of the stomach.

Sympathetic innervation is provided by branches of the superior and inferior cervical ganglia in the neck and the splanchnic nerves and the celiac plexus in the chest and abdomen. Sympathetic nerves are also present in the myenteric plexus. They do not have a motor function but mainly modulate the activity of other neurons. In Fig. 5 are shown the oesophageal nervous pathways.

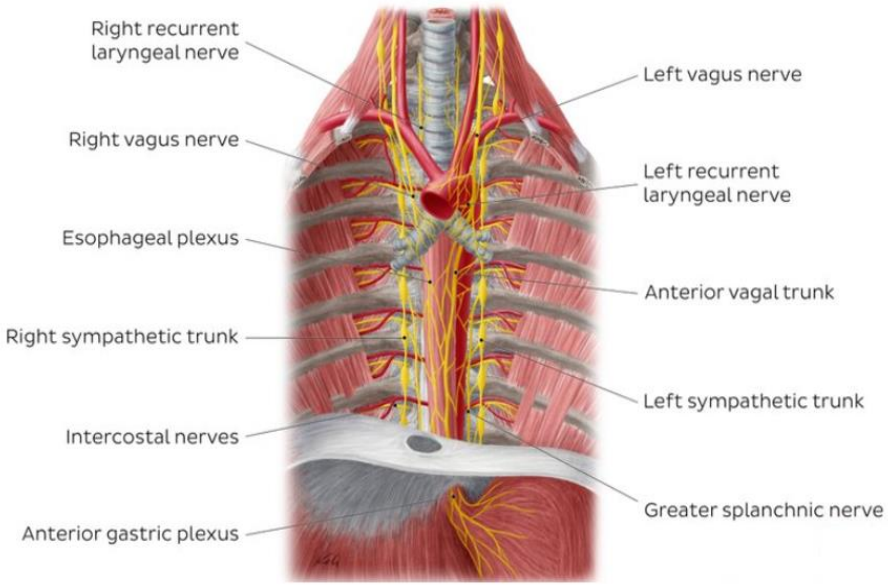


Figure 5: Nervous pathways of the oesophagus. Adapted from 'Esophagus' by Adrian Rad BSc (Hons), Kenhub, www.kenhub.com/en/library/anatomy/esophagus

1.3.3 Oesophageal atresia

Oesophageal atresia is a congenital medical condition (birth defect) that affects the alimentary tract, occurring in approximately 1 in 3000 live births [66]. It causes the oesophagus to end in a blind-ended pouch rather than connecting normally to the stomach. It comprises a variety of congenital anatomic defects that are caused by an abnormal embryological development of the oesophagus. It is characterized anatomically by a congenital obstruction of the oesophagus with interruption of the continuity of the oesophageal wall.

This birth defect arises in the fourth foetal week, when the trachea and oesophagus should begin to separate from each other.

Any attempt at feeding could cause aspiration pneumonia as the milk collects in the blind pouch and overflows into the trachea and lungs. Furthermore, a fistula between the lower oesophagus and trachea may allow stomach acid to flow into the lungs and cause damage. Because of these dangers, the condition must be treated as soon as possible after birth.

Oesophageal atresia is the most common GI atresia. Other congenital malformations are present in up to 50% of cases. Two syndromes in particular are associated with oesophageal atresia [67]:

- VACTERL (vertebral anomalies, anal atresia, cardiac malformations, tracheoesophageal fistula, oesophageal atresia, renal anomalies and radial aplasia, and limb anomalies).
- CHARGE (coloboma, heart defects, atresia of the choanae, retardation of mental and/or physical development, genital hypoplasia, and ear abnormalities).

There are 5 major types (A, B, C, D, E) of oesophageal atresia, according to the classification made by the American surgeon R. Gross in 1953. (Table 1.5) [68]

Treatment

Treatments for the condition vary depending on its severity. The most immediate and effective treatment in the majority of cases is a surgical repair to close the fistula/s and reconnect the two ends of the oesophagus to each other. However, in some cases the gap

Table 1.5: Listing of oesophageal atresia variants with relative incidence. Adapted from Robert E. Gross. In: P.P. Rickham (eds) *Historical Aspects of Pediatric Surgery. Progress in Pediatric Surgery, vol 20*. Springer, Berlin, Heidelberg,1986

Classification by Gross	Name	Description	Frequency
Type A	"Long Gap", “Pure” or “Isolated” Oesophageal Atresia	Characterized by the presence of a “gap” between the two oesophageal blind pouches with no fistula present.	7%
Type B	Oesophageal Atresia with proximal TEF (tracheoesophageal fistula)	The upper oesophageal pouch connects abnormally to the trachea. The lower oesophageal pouch ends blindly.	2-3%
Type C	Oesophageal Atresia with distal TEF (tracheoesophageal fistula)	The lower oesophageal pouch connects abnormally to the trachea. The upper oesophageal pouch ends blindly.	86%
Type D	Oesophageal Atresia with both proximal and distal TEFs (two tracheoesophageal fistulas)	Both the upper and lower oesophageal pouch make an abnormal connection with the trachea in two separate, isolated places.	<1%
Type E	TEF (tracheoesophageal fistula) ONLY with no Oesophageal Atresia, H-Type	Oesophagus fully intact and capable of its normal functions, however, there is an abnormal connection between the oesophagus and the trachea.	4%

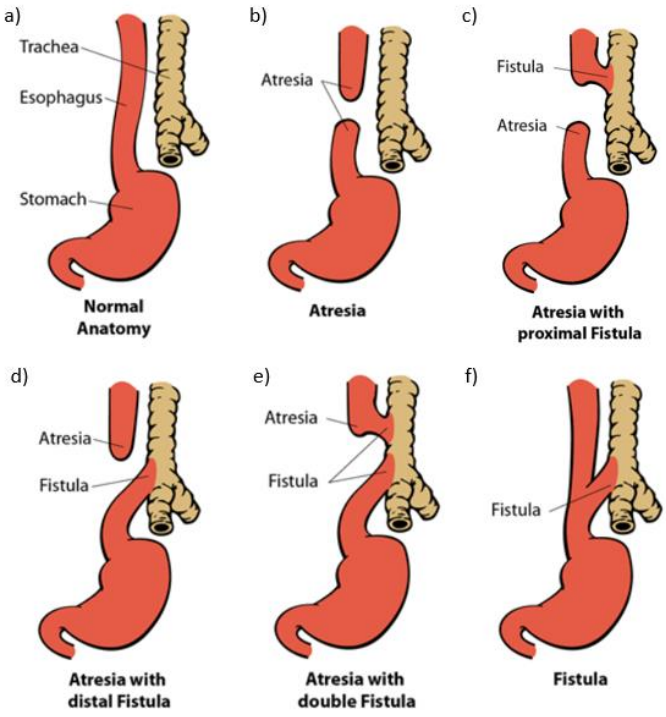


Figure 7: The different types of oesophageal atresia. Adapted from 'Esophageal Atresia & Tracheoesophageal Fistula', Department of Surgery, University Of California San Francisco, *www.surgery.ucsf.edu/conditions--procedures/esophageal-atresia.aspx*

between upper and lower oesophageal segments may be too long to bridge, and the optimal treatment in such situation remains controversial [69]. The surgeon J. Foker, from University of Minnesota, developed a technique which may be utilized to elongate and then join together the short oesophageal segments [70].Using the Foker technique, surgeons place traction sutures in the tiny oesophageal ends and increase the tension on these sutures daily until the ends are close enough to be sewn together. The result is a normally functioning oesophagus, virtually indistinguishable from one congenitally well formed. Unfortunately, the results have been somewhat difficult to replicate by other surgeons and the need for multiple operations has tempered enthusiasm for this approach.

Traditional surgical approaches include gastrostomy followed by gastric pull-up, colonic transposition and jejunum transposition.[71] Gastric pull-up has been the preferred approach at many specialized centers, including Great Ormond Street Hospital (London)[72]. Gastrostomy, or G-tube, allows for tube feedings into the stomach through the abdominal wall. Often a cervical oesophagostomy will also be done, to allow the saliva which is

swallowed to drain out a hole in the neck. Months or years later, the oesophagus may be repaired, sometimes by using a segment of bowel brought up into the chest, interposing between the upper and lower segments of oesophagus.
In any case, post-operative complications often arise, including a leak at the site of closure of the oesophagus. Sometimes a stricture, or tight spot, will develop in the oesophagus, making it difficult to swallow. This can usually be dilated using medical instruments. In later life, most children with this disorder will have some trouble with either swallowing or heartburn or both. Oesophageal dysmotility occurs in 75-100% of patients.
In any case, oesophageal replacement remains associated with complications and poor quality of life. Tissue engineering could, in the near future, become a valid therapeutic tool to address the various problematics [73].

1.4 Oesophageal Tissue Engineering

In treating severe congenital and acquired oesophageal defects, the current surgical approaches are complex and often lead to serious complications. Thus, regenerative medicine techniques, which extend the boundaries of reconstruction and do not, in most applications, require immunosuppression, present attractive alternatives. Regenerative medicine has been used to describe the use of natural human substances, such as genes, proteins, cells, and biomaterials to regenerate diseased or damaged human tissue. in order to restore normal function. Tissue engineering with the end-point of organogenesis has been successful through a combination of appropriate cells with a scaffold as well as the use of only one of these two components, for example in the repair of urethra and skin. There are two broad categories of intervention in tissue engineering: acellular scaffolds and cell seeded scaffolds. [74].

Acellular scaffolds:

Transplanted acellular scaffolds have the aim of hosting epithelial and smooth muscle cells that migrate from surrounding tissue to repopulate the new conduit. Acellular scaffolds studied to date conform to one of three categories: synthetic, collagen alone and

decellularized matrix. Synthetic scaffolds such as polyethylene plastic [75-77] and silicon [78,79] have been used for oesophageal replacement, but the nature of the materials did not allow cellular migration and led to poor results in animal models. Polyvinylidene fluoride (PVDF) and polyglastin-910 (Vicryl®) were compared for the regeneration of patch defects in rabbits, and PVDF was shown to lead to improved results with an absence of strictures and neoepithelialization [80]. However, in a different study, the combination of Vicryl® and collagen brought about positive results both for patch and tubular defects in dogs, with a low mortality of 8.3% [81]. The successful use of synthetic polymers in other organs such as the trachea [82] suggests that this approach may appear attractive and further development of appropriate materials is needed.

Collagen scaffolds have been used to produce porous tubular structures [83-88], showing greater epithelial and muscle cell densities with respect to synthetic scaffolds, but only in short (5cm) surgical created defects [86], while in longer defects (10 cm) poor cellular migration in the muscle layer was observed [85]. Moreover, when the same methods were used to replace intra-thoracic portions of the oesophagus in dogs, muscular regeneration was completely absent, something the authors attributed to the lack of a vascular supply in the thorax [85]. In an attempt to address this, the scaffold was wrapped in omentum [88]. as has been successfully applied to tracheal tissue engineering [89,90]. However, muscular regeneration remained absent, whilst an increase in mid-portion stenosis and mortality was observed[88].

Decellularized matrices are derived from human and animal organs and tissues that have been treated to remove cells and immunogenic material [91]. Importantly, however, they retain the macro and micro-architecture of the tissue of origin, and the molecular components of its natural extracellular matrix [92-95]. They have the added hypothetical advantages over synthetic scaffolds of not producing potentially toxic degradation products or inducing inflammation characteristics that may be important in the prevention of stenosis [28,96,97]. Decellularized scaffolds that have been used for oesophageal organs originated from the oesophagus as well as from other tissues such as the small intestinal submucosa (SIS) [83-86,99-103]. Significant heterogeneity exists among studies, both with respect to the type of scaffold, extent of surgery and species used, which partly explains the range of results reported. Thus, regeneration of the muscularis propria layer is seen to take place in some studies [99,100,104] but not others [105]. Studies that have attempted tube-interposition with

SIS report the development of oesophageal stenosis and increased mortality [100, 106, 107]. By contrast, studies applying SIS as a patch repair demonstrated encouraging results [100, 106-109]. Hypothetically, decellularized oesophageal tissue should retain the signals, both chemical and structural, that will direct the appropriate migration and differentiation of host cells, in a way unlikely to occur with scaffolds originating outside the oesophagus, such as SIS [102].

Cell-seeded scaffolds

To reduce complications arising from acellular approaches, scaffolds can be seeded with cells prior to transplantation. The two main cell types that are important for oesophageal tissue engineering are those that will reconstitute the epithelium and the muscle layer on the luminal and extra-luminal sides respectively. Also important in the formation of a functional oesophagus are the vascular and neuronal cell components. A number of in vitro experiments have examined the seeding and culture of oesophageal epithelial cells and different scaffolds to assess the optimal combination. In one of the studies, the growth of human oesophageal squamous cells on human decellularized oesophagus, porcine decellularized oesophagus, human decellularized dermis, and collagen was compared. [110]. Interestingly the porcine matrix and collagen gave better results leading to the formation of a mature stratified epithelium. The seeding of rat oesophageal epithelial cells (ECC) showed different a different behaviour using 3-D or 2-D scaffolds. in the form, cells were shown to be viable up to 8 weeks in vitro but they couldn't fully integrate within the scaffold, remaining on the surface [111]. Seeding of sheep EEC on the same 3-D collagen scaffold resulted in the absence of epithelium sheet formation, which was attributed to cellular penetration into the scaffold and loss of cell-to-cell contact [112]. However, when the same cells were seeded on the 2-D collagen scaffolds a single layer of epithelium was evident following 3 wk of in vitro culture that remained viable up to 6 wk. Positive selection of the epithelial population could increase proliferative capacity, as demonstrated in another study [113]. Ovine EEC for expression of pancytokeratin (PCK) were selected. The PCK-negative subpopulation had minimal cell attachment on the collagen scaffolds, whereas the PCK-positive cells had a uniform distribution. In vivo experiments using EEC-scaffold constructs, similarly to results

in acellular approaches, have shown more promise for regeneration of partial rather than circumferential defects in rats and dogs [114-116]. An innovative approach recently described seeded cells on a temperature-responsive dish that became hydrophilic at 20 ℃ and allowed harvesting of a single-cell sheet [114]. When the cell sheets were transplanted in dogs that had undergone endoscopic submucosal resection, complete wound healing was observed at week 4 with no signs of stricture and an intact epithelium [115]. In a further study mucosal epithelial cells from oral biopsy were obtained, or oesophageal organoid units created following digestion of rat oesophagi [116]. These were seeded onto scaffolds and implanted as complete oesophageal substitutes, but histology of the resultant muscle layers showed poor architecture. To overcome the limitations of using EEC in isolation, oesophageal constructs prepared using EEC-seeded collagen scaffolds were placed on the latissimus dorsi muscle of athymic mice with the intention to harvest and tabularise the muscle once the epithelial side has matured [117, 118]. It was found that an increase in the number of epithelial layers from 2 when EEC seeded alone, to 18 when co-seeded with fibroblasts, occurred [119]. In a more recent study, both epithelial and fibroblast cells were seeded on a bed of smooth muscle cells (SMC) embedded in a collagen gel in vitro, prior to transplanting them on the latissimus dorsi of athymic rats [120]. Others also aimed to combine different cell lines and scaffolds into one tubular structure in dogs [121]. They used oral keratinocytes and fibroblasts cultured on human amniotic membrane and SMC seeded on poly (glycolic acid). These two scaffolds were then rolled together and implanted into the omentum for 3 weeks following which they were transplanted into a 3-cm intrathoracic oesophageal defect. Both muscular and epithelial layers were present at 420 d of follow-up, although no peristaltic activity was observed. The vascularization problem was in different cases addressed wrapping a seeded scaffold in the omentum but giving suboptimal results.

In a study, mechanical stimulation of smooth muscle cells was performed by the use of a bioreactor, thus highlighting the importance of a multidisciplinary engineering approach [122]. In a more recent study done in 2018 by Urbani et al. at UCL Great Ormond Street Institute of Child Health, a glass bioreactor (Fig. 8) was developed to reconstruct a layered full-circumferential oesophageal tissue in vitro, addressing some major challenges in organ engineering, namely (i) development of multi-strata tubular structures, (ii) appropriate repopulation/maturation of constructs before transplantation, (iii) cryopreservation of

bioengineered organs and (iv) in vivo pre-vascularization. [123]. In the study a decellularized rat oesophagus was seeded with human mesoangioblasts (hMABs), mouse fibroblasts (mFBs), enteric murine neural crest cells (mNCCs) and rat oesophageal epithelial cells (ROECs). The construct was cultivated in a bioreactor-based dynamic culture and also transplanted in the rat omentum to promote vascularization.

MABs were differentiated in smooth muscle cells, constituting most of the muscular part of the organ, FBs were helping a more homogenous distribution of MABs all over the construct, NCCs were chosen to stimulate nervous pathways growth and endothelial cells were deputed to cover the luminal part of the organ. This study represents the most recent attempt of the reconstruction of a multi-strata recellularized oesophagus, and the main work exposed in this thesis represents a follow-on attempt in oesophageal tissue engineering.

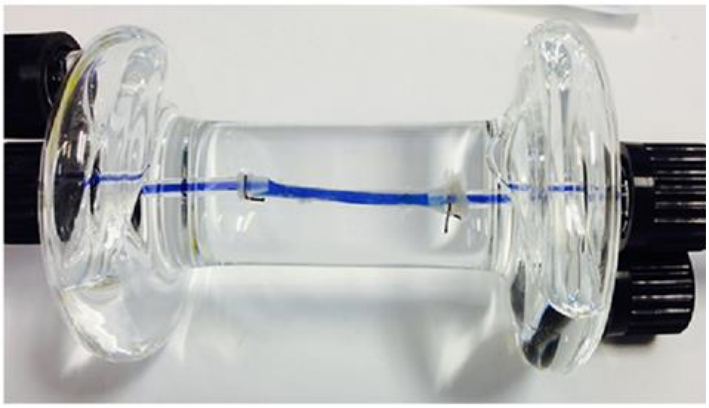


Figure 8: The bioreactor used in the study of Urbani et al. [123] consenting the suturing of the two ends of the oesophageal scaffold to two glass rods. The glass rods and the tied construct are let enter inside the glass main structures and the rods can go through perforated lids, which are then tightened to immobilize the scaffold.

1.5 Aim of the thesis

The main aim of the work presented in this thesis was the development of a novel bioreactor for oesophageal dynamic culture, with the use of a decellularized piglet oesophagus as a scaffold seeded with both human mesoangioblasts and human fibroblasts and prevision to add human epithelial cells. The bioreactor chamber designed is suitable for both decellularization and recellularization. The design, unique so far, and mode of use of the bioreactor was chosen after having examined the previous literature, especially the

previous work developed in our group by Urbani and colleagues [123]. This work, conducted at the University College of London Institute for Child Health is part of the development of this study, aiming at producing a complete human bioengineered oesophagus.

Chapter 2

Material and methods

2.1 Bioreactor

The rectangular chamber and the lid were designed using the SolidWorks software. The projects files were saved in .dxf format and successively opened with Adobe Illustrator and converted in .ai format. The files were then opened with ArtCAM software (Autodesk) and converted in a 3D project. The 3D files were thus sent to the Computer Numerical Control (CNC) machine terminal, from where it was possible to start the physical manufacturing of the objects. The CNC used (Roland Modela Pro II) belonged to the Institute of Making, University College of London.

The materials used were a PTFE (Teflon) RS PRO 600x300x60 sheet and a PC (Polycarbonate) RS PRO 300x300x8 sheet. Both the sheets were fixed on the CNC wooden work-base using a cordless screwdriver, adding 8 screws of 8 cm length (shark + thread). An 8 mm diameter ball-nose bit was employed by the CNC for the internal carving of the parallelepipedal space deputed to host the organ. A flat-end 8 mm bit was used to cut the external perimetral contour, separating the chamber shape from the rest of the PTFE sheet, and a final flat-end bit of 3 mm was responsible for the upper surface holes necessary for the screws. The 8 mm flat-end bit was also employed to cut the external shape of the lid and the central hole of the lid from the polycarbonate sheet. A 5 mm flat-end bit was used for the lid holes. The holes at the shorts sides of the PTFE chamber were performed with a drill press, keeping the chamber appropriately clamped to a working bench. Three of the planned four holes were drilled with a 5 mm drill bit, the other one with a 3 mm drill bit. The holes (except the 3 mm diameter one) were successively threaded with a ¼ - 28 UNF tap (RS), broadening the holes diameter to approximately 6.35 mm (1/4 inches). The central hole on the PC lid was also threaded with a G 1/8 BSPP tap reaching a diameter in mm of 9.72. The 3 mm diameter hole was not threaded. During CNC machining, auxiliary structures called bridges were helping both the chamber and lid to stay in place when the bit was cutting near the bottom of the working bench.The bridges were portions of the original material sheet

that were not cut during the passage of the drill bit along the external perimeter of the chamber or of the lid. Such structures were successively removed with saws and sand-belts (Institute of Making). Sand-belts were also used to refine the edges. The bioreactor was designed to be GMP compliant.

2.2 Cells

In this work, the cells cultured for the organ seeding were human mesoangioblasts (hMABs) and human fibroblasts (hFBs). The following sections regard separately the expansion of both the cell types. The culture of hMABs was carried on following the model of an already established protocol [151]. The followed protocol for hFBs was performed under dictation of phD collaborator in the Surgery department of GOSH.

Human mesoangioblasts (MABs)

Human mesoangioblasts (hMAB) were isolated from paediatric skeletal muscle biopsies from patients aged from 1 week to 8 years old, with informed consent, during surgeries at the Great Ormond Street Hospital, London, in accordance with ethical approval by the NHS Research Ethics Committee, REC Ref: 11/LO/1522. The Committee was constituted in accordance with the Governance Arrangements for Research Ethics Committees and complied fully with the Standard Operating Procedures for Research Ethics Committees in the UK. The cells were cultured in an incubator environment at 37°, 5% O_2 and 5% CO_2. The medium used to culture the cells was Megacell DMEM (Dulbecco's Modified Eagle Medium, Sigma) supplemented with 5% FBS (Fetal Bovine Serum,Life Technologies), 1% Penicillin-Streptomicin (Life Technologies), 1% L-glutamine (Life Technology), 1x non-essential aminoacids (Sigma), 50 µM b-mercaptoethanol (Life Technologies) and 5 ng/ml bFGF (Invitrogen). Growth medium was changed every other day. Cells were passaged at 60% confluence up to 10 passages. Medium was discarded, cells were gently rinsed with PBS -/- (Life Technologies) and incubated with TrypLE for three minutes at 37°C. Once the cells detached from the pate and went in suspension, TrypLE was inactivated with a blocking medium, composed by DMEM (Sigma) with 10% FBS solution. The cells were collected in Falcon tubes and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, and

the cells were resuspended in Megacell medium and plated. 1:10 splitting was usually performed.

Human fibroblasts (hFBs)

hFBs were obtained as anonymised cryopreserved samples from the ICH Surgery department in Great Ormond Hospital for Children. The frozen cells were thawed in waterbath for 1-2 minutes, then 1 ml DMEM was added dropwise to the cryotube. The DMEM used for hFBs culturing was supplemented with 20 % FBS (Life Technologies), 1% L-glutamine (Life Technologies) and 1% P/S (Life Technologies). Successively, a 15 ml falcon tube with 5 ml of DMEM was prepared, and the content of the cryotube was added dropwise. At this point the cells were centrifuged for 5 minutes, the supernatant discarded, and the pellet resuspended in 1 ml of DMEM. 1 ml of DMEM was added to the plate before adding of cells. Cells were passaged at 70 % confluence. The medium already present in the plate with cells was aspirated and the cells were washed in 1 ml of DPBS (Life Technologies). In the plate 700 µl of TrypLE (Life Technologies) was added per well. The plate with detached cells in suspension was put in the incubator at 37° for 4 minutes. Then, 700 µl of blocking medium (DMEM + 10% FBS) was added to the wells to inactivate the trypsinizer, the solution was resuspended to break eventual clumps and the cells were collected in Falcon tubes and centrifuged at 1200 rpm for 4 minutes.
The supernatant was discarded, and the cells were resuspended in DMEM medium and plated. 1:10 splitting was performed also in this case.

2.3 Dynamic culture

Perfusion system

A suitable perfusion system was applied to the manufactured chamber. The components of the perfusion set up consisted in a i50 iPumps peristaltic pump, Masterflex Pharmed BTP silicone tubes (internal diameter 1.6 mm, Cole-Parmer UK), polypropylene adapters (Cole-Parmer,UK), 3-way stopcocks (Vygon, UK), a g18 screw cap borosilicate reservoir bottle (Sigma, UK) and the assembled bioreactor. The silicone tubes linked together the bioreactor

chamber, the reservoir and the peristaltic pump, by using fittings. The types of fittings used were 1/4 – 28 UNF Male Luer (Masterflex® , Cole Parmer,UK), Female Luer to Hose Barb adapter (Masterflex®, Cole-Parmer, UK), Male Luer to Hose Barb adapter (Masterflex®, Cole-Parmer, UK), G1/8 BSPP Male Luers (Masterflex®, Cole-Parmer, UK) and 3-way stopcock (Vygon, UK). All the fittings were provided with sealing silicone o-rings (OR5X2, Hooper LTD, UK), to avoid leakages at the connecting sites. A silicon o-ring was also present in the groove carved on the PTFE chamber superior surface, to keep the sealing with the bioreactor assembled. A 4 mm diameter Foley urethral catheter tube was used for the suturing of one end of the oesophagus. A Foley catheter tube is equipped with a drainage port and a balloon port. The tube was cut some centimetres before the ports junction to allow the embedding of the fitting where one oesophagus end was sutured. The bioreactor chamber and lid were assembled together, autoclaved and tested with the Dulbecco's Medium Eagle Medium (DMEM; Life Technologies, UK) with 1% of penicillin and streptomycin (P/S; SigmaR, UK) for 72 hours in the incubator in the absence of cells and scaffold. The peristaltic pump was set at 12.3 rpm (3 ml/min). A Millex Syringe filter (Sigma, UK) was used for the filtration of air.

Organ harvesting

All animal procedures were conducted in accordance with ethical approval and UK Home Office Project Licence PPL/70/7622 and 70/7478. 3 kg piglets were used for oesophageal harvesting.

Decellularization protocol

The decellularization was performed according to the established protocol, described by Maghsoudlou [149] and Totonelli [150], through two cycles of detergent-enzymatic treatment (DET). The oeshageal lumen was perfused with continuous fluid delivery (iPumps i50) at 3 ml/min. Each DET cycle was composed of MilliQ water for 24 h, 4% sodium deoxycholate (SDC, Sigma) for 4 h, and 2000 Kunitz DNase-I (Sigma) in 1 M NaCl for 3 h. The process was repeated for two cycles.

The decellularized oesophagi were gamma irradiated and stored at 4℃ in sterile PBS with 1% Penicillin-Streptomycin (Sigma, UK).

Cells seeding

Human mesoangioblasts (hMABs) and human fibroblasts (hFBs) were trypsinised and resuspended in a solution of PBS, containing 0.5 ng ml−1 Collagen type I (Sigma) and 0.1 ng ml−1 Fibronectin (Sigma) and kept on ice until seeding. The ratio of concentration of hMABs and hFBs was 85:15 as reported by Urbani et al. [123]. The volume of cell suspension was calculated to inject 1×10^6 cells every 5mm length of scaffold. 3–3.5 cm of rat oesophageal decellularized tubular scaffolds was seeded. Cells were microinjected every 3–4mm with an insulin syringe (MyJector)/27 G needle at multiple sites along 4 distinct longitudinal lines. Multiple microinjections were performed manually under a stereomicroscope to ensure cell delivery to the muscle layer. For easy handling and ensuring constant tight tension whilst microinjecting, a 6 F nasogastric tube (Enteral) was inserted into the scaffold first.

2.4 Analyses

DNA quantification

PureLink Genomic DNA MiniKit (Invitrogen) was used to isolate DNA from the acellular oesophageal scaffolds following the manufacturer's instructions as previously described [124,127]. DNA samples were measured spectrophotometrically (Nanodrop).

Histology

The oesophagi were fixed in 4% paraformaldehyde (PFA; Sigma, UK), dehydrated in 30 % sucrose O/N and embedded in 7.5 % Gelatin/ 15 % Sucrose, frozen and sectioned through a cryostat (Leica Cryostat, UK). Prior to staining, slides were de-gelatinzed in 1 X PBS in a 37°Cwater bath for 30 minutes. Then, they were left in haematoxylin (H&E; Leica

Biosystems, UK) for 5 mintues, rinsed in MilliQ water twice and then in alcoholic acid (ETOH 70%-HCl 1% solution). The sections were then counterstained in eosin (H&E, Leica) for 5 minutes, washed again in MilliQ water for 5 minutes, rehydrated in graded EtOH (70 %; 90%; 100%). Successively, the slides were stained with Masson's Trichrome (MT; Leica, Raymond A Lamb, BDH Chemicals Ltd, UK).

Haematoxylin and Eosin stain is one of the principal histological procedures performed. Eosin is an acidic, negatively charged dye, staining basic (or acidophilic) structures red or pink. In such a way, cytoplasm is stained. Haematoxylin can be considered as a basic (after coupling with Al salts mordants) dye used to stain acidic (or basophilic) structures a purplish blue. Thus, cells nuclei are stained in purple [128].

Masson's Trichrome is a three-colours stain used in histology. Using acid-base chemistry, three dyes are employed to selectively stain muscle, collagen fibers, fibrin, and erythrocytes. Bouin's solution is used first as a mordant to link the dye to the targeted tissue components. Nuclei are stained with Weigert's haematoxylin, an iron haematoxylin, which is resistant to decolourization by the subsequent acidic staining solutions. Biebrich scarlet-acid fuchsin solution stains all acidophilic tissue elements such as cytoplasm, muscle, and collagen. Subsequent application of phosphomolybdic/phosphotungstic acid is used as a decolourizer causing the Biebrich scarlet-acid fuchsin to diffuse out of the collagen fibers while leaving the muscle cells red. Application of aniline blue will stain the collagen, after which, 1% acetic acid is applied to differentiate the tissue sections [128].

Immunofluorescence and immunohistochemistry

Tissue samples were fixed in 4% PFA, washed in PBS, dehydrated in 30 % sucrose overnight, embedded and frozen in O.C.T (Sakura Finetek Ltd, UK) with ice-cold isopentane (Sigma, UK) and and stored at – 80 °C. 7–10 μm thick sections were cut (Leica cryostat, UK), and slides stored at−20 °C. Cells and sections were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, washed and blocked with 5% Goat Serum for 1 h at room temperature. Primary and secondary antibodies were diluted in 1% Goat Serum/PBS/0.01% Triton X-100. Sections from in vivo experiments were labelled using a kit specifically designed to reduce endogenous IgG staining (M.O.M, Vector). All primary antibodies were applied overnight at 4 °C. Slides were incubated with Alexa Fluor secondary

antibodies (Invitrogen) for 45 min at room temperature, washed and mounted with Vectashield + DAPI (Vector Labs). Cells were incubated with Hoechst diluted 1:1000, washed and maintained in PBS until imaged. Paraffin-embedded sections were pressure-cooked for 3 min in Sodium Citrate Buffer (pH 6.0) followed by incubation in peroxidase blocking solution for 5 min and washing in Tris Buffered Saline pH 7.6 for 5 min. Following 1 h incubation, primary antibodies (listed in Supplementary Table 1) were detected using an avidin-biotin-based system (Vector). Images were acquired with a Zeiss LSM 710 confocal microscope (Zeiss). hMAB and mFB proportions were calculated as the number of hNuclei+ and hNuclei- cells over the total number of DAPI+ cells in random sections from different regions of recellularized scaffolds immunostained for hNuclei and DAPI.

Collagen

A commercial collagen assay kit (QuickZyme, Biosciences, NL) was used to quantify the collagen content of fresh and decellularized oesophagus. The sample was hydrolized with 6M HCL for 20 hours at 95 % and hydrolysed with 6M HCl for 20 hours at 95˚C, and the hydrolysates were incubated with a chromogen solution for 1 hour at 60 °C to develop color. The color intensity was directly proportional to the amount of hydroxyproline within the acid-hydrolyzed sample, which could be a direct measure of the total collagen. The resulting solutions were measured spectrophotometrically at 555nm, and the collagen quantity was calculated from a standard curve plotted with known collagen hydrolysate concentrations.

Elastin quantification

The elastin content of native and decellularized tissue was quantied using a kit (Biocolor, UK) according to the manufacturers instructions. The samples were homogenized, and elastin was solubilized in 0.25 M oxalic acid. Two consecutives incubations were performed at 95C to ensure complete extraction of elastin. Extracts were incubated with 5,10,15,20-tetraphenyl-21H,23H-porphine tetrasulfonate (TPPS) dye. It is based on a quantitative colorimetric determination. The absorbance was determined at 555 nm spectrophotometrically (Tecan Infinity, US). Elastin concentrations from a standard curve were used to calculate the elastin content of the tissue.

Chapter 3

Results

3.1 Bioreactor

The developed bioreactor increases the handiness and portability with respect to the previous custom dual glass chamber used by Luca Urbani et al. [123].
Besides, it easily allows to be adjustable on the basis of the oesophagus length, and allows simples introduction, handling and fixation of the organ inside the chamber.

Design

The bioreactor consists of a PTFE structure, with a parallelepiped rectangular shape, presenting a carved hollow chamber with equal shape. PTFE was chosen for the manufacturing because of its hydrophobicity and biological inertness, thus making it compatible with biological applications. Importantly, PTFE can be autoclaved, sterilised with ethanol or UV radiations and it possesses high chemical resistance to corrosive solutions. The bioreactor (Fig. 9,10,11) has dimensions 200 x 64 x 42 mm, while the internal carved chamber has dimensions 160 x 30 x 35 mm. The short side faces present 2 holes each. Three of them were threaded with a 1/4 – 28 UNF tap and they have a diameter of 6.35 mm (1/4 – 28 UNF standard, 1/4 represents the diameter expressed in inches). The other hole has a diameter of 3 mm and is not threaded. The upper face of the structure presents a groove, with a 3 mm width and a 1.7 mm depth, deputed to house a silicone o-ring (Internal Diameter: 68 mm, thickness: 3 mm) interposed between the PTFE chamber and the PC lid. The upper face also hosts 10 holes, of 4 mm diameter each, threaded with an M4 (metric standard) tap. The lid consists in a parallelepiped rectangle build in polycarbonate, with dimensions 200 x 64 x 8 mm. The lid was drilled in 10 points, coincident with the corresponding PTFE upper holes, with the use of an M5 drill bit creating 5 mm diameter holes. The holes in the lid were drilled 1 mm wider than the correspondent ones on the

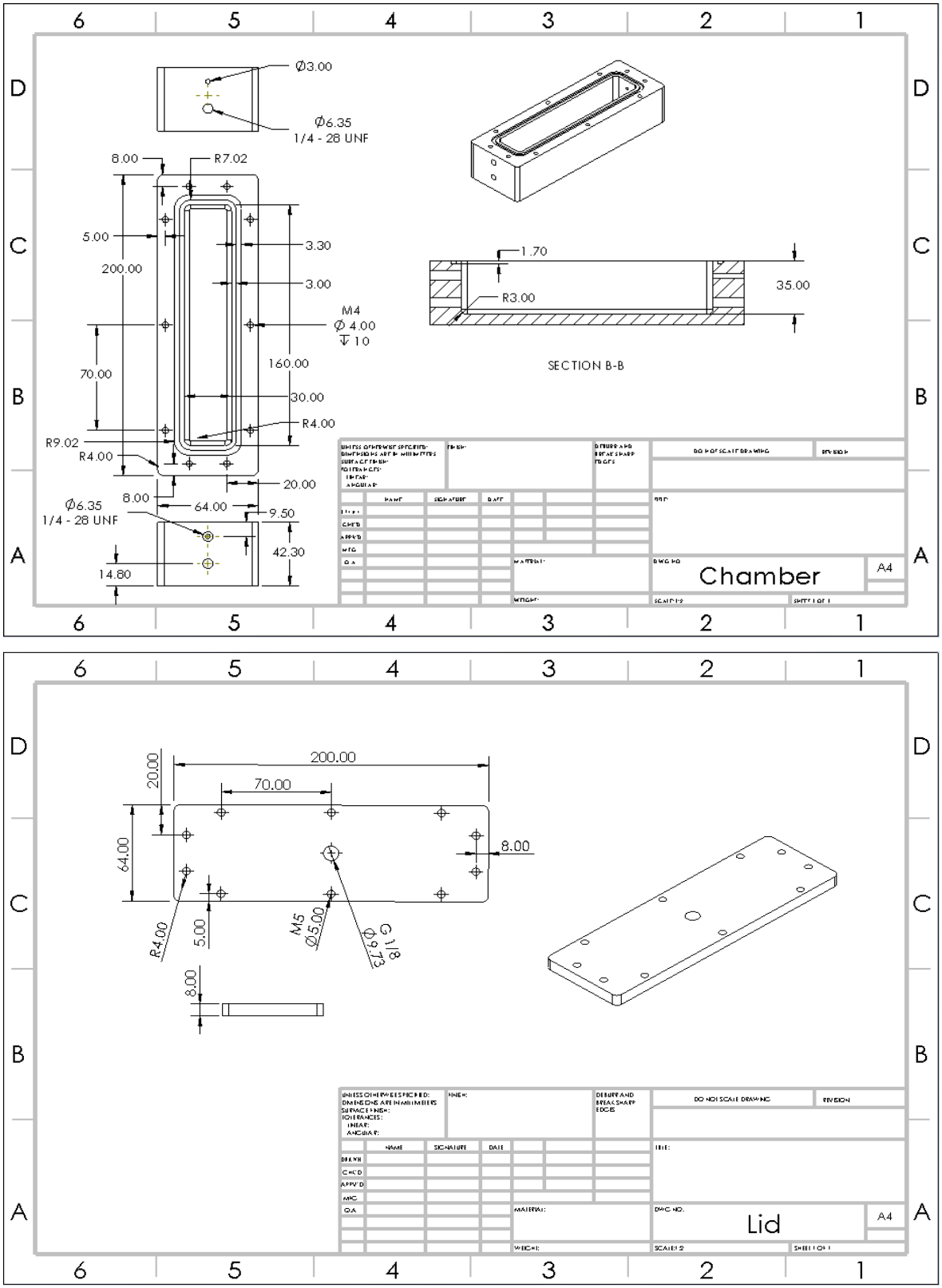


Figure 9: CAD drawings performed with SolidWorks®, showing the various dimensions of the PTFE chamber and the PC lid.

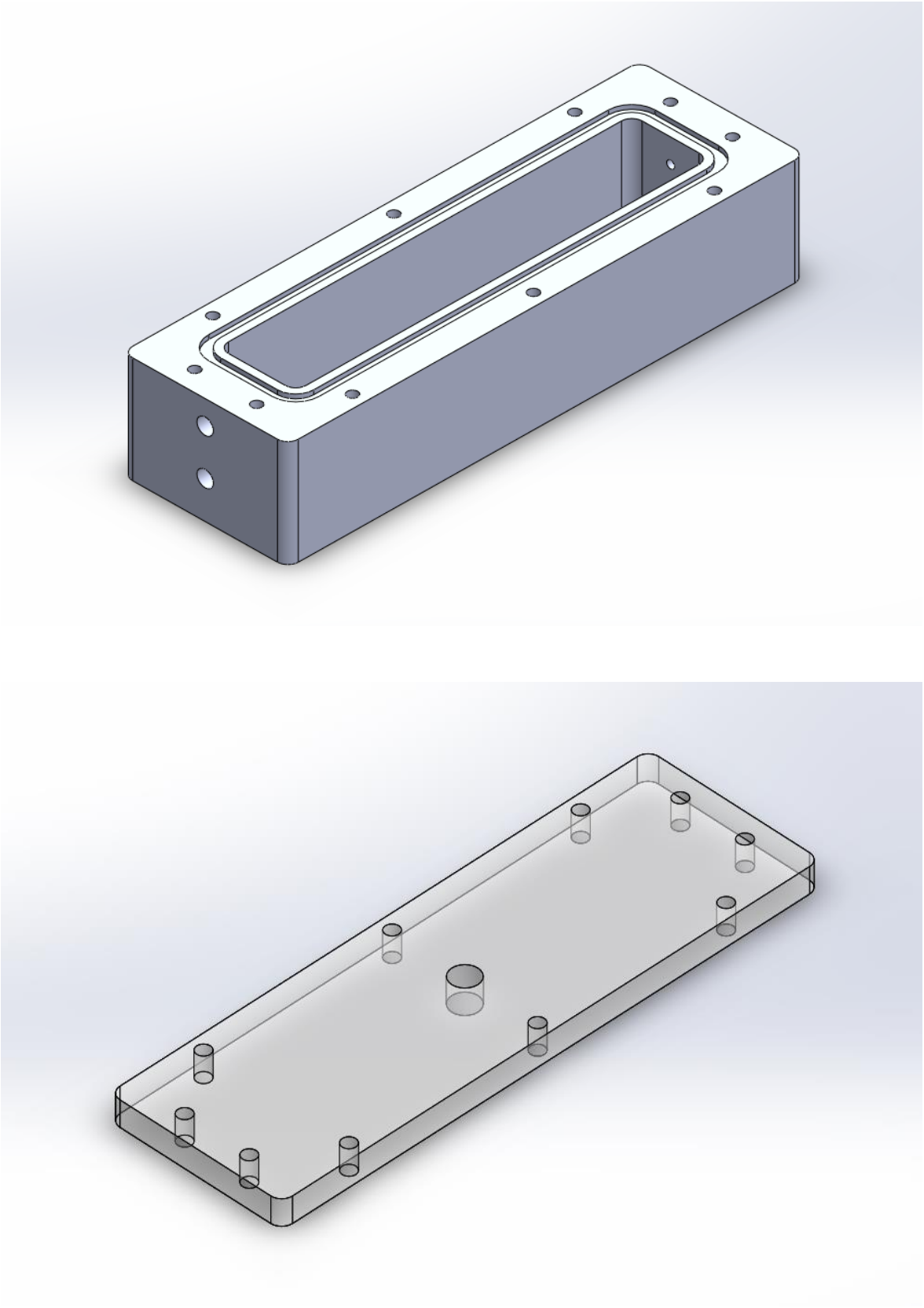


Figure 10: 3D model representation of the bioreactor parts designed in SolidWorks®.

chamber in order to ease the passage of the stainless-steel bolts. At the centre of the lid an additional hole is present, which has a diameter of 9.73 mm (G1/8 according to BSPP, British Standard Pipe Parallel). This hole was threaded with a G1/8 BSPP tap (RS).

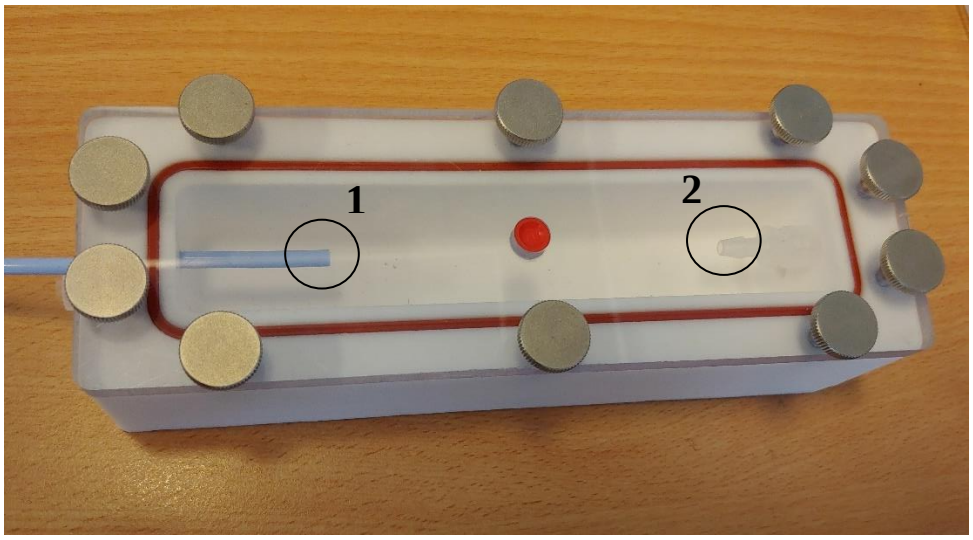


Figure 11: Main chamber assembled with the lid using stainless-steels bolts. The circled parts are the sites where the two ends of the oesophagus were sutured, namely 1) a urethral catheter tube end and 2) the barbed end of a female hose barb luer fitting

3.1 Perfusion system

The hydraulic system consists in a closed-loop circuit that allows a continuous perfusion of the seeded scaffold. The circuit is composed by the bioreactor chamber, a peristaltic pump and a reservoir bottle. These items are connected via medical-grade tubes, permeable to oxygen and autoclavable.

The hydraulic seal of the system was tested keeping the system at room temperature, filled with flowing MilliQ water circulating at 10 ml/min for 3 days (Fig. 12). A second trial was performed together with other two bioreactors (Fig. 13), which were already been used for tracheal bioengineering. In this latter system, the downstream bioreactors tubes are linked to Terumo BCT Inc 1000 ml transfer bags (Fischer Scientific, UK), instead of just letting water pour into the sink: this was done in order to exploit the negative pressure that the bags created when filled, thus increasing the force applied to internal chambers walls and lid surfaces, giving further stress to observe eventual spillages. In both the experiments, no significant leakage was detected.

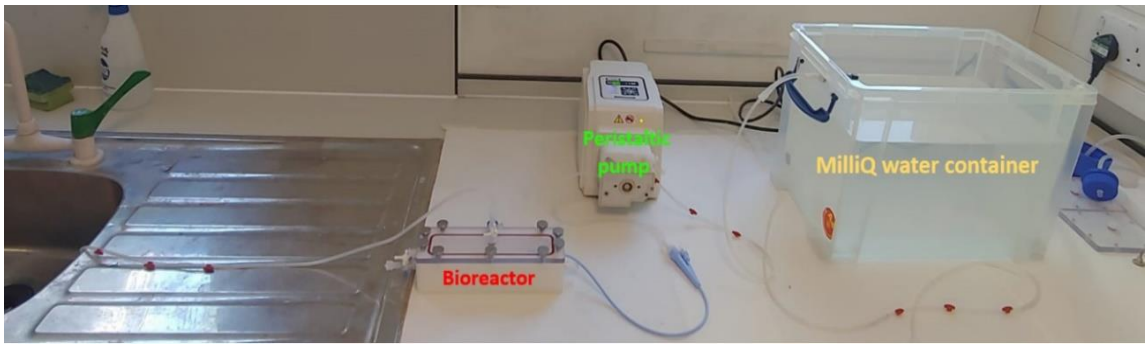


Figure 12: Components of the perfusion system in the hydraulic sealing trial. The MilliQ water was driven by the peristaltic pump into the bioreactor, from which it flowed out from a 3-way stopcock screwed on top of the lid. In this way, it was possible to assess the presence or the absence of eventual leakages coming from the contact points of the chamber with the lid or from the fittings screwed in the structures.

Figure 13: Further trial of hydraulic tightness (together with other two tracheal tissue engineering bioreactors). The outflow is collected in transfer bags able to exert a negative pressure inside the chamber, increasing the possibility of leakage

Successively, an experiment was performed to assess the sterility. The chamber was filled with DMEM (Dulbecco Modified Eagle Medium) which was let flowing with a rate of 3

ml/min for 4 days inside an incubator (Fig. 14). After 2 days, the medium had turned yellow, revealing an intruding presence of bacterial colonies. All the items were then washed and let decontaminating in a solution of MilliQ water and Mucasol (Sigma, UK) for 2 days. The experiment was repeated, this time letting the system in the incubator for a period of 7 days, and no contamination was detected.

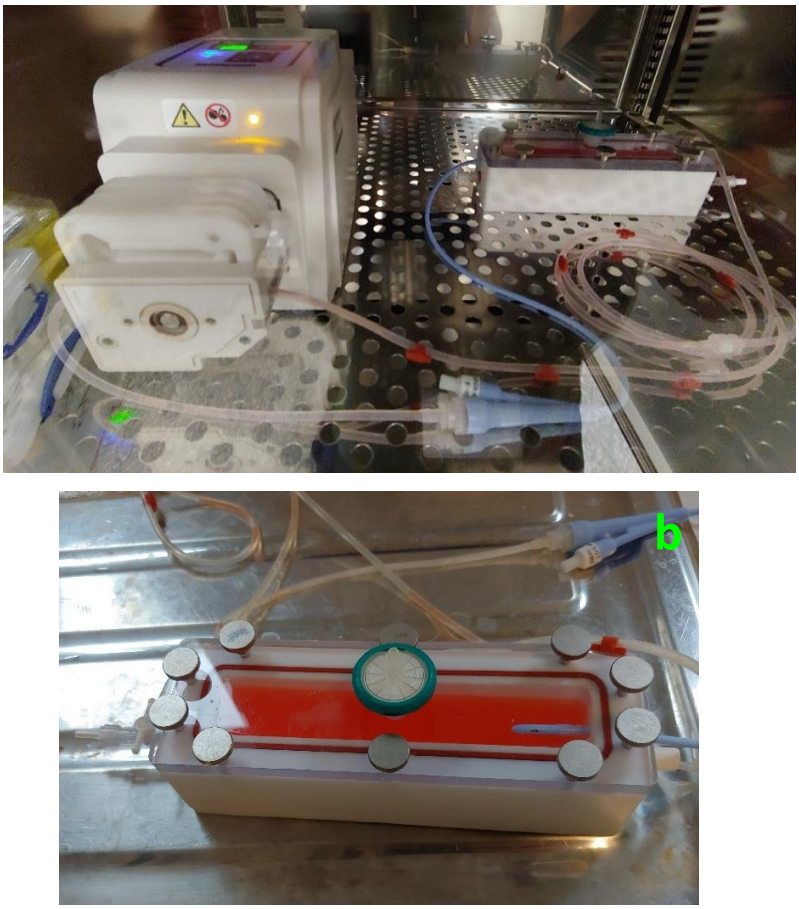


Figure 14: Sterility test performed in the incubator (a). The chamber was filled with culture medium and this last was made recirculating with a peristaltic pump. Letting the structure inside the incubator, it was possible to notice every day if the colour of the medium had changed. In (b) the bioreactor was taken out from the incubator after the first sterility trial. The medium had started changing colour, from intense red to orange, possible proof of bacterial contamination. Such proof was then validated with observation of a medium sample at microscope.

Finally, the whole perfusion system set-up including the seeded oesophagus fixed to the bioreactor is presented in Fig. 15.

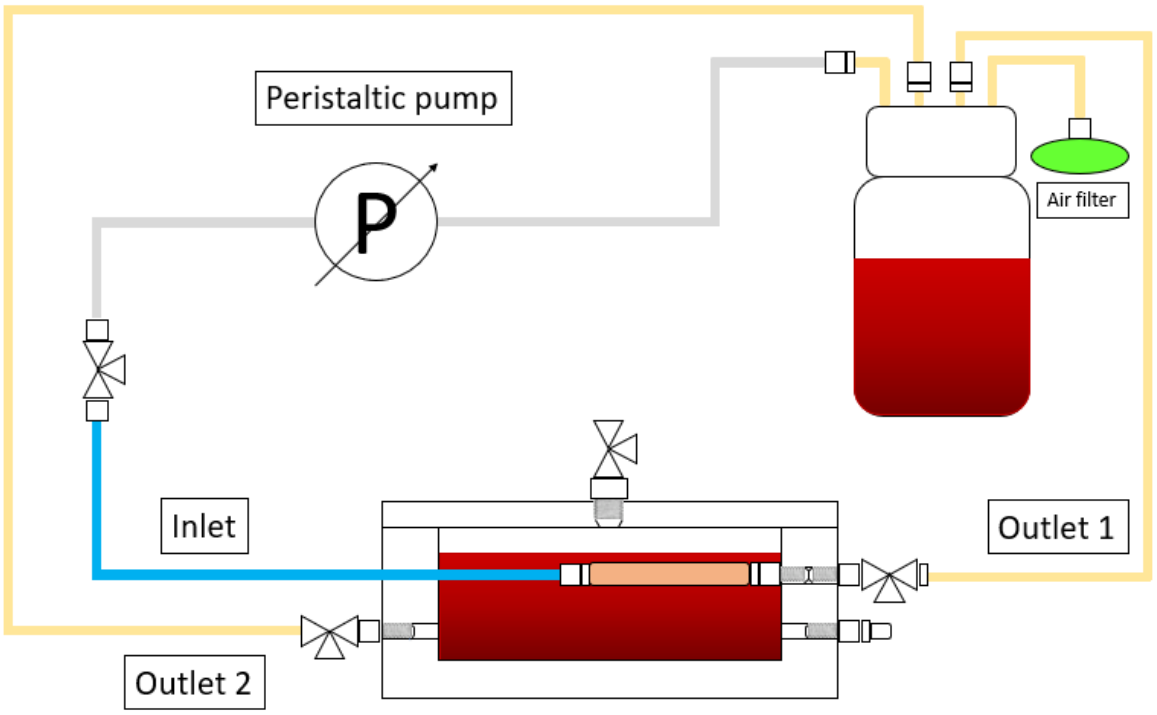


Figure 15: Schematic representation of the perfusion system used for the seeded oesophageal scaffold culture, comprising the bioreactor containing the oesophagus, a peristaltic pump, a reservoir bottle, an inlet channel, two outlet channels and an air filter linked to the reservoir. From the reservoir, the peristaltic pump withdraws culture medium which flows through the Inlet channel and perfuses the luminal wall of the oesophagus. Then, the medium flows out through the Outlet 1 channel back to the reservoir. The Outlet 2 channel is directly connecting the chamber to the reservoir. The Outlet 2 tube is linked to the chamber by a 3-way stopcock, which allows to fill the chamber with medium at the beginning of the culture and to change the old medium with fresh one during the course of the culture. Besides, a different culture medium from the one used to perfuse the oesophagus lumen can be used if necessary. During the culture, if the medium inside the reservoir and the chamber are at the same level, the fluid remains at hydrostatic equilibrium, according to the principle of communicating vessels. Besides, in the event that any leakages derive from the perfused luminal part of the oesophagus, the level of the fluid would increase both in the chamber and in the reservoir, thus preventing a saturation of the fluid in the chamber.

3.3 Decellularization

After the piglet oesophagus harvesting (Fig. 16), one of the extremities of the organ was sutured to the catheter tube with a pouch tie and connected to the decellularization system, composed by a bioreactor hollow chamber and a peristaltic pump, while the other end was let free. The 3D ECM-derived scaffold was generated through the application of the Detergent Enzymatic Treatment (DET) protocol, according to the one developed by Maghsoudlou et al. [124]. The first phase of the process was characterized by the perfusion

of MilliQ water for 24 hours, after which it was possible to appreciate a first gradual changing in the organ colour The detergent treatment through 4% SDC and subsequent enzymatic digestion with DNAse made the oesophagus transparent (Fig. 17).

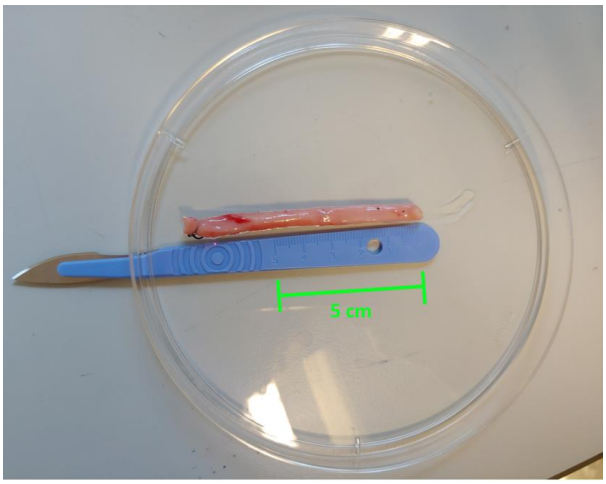


Figure 16: The piglet oesophagus, with a length of almost 8 cm right after the harvesting. The suture is marking the proximal end.

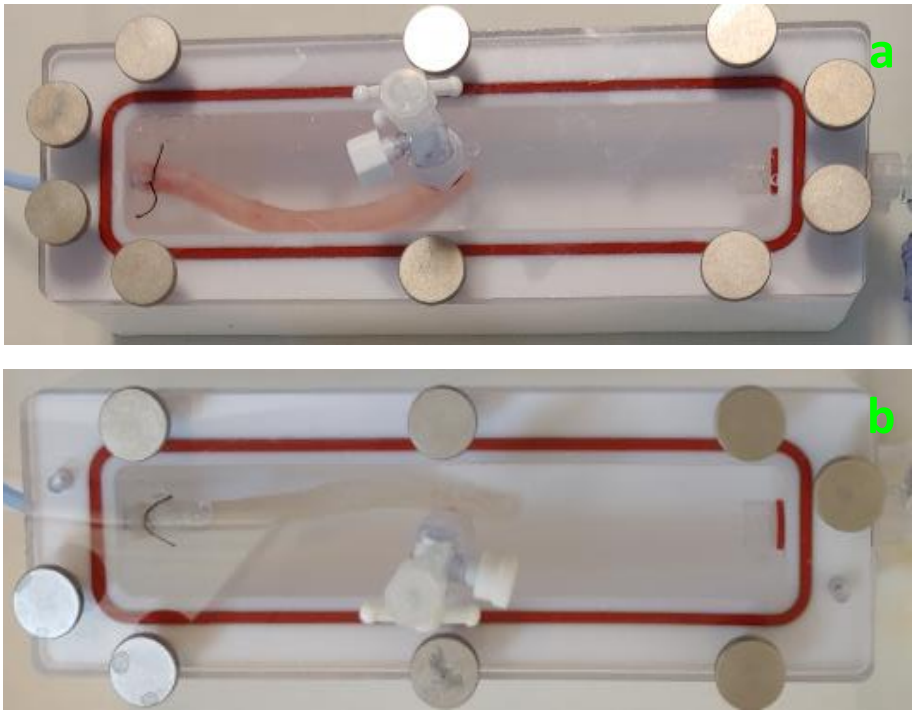
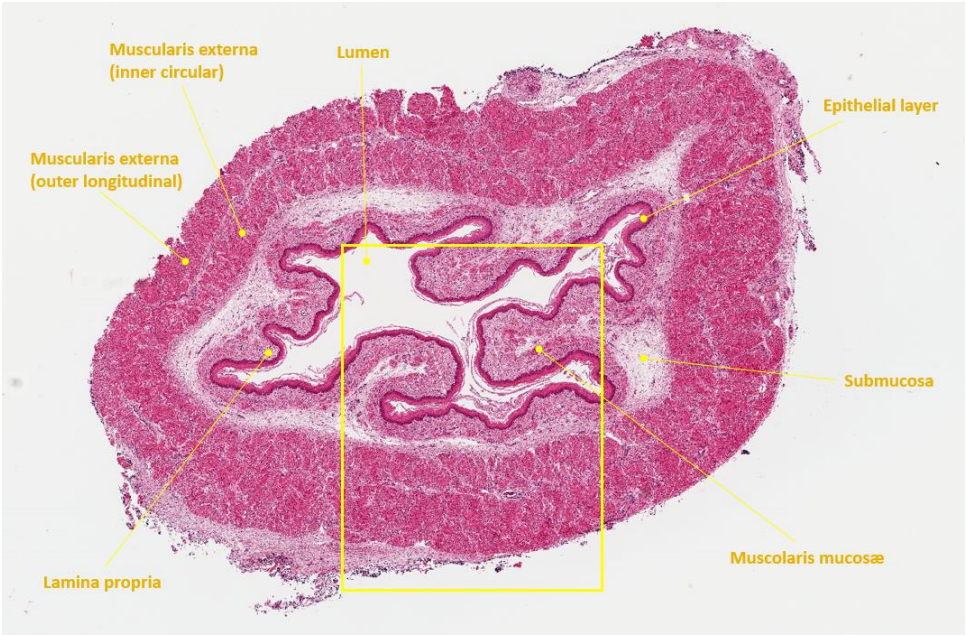


Figure 17: The oesophagus after (a) 24 hours from the beginning of the decellularization and (b) at the end of process.

Subsequently, Haematoxylin and Heosin histological analysis was performed in order to evaluate the presence of cells



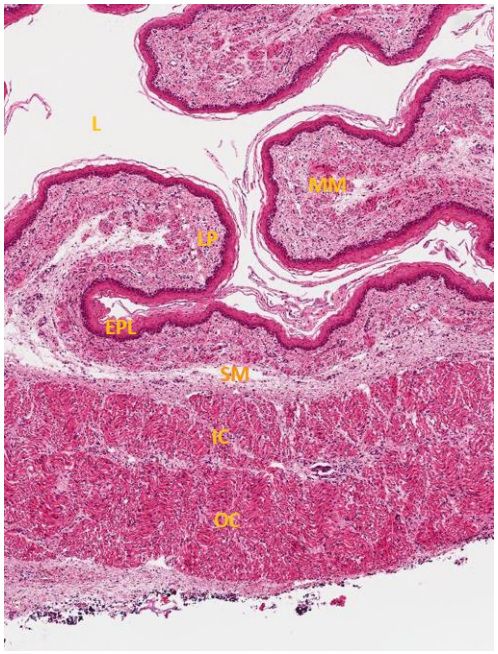


Figure 18: Whole native oesophageal section and magnification of a portion. The visible structural layers are: L = lumen; EPL = epithelial layer, LP = lamina propria, containing lymphatic capillaries, blood capillaries and loose connective tissue. It also contains lymphoid aggregations; MM = muscularis mucosae, composed of a double layer of smooth muscle cells; SM = submucosa, highly vascular, contains loose connective tissue and oesophageal glands secreting mucus; IC = muscularis externa, formed by inner circular layers; OC = muscularis externa, formed by outer longitudinal layers; muscularis externa is constituted by skeletal muscle cells in the upper third of the organ, by a mixture of skeletal and smooth muscle cells in the middle one-third, and by smooth muscle cells alone in the lower third.

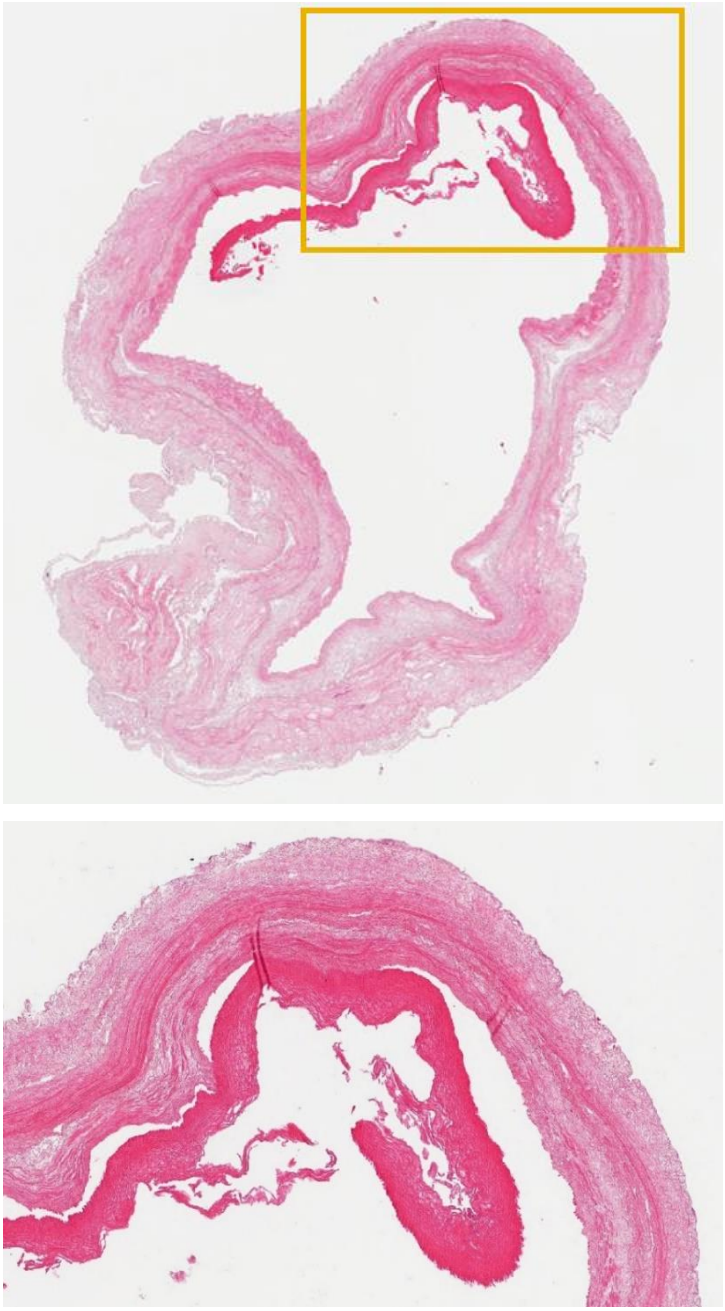


Figure 19: Haematoxylin and eosin staining of section cut from the oesophageal scaffold after decellularization. Pale eosin stain (pink/reddish) reveals that most of cellular protein content was discharged. No visible sign of cells nuclei stained in purple by haematoxylin are notable, proving that no alive cells from native tissue remained after the process.

Hemalum (a molecular structure composed by aluminium ions and oxidized h*a*ematoxylin) dyes the nuclei in blue, whilst eosin colors intracellular proteins and collagen. The sections analyses and imaging were performed in collaboration with Savvas Savvidis, graduate student in the De Coppi group. Fig. 18 shows details of the different layers of the native oesophageal tissue section. In Fig. 19 the section presented underwent decellularization in

the bioreactor designed in this thesis work. It is evident in the picture that most of the cells of the native tissue have been removed and the overall structure has undergone a significant change. The eosin stain results decisively paler than in the native counterpart, thus proving the removal of cellular protein content. It's also notable the absence of nuclei purple stain by haematoxylin. Dna quantification was assessed, demonstrating a lower concentration in decellularized tissue with respect to the native one (data not shown). Collagen and elastin content were then evaluated, respectively with QuickZyme (Biosciences, NL) and with Biocolor (UK) assay kits. The increased collagen content shown in Fig. 20 was attributable to the evaluation of collagen quantity in µg normalized by the tissue weight in mg, due to reduced cellular content following decellularization process. A slight reduction in elastin content was detected.

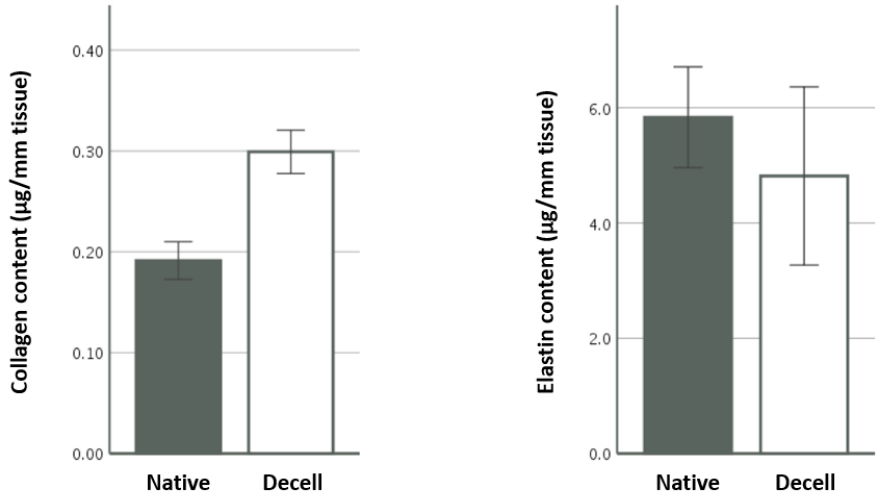


Figure 20: Graphs with results of analysis of collagen and elastin content. Collagen is represented as increased because other cellular components have been removed with decellularization process so its amount in weight compared to the total tissue weight is higher than before the process. A slight reduction of elastin is visible after decellularization. It is quite difficult to maintain the exact pre-existing architecture of the native organ. Improvements in decellularization process constitutes one of the most important aspects of tissue engineering.

3.4 Recellularization

The cells utilized for the seeding were human mesoangioblasts and human fibroblasts, harvested at the Great Ormond Street Hospital from skeletal muscles biopsies. In Fig. 21 are shown pictures taken during cell cultures.

The hMABS directly withdrawn from cell cultures used to seed the oesophagus were selected from the at passages 7 – 11, while fibroblasts were taken from passages 39-42.
The decellularized organ was seeded with a mix of hMABs and hFBs in ratio 85:15, through microinjections of the organ muscle wall, interspaced of 4-5 millimetres, on 4 different longitudinal lines. A nasogastric tube was passed through the lumen (Fig. 22) to mantain the hollow cavity and ensure wall tension during seeding. The scaffold was then deposited on a plate filled with Megacell medium and left to rest inside an incubator at 37° for 2 hours.

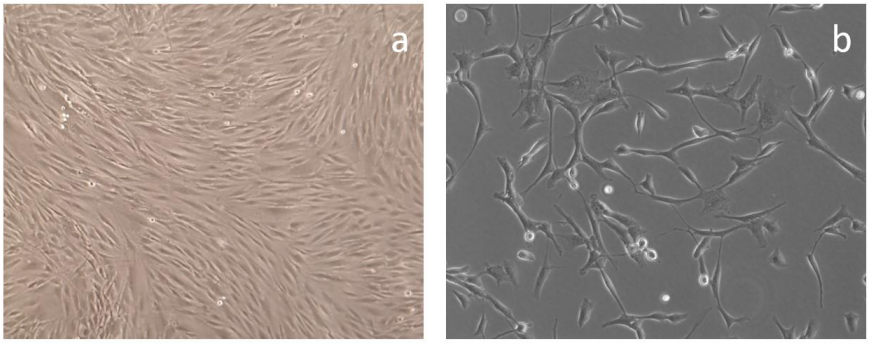


Fig 21: Live pictures of cells during culture. a) fibroblasts at full confluence at passage 10; b) hMABs at 50-60% confluence at passage 3.

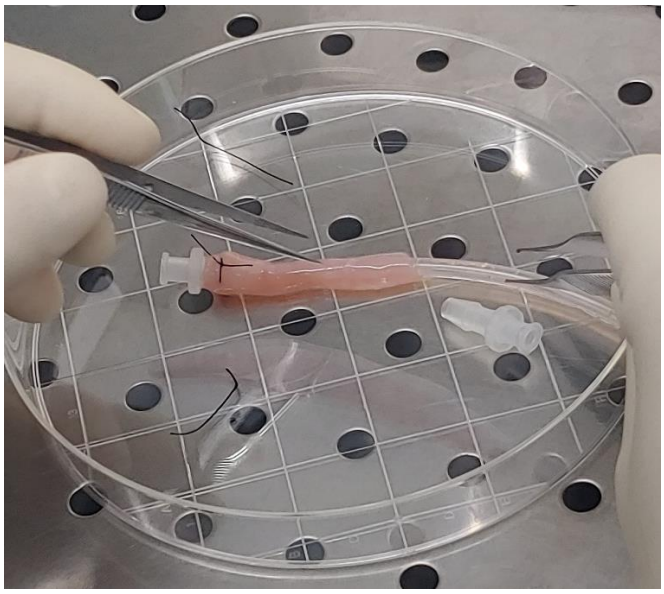


Figure 22: The harvested oesophagus sutured at a nasogastric tube. The tube prevented the collapse of the organ while microinjections of hFBs and hMABs were effectuated on the external side. The plate was successively filled with medium and put into the incubator, so that the cells could have the possibility to spread and start engrafting, prior to transfer into the newly designed bioreactor.

The seeded oesophagus was successively fixed to the bioreactor (Fig. 23,24). The organ was sutured at both ends to a female luer to hose barb adapter fitting coupled to a male luer fitting. At one end of the organ, the male fitting was screwed on the internal surface of the chamber, at one of the two short sides.

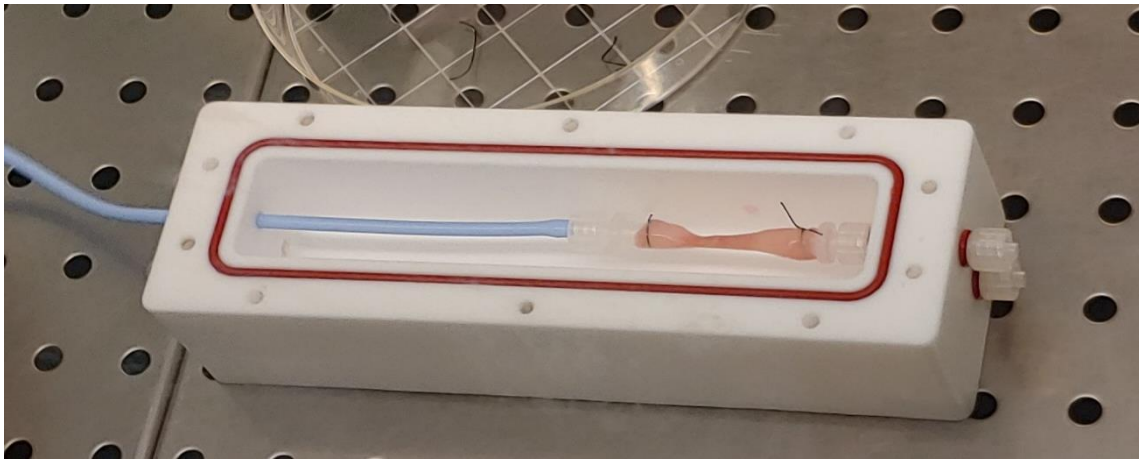


Figure 23: The oesophagus sutured at both ends in the bioreactor, prior to the application of other fittings and to connection of the chamber with the rest of perfusion system

On the external side of this wall a threaded male luer fitting was coupled to a 3-way stopcock, which linked a medical grade tube to the reservoir bottle. This tube constituted the first outlet channel of the bioreactor. On the same side surface, the hole at 18 mm below the upper screw was occupied by a threaded male fitting whose luer part was capped.

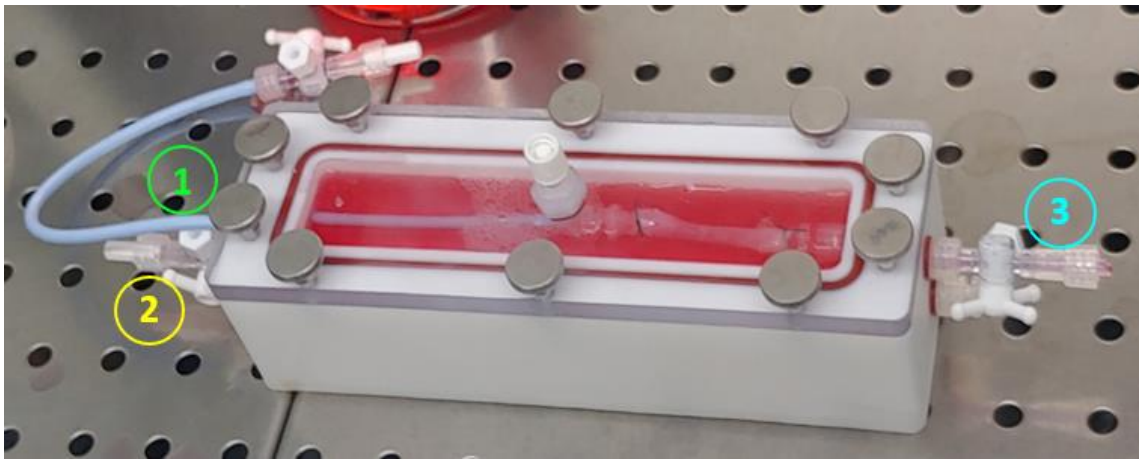


Figure 24: Seeded oesophagus in the bioreactor after filling of medium in the chamber. In (1) is the inlet channel, from where the medium perfuses the organ and flows out through the first outlet channel (3).The medium filling the chamber and surrounding the oesophagus was instead introduced using the stopcock in (2), that also represents the site of the second outlet channel.

On the opposite side surface of the chamber, a Foley silicone catheter tube was crossing the wall. The tube bladder opening end was pulled through the 3 mm upper hole and was connected to the proximal end of the oesophagus. Externally to the chamber, the catheter was cut some centimetres before the balloon and drainage ports fork, so the single end was connected to a male barbed luer which was in turn embedded to a 3-way stopcock. The stopcock was then linked to another silicone tube that reached the reservoir bottle. This latter tube was fixed into a cartridge of the peristaltic pump in the final set-up of the system inside the incubator. This tube constituted the inlet channel of the chamber, consenting the medium flow to perfuse the luminal surface of the organ. The catheter tube diameter was designed 1 mm larger than the hole through which it passed, thus preventing eventual leakages of medium from the inside because of its sealing action. At the same time, the tube was not too

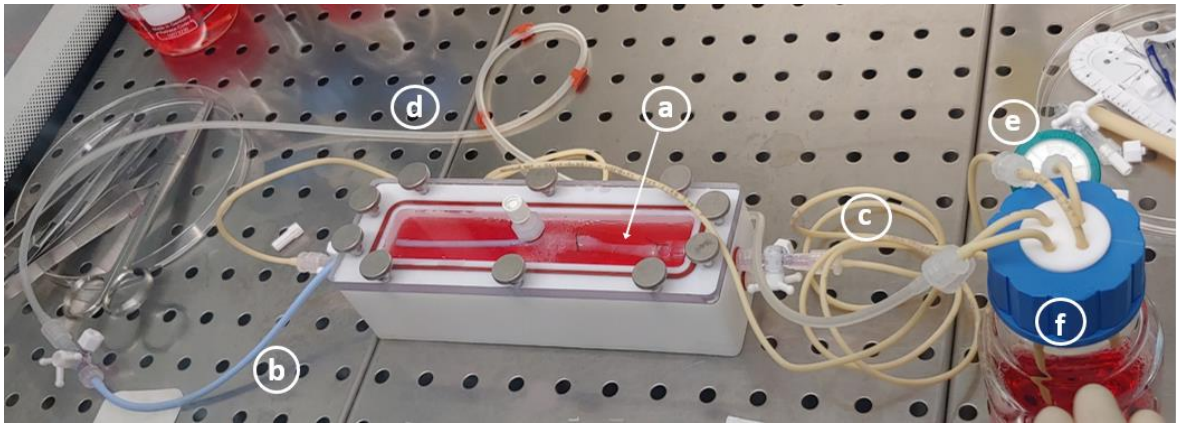


Figure 25: The dynamic perfusion system assembled. The peristaltic pump was added later when all the items were put into the incubator. (a) is the seeded oesophageal scaffold; (b) is the catheter tube to which an organ's end is sutured; (c) are the medical grade tubes where the medium circulate between the chamber and the reservoir; (d) is a silicone tube, connected to the urethral catheter, which was successively clamped to a cartridge in the peristaltic pump to allow the flow of medium. On its other end, such tube was connected directly to the reservoir, so that consented the direct luminal perfusion of the organ; (e) is the air filter, to allow oxygen to enter the reservoir; (f) is the borosilicate reservoir bottle.

large to remain stuck, so it could be pulled towards the internal environment of the PTFE block, or retracted back. This makes possible the culture of oesophagi of different length. On the same surface, below the 3 mm hole, a threaded male luer fitting was occupying the remaining wall hole. This male luer fitting was coupled to a 3-way stopcock. The stopcock was connected to a medical grade silicon tube that directly reached the reservoir. This latter silicon tube constituted the second outlet channel of the chamber. The 3-way stopcock

allowed to fill the chamber with medium at beginning of the culture and allowed fresh medium change. The level of medium in the chamber can be maintained if it is at the same height of the medium in the reservoir, thanks to the creation of hydrostatic equilibrium, according to the principle of communicating vessels. Besides, if the culture conditions require it, using the stopcock it is also possible to insert a different medium from the one used for the luminal perfusion. Furthermore, the second outlet channel results precious in the event of a leakage coming from the organ's perfused luminal part, because the increase in medium level wouldn't be confined to the chamber but it would also interest the medium in the reservoir, thus avoiding a complete filling of the chamber.

Stainless-steel bolts were added to assemble to chamber and the lid. A G1/8 threaded fitting was screwed on the centre of the lid, with a 3-way stopcock successively added. The reservoir bottle was provided with syringe air filter to allow the passage of air. Successively, the peristaltic pump was added to the described system, completing the dynamic culture perfusion system and everything was put inside the incubator at 37º, 20% O2 and 5% CO2. Fig. 25 presents the system assembled in a laminar flow cabinet hood, while and experiment is being executed, prior the adding of the pump.

The seeded scaffold was cultured in dynamic conditions for 11 days, after which H&E staining, immunofluorescence staining and Masson's Trichrome analyses were conducted, in order to evaluate if an effective expansion of cell population and successive start of a growing tissue had occurred.

Haematoxylin and Eosin analysis revealed that cells had indeed proliferated and populated certain areas of the scaffold, as it is possible to see in Fig. 26, where the purple spots are indicative of the haematoxylin stained nuclei and the pink/reddish spots provide indication of the presence of cytoplasmic material of cells. In the image it is possible to notice that the cells have been grouped in clusters. Wherever cells aggregated in that fashion, the stain colour results darker, which indicates a recovery in cell protein content. Besides, where such cells conglomerates are present, the remodelling of the organ architecture occurs.

After H&E, Masson's Trichrome analysis were conducted to assess the presence of cells nuclei, cytoplasmic and ECM material. The analysis was performed on another section of

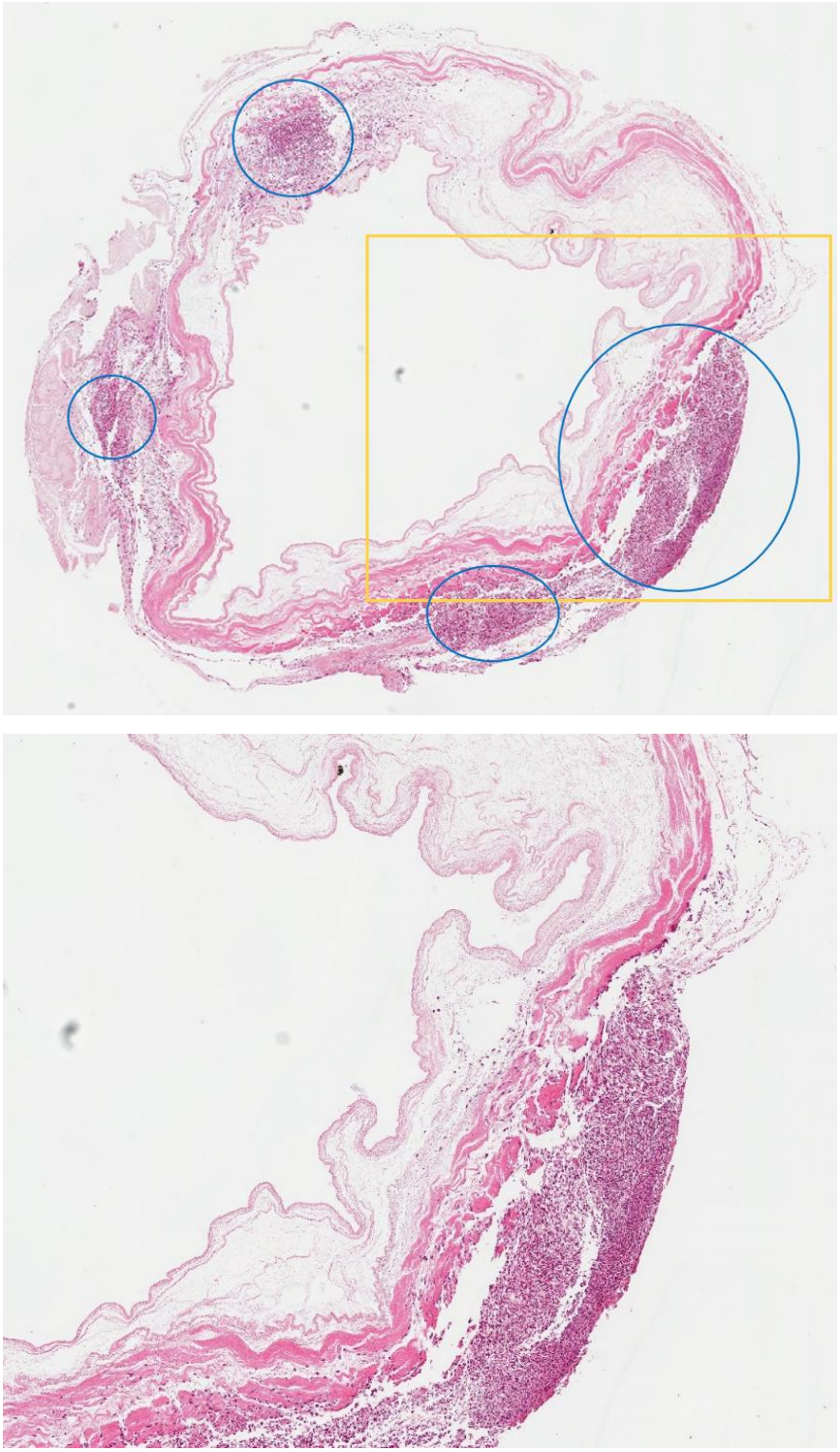


Figure 26: Haematoxylin and Eosin staining of a section obtained from the recellularized oesophagus and relative magnification. The cells distribution in clusters is highlighted by blue circles. The stain in these zones assumes a darker colour due to the production of cytoplasmic proteins, compared to the zones where cells are absent. The clustered cells are also associated to the remodelling of the pre-existing organ's architectures. The zones where cells have not proliferated show a pale colour and no sign of remodelling.

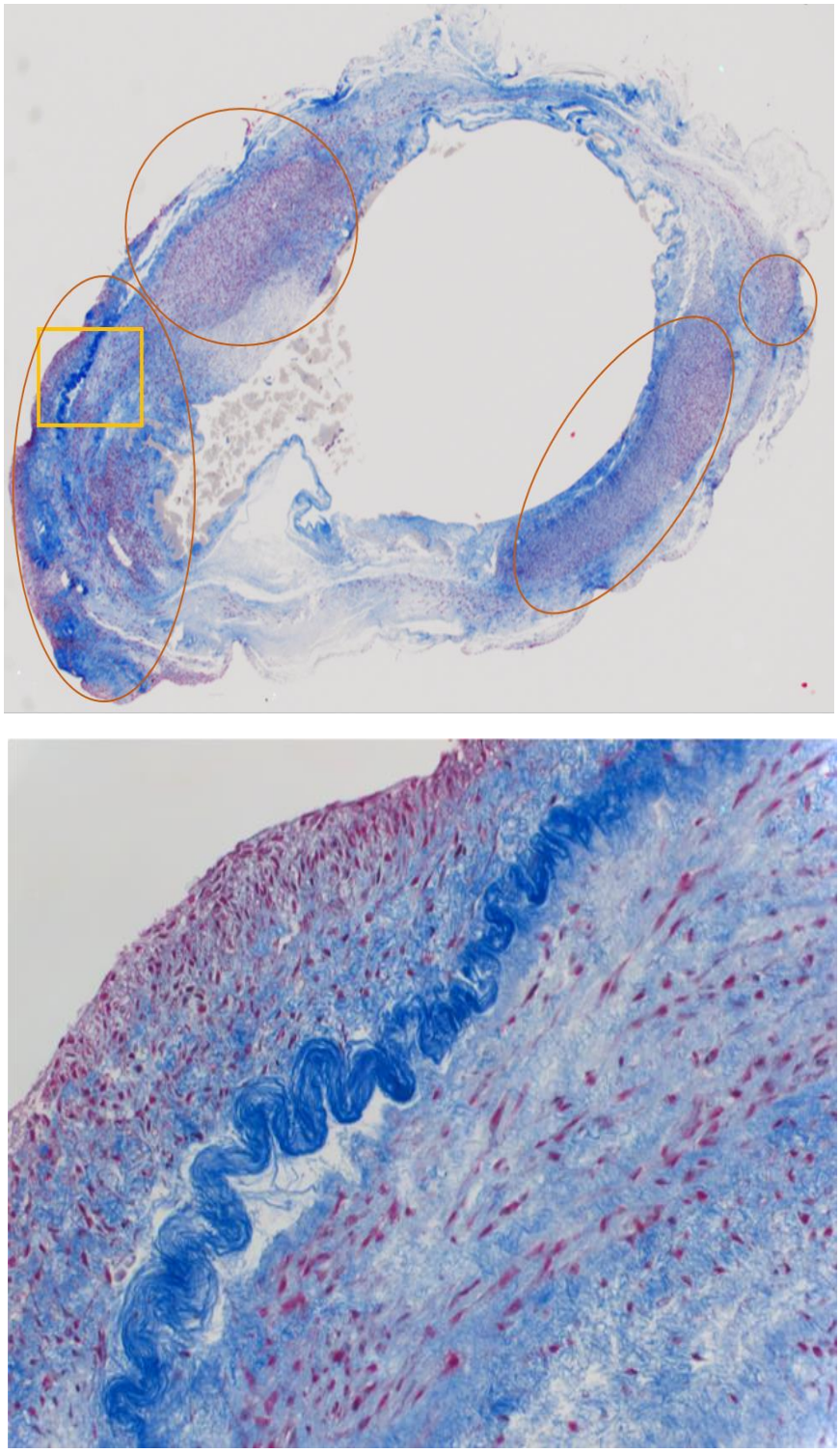


Figure 27: Masson's Trichrome staining of a further section taken from the recellularized oesophagus, with relative magnification. In circled areas are marked the zones with highest cells repopulation density. Red stain highlights muscle cells and blue stain reveals the presence of collagen. In some areas a dense concentration of cells collagen is visible, thus suggesting that a reconstruction of the organ's internal structures had started.

the tissue, with the results shown in Fig. 27. In the image the red-stained spots denote the presence of muscle cells, while blue-stain is representative of collagen content. Therefore, MT analysis also shows that the areas where cell proliferation had occurred were associated with organ's architectural remodelling, whereas no sign of structural retrieval was notable in zones devoid of cells.

At the end of bioreactor culture, immunofluorescence analysis was performed. The staining was effectuated against SM22 marker, a protein expressed specifically in adult smooth muscle cells, and against Calponin, a calcium-binding protein responsible of inhibiting ATPase activity in smooth muscle cells. Smooth muscle cells are highly abundant in muscular layers of oesophagus, so it was determinant to evaluate its presence in the recellularized organ. Nuclei of cells were counterstained with DAPI. The analysis showed the presence of relative markers, indeed proving that differentiation of hMABs into smooth muscle cells, had occurred (Fig.28).

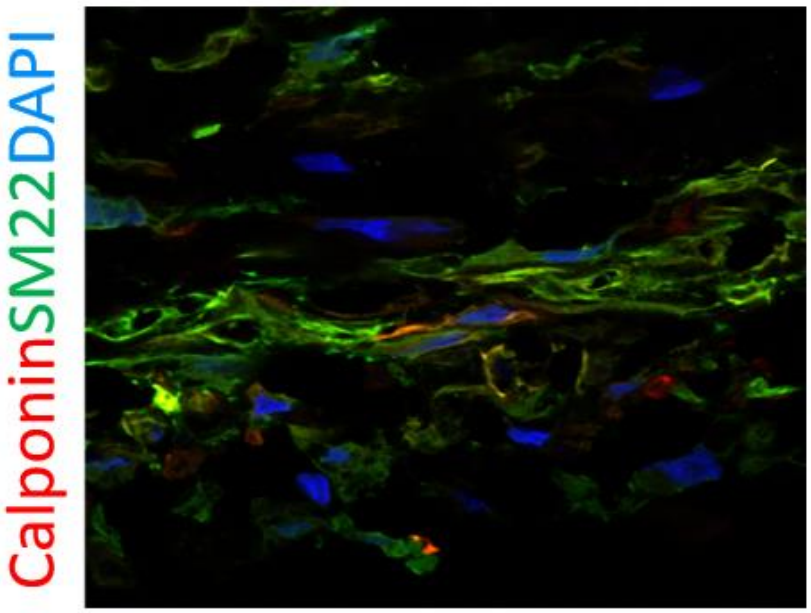


Figure 28: Immunofluorescence staining for DAPI, Calponin and SM22 markers. DAPI stains the nuclei of cells, thus revealing that viable cells were present. Calponin and SM22 are two proteins particularly abundant in smooth muscle cells. Their presence was a demonstration that seeded hMABS in the scaffold had undergone differentiation in smooth muscle cells.

Chapter 4

Additional work

The main work of this thesis was focused on the development of a novel bioreactor for oesophageal tissue engineering. However, the applications of the realized bioreactor may be extended in the future to the whole class of luminal organs. Considering the host group's broad tissue engineering fields of interest, together with the main work an additional project was carried on, with the aim of developing a similar bioreactor for parenchymatous organs tissue engineering. The work executed and described in the following paragraphs details the relative design and construction of such bioreactor.

4.1 Luminal and parenchymatous organs

Most organs are composed of several tissue types. Usually, a single tissue is of major functional importance and the other tissues provide structural and physiological support.
The primary tissue of an organ is referred to as the parenchyma. An organ is comprised of the four basic tissue types: epithelia, connective tissue, muscular tissue nervous tissue. The supportive elements also consist of the same classes of tissues and within a single organ are collectively termed the stroma.
The parenchymal and stromal components of most organs are arranged in one of two general patterns. If an organ is disposed around a lumen or cavity then it is considered a luminal or hollow organ. If the tissues that make up an organ are present in a more anatomically localized and solid form, then the organ is regarded as a parenchymatous or compact organ. In luminal organs, the component tissues are arranged in concentric layers about the lumen in a specific manner. The tissue that interfaces directly with the luminal space or cavity is by definition an epithelium and comprises the parenchyma. The stroma consists of various alternating layers of muscular and connective tissue in which stromal vascular and nervous elements may be found. Luminal organs are distinguished by their regional differences in function, and the often subtle structural modifications associated with their specific

functional role. Examples of luminal organs are oesophagus, trachea, stomach, intestine, bladder and urethra. Parenchymatous organs possess an extensive connective tissue framework, the stroma, that encloses and supports the parenchyma. The outermost connective tissue investment is called the capsule which defines the outer limits of the organ and is responsible for its anatomic localization at the macroscopic level. As a result, most parenchymatous organs can easily be dissected out and described as individual units. From the capsule, additional strands of connective tissue, termed trabeculae or septae, extend into the organ dividing it into compartments. Compartmentalization may be complete or incomplete. Often an indentation is evident on the capsular surface. This region is termed the hilus and serves as the point for the entry or exit of various vascular and nervous elements which contribute internally to the stroma. Usually a single artery enters at the hilus. Its major branches are found within the trabeculae or septae and the small terminal arteries extend into the parenchymal compartment where they give off capillaries. Veins and nerves follow the pattern of the arteries. Parenchymatous organs suspended within body cavities may also be covered externally by a serosa. The parenchyma of compact organs is arranged in masses, cords, follicles, tubules, ducts or strands. In the kidney, for example, the parenchyma consists largely of the tubules of the nephron and the ducts which collect and transport the excretory product. In the thyroid, the parenchyma consists of epithelial lined follicles filled with colloid and in the liver the epithelial cells of the parenchyma are present in a pattern of radiating cords [130].

4.2 An overview on parenchymatous organs tissue engineering: liver and kidney

The need for a valid alternative to organs transplantations, has brought the global research to investigate in the field of tissue engineering in order to address the problems that current procedures cannot be adequately deal with, namely lack of organs for transplanting due to the increasingly higher demand and constant pharmacologic immune-suppressive therapy to limit body's natural immune rejection reactions.

Devices such as Bioartificial Liver (BAL) and Bioartificial Kidney (BAK) have been used as temporary alternative to liver transplant, addressing specifically end-stage diseases

(ESLD, End-Stage Live Disease, and ESKD, End-Stage Kidney Disease) and acute failures (ALF Acute Liver Failure, and AKF, Acute Kidney Failure). They have been reported to sustain organ function, bridging the period between failure and transplantation of a new organ or, in some cases, until a recovery of the native organ through endogenous regeneration. BAL and BAK devices provide an extracorporeal support based on blood dialysis to remove metabolic wastes. BAL mainly incorporates functional primary porcine hepatocytes due to limitations in amount and availability of human primary hepatocytes functional hepatocytes. For such reason, it is necessary to use a barrier to separate the blood and hepatocyte compartment [131]. The engineering challenge related to BAL systems is about hepatocytes viability. It is necessary to provide adequate oxygenation to hepatocytes in order to avoid their function loss and apoptosis. The main criticality is that, to date, no BAL system has been demonstrated to improve the survival in patients with acute liver failure. There is still no scientific evidence of a potential more favourable risk/benefit ratio compared to liver transplantation [132].

Similarly to BAL, BAK is composed of a membrane coated with cultured proximal porcine tubule cells [133, 134] These cells support unidirectional transport, and when added in series to a traditional hemofiltration circuit, the resultant bioartificial kidney had appeared to significantly improve outcomes of acutely uremic dogs in models of endotoxin-mediated sepsis [135], but it has significant limitations in terms of morbidity, mortality, and cost [136].

Organs tissue engineering was adopted to overcome the problems of transplants and artificial substitutes. Many different attempts to obtain a functional organ were made, in terms of scaffold and type of cells choice and perfusion system.

In liver bioengineering, synthetic 3D scaffolds have been used, but they lack an accurate functional reproduction of the native organ vasculature tree [137], as well as of the required complex architecture and dynamic characteristics [138]. Whole organ decellularization (Fig. 29), after harvesting from donor mices, has so far given the more optimistic results, despite the main problems of such procedure, such as loss of organ volume and imperfect retaining of its architecture [139]. Regarding cells type selection, the use of primary hepatocytes could appear the most direct and obvious way, but they showed limited availability, expandability and phenotipic and functional instability, when cultured *in vitro*. [140]. Similar problems were come out using cancer-derived cell lines [141]. A lot of focus has been set on the use of induced pluripotent stem cells (iPSCs), which are able to differentiate in various cells kind

through soluble factor supplementation. These cells have been used to obtain hepatocyte-like cells with promising results but showing a phenotype more similar to fetal cells than adult human hepatocytes [142]. 3D liver organoids have been cultured considering the necessity

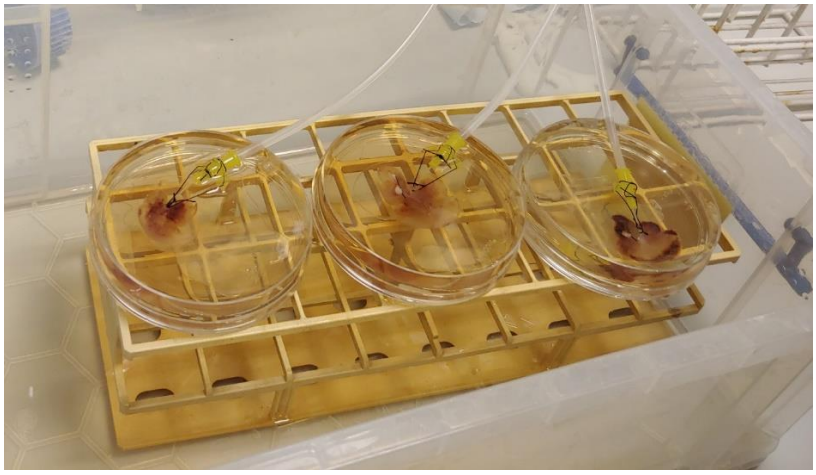


Figura 29: Rodent livers decellularization performed at Surgery department of UCL Great Ormond Institute for Child Health

for rapid cell expansion necessary in clinical application, since they allow high densities of long-term culturing condition, supporting functional maturation and longevity of hepatocytes-like cells [131, 143].

Most of the bioreactor-based dynamic perfusion system for liver tissue engineering adopts a chamber where the organ is perfused through an inlet linked to a vascular conduit (usually inferior vena cava or portal vein), letting the medium flow out the container through an outlet. In this way, the media recirculates continuously, thanks to a peristaltic pump, mimicking the blood flow, and inducing a mechanical stimulation to the cells. In more complex examples, independent perfusion inlets linked to both hepatic artery and portal vein, appropriately cannulated, are present. The adding of an oxygenator membrane to flowing medium with 95% O_2 and 5% CO_2 can further enhance the correct growth of cells [144].

When it comes to kidneys, of the earliest attempts of *in vitro* kidney tissue engineering involved the isolation and in vitro propagation of cells derived from both the undifferentiated MM (metanephric mesenchyme and unbranched UB (ureteral bud), which were shown to be able to origin branched structures and polarized tubules. [145]. The functional capacity of these cell-derived tubules was not characterized, but such approach was relevant for tissue

engineering further development because a number of the growth factors that regulate in vitro tubulogenesis in cell culture are also regulated during renal recovery from injury [146]. According to whole organ engineering, a study showed the possibility to culture separately MM and UB and then combining them, mimicking the structure that such tissues build in fetal renal development, although no functional characterization was performed. [147]. A whole human harvested metanephros (mature and functional kidney whose organogenesis depends on interactions between the MM and the UB), transplanted intraperitoneally into mice showed viability for 60 days, and express a genetic profile similar to "normal" human kidneys [148]. Sullivan et al. developed a method for the decellularization of whole porcine kidneys using a high-throughput system [149]. In 2018 Przepiorski et al. conducted an experiment where they developed a protocol for the generation of kidney organoids from iPSCs differentiation, using a spinner-flask bioreactor [150].

4.3 A bioreactor for parenchymatous organs bioengineering

4.3.1 Material & Methods

Bioreactor's design

Alike the oesophageal bioreactor, the chamber and lid of the parenchymatous organs bioreactor were manufactured at the Institute of Making (UCL) using a CNC machine (Roland Modela Pro II). The chamber and lid were designed with the SolidWorks software, saving the project files in .dxf format, which were then opened in Adobe Illustrator, saved in .ai format and opened in ArtCAM. The files were sent from ArtCAM to CNC terminal.
The material used were PTFE (Teflon) RS PRO 600x300x30 and PC (Polycarbonate) RS PRO 300x300x8. The chamber had to be 26 mm high, while the original PTFE sheet was 30 mm high, so before starting the shaping of the structure, an initial filing of 4 millimetres with a 10 mm diameter bit was performed by the CNC. Successively, a 6 mm flat-end bit was used for the carving of the internal circular space, and an 8 mm flat-end bit was used for the external perimetral circular cut. The circular groove on the top surface was created with a 2 mm flat-end bit. A 3 mm drill bit was used for the creation of the holes for screws on the

upper surface. The holes on the lateral surface of the PTFE cylinder, as well as the hole going through the PC lid, were made by hand with a cordless drill using a 5 mm (M5) drill bit. These holes were then threaded with a 1/4 - 28 UNF tap. A saw and a sand-belt were used to cut off bridges and refine the structures edges.
In order to assemble the chamber and the lid together, 6 stainless steel knurled screws with collar (Wyxroyd) were utilized. The bioreactor was designed to be GMP compliant.

Perfusion system

After the realization of the bioreactor, a perfusion system was set-up only in the context of a hydraulic sealing test. The set-up comprehended the bioreactor chamber, an i50 iPumps peristaltic pump, Masterflex® Pharmed BTP silicone tubes (internal diameter 1.6 mm, Cole-Parmer UK) , polypropylene fittings and a Terumo BCT Inc 1000 ml transfer bag (Fischer Scientific, UK). The fittings types used were: 1/4 – 28 UNF Male Luer, (Masterflex® ,Cole-Parmer UK), 1/4 – 28 UNF Female Luer to Hose Barb Bulkhead, (Masterflex® ,Cole-Parmer UK), Female Luer to Hose Barb adapter (Masterflex® , Cole-Parmer, UK), Male Luer to Hose Barb adapter (Masterflex® Cole-Parmer, UK), Female Luer to Hose Barb Straight Slip luer (Masterflex® Cole-Parmer, UK). The threaded fittings screwed on the external surface of the cylinder were provided with silicone o-rings (OR5X2, Hooper LTD, UK) to hold a correct sealing. The device was assembled, autoclaved and subjected to a hydraulic sealing test. For the test, the assembled bioreactor was linked to the peristaltic pump and to the transfer bag. The pump was driving MilliQ water withdrawn from a container through a silicone tube connected to a medical grade silicone tube that reached the bioreactor in correspondence of one of the two channels present on cylinder external surface. The water flowed out through the second channel, by another medical grade silicone tube connected to the transfer bag. The water flow was set to 10 ml/min.

4.3.2 Results

Bioreactor Design

The bioreactor chamber was realized in a cylindrical shape, and also in this case PTFE was chosen being hydrophobic, biologically inert, and being able to be autoclaved and sterilized with ethanol or UV radiations.

The PTFE chamber presents itself as a cylinder with a 93 mm diameter and a 26.75 mm eight. Internally, it is carved starting from the upper surface in another cylinder with a 65 mm diameter and a 20 mm height. In such a way, the whole structure is almost a hollow cylinder, but it keeps a 6.75 mm thick bottom. The cylinder wall presents two holes, one front of the other but vertically staggered between them. The holes present a 1/4 - 28 UNF thread, necessary for the corresponding luer fittings. The top surface of the cylinder presents 6 equidistantly spaced holes, 60° from each other, with a threaded diameter of 4 mm (M4). On the same surface a 3 mm wide and 1.7 mm deep groove is present, , in order to house an o-ring to maintain the hydraulic sealing.

The lid consists in a cylinder build in polycarbonate, with a diameter of 93 mm and a height of 8 mm. The lid is drilled in 6 equidistant M5 points, coincident with the corresponding PTFE chamber upper holes. They were made slightly broader than the PTFE ones to facilitate the entry of the screws. The lid presents one M5 hole with a 1/4 - 28 male luer screwed on it. A three-way stopcock can be set on the luer in order to consent air filtration.

On one side of the external surface of the PTFE cylinder a male luer with 1/4 - 28 UNF thread is screwed, in correspondence with one of the holes, constituting the outlet drain. Externally, a female hose barb luer connects the male luer to a medical grade silicone tube. On the other side of the cylinder external surface a threaded barbed female luer is screwed, in correspondence to the upper wall hole, and it is externally connected to a silicone tube by a male luer. Both the threaded fittings are surrounded by the sealing o-rings. Inside the PTFE chamber, a segment of medical grade silicone tube (whose length can be variated, according to experiments requirements) is embedded on the hose barb of the upper threaded female luer. The other side of the segment is linked to the female slip barbed luer. The female luer slip part can be used to embed a cannulated parenchymatous organ, allowing the culture medium to flow inside, perfuse the organ, and flow out through the lower hole of the PTFE chamber. A male threaded luer is present at the centre of the lid, allowing the passage of air. Such passage can be regulated through the further addition of a 3-way stopcock on top of the luer.

The SolidWorks® drawings and 3D parts of the bioreactor are shown in Fig. 30-31. In the designs made with the software lateral holes were not defined, since they were later created manually after deciding their most opportune position. The assembled realized bioreactor is shown in Fig. 32.

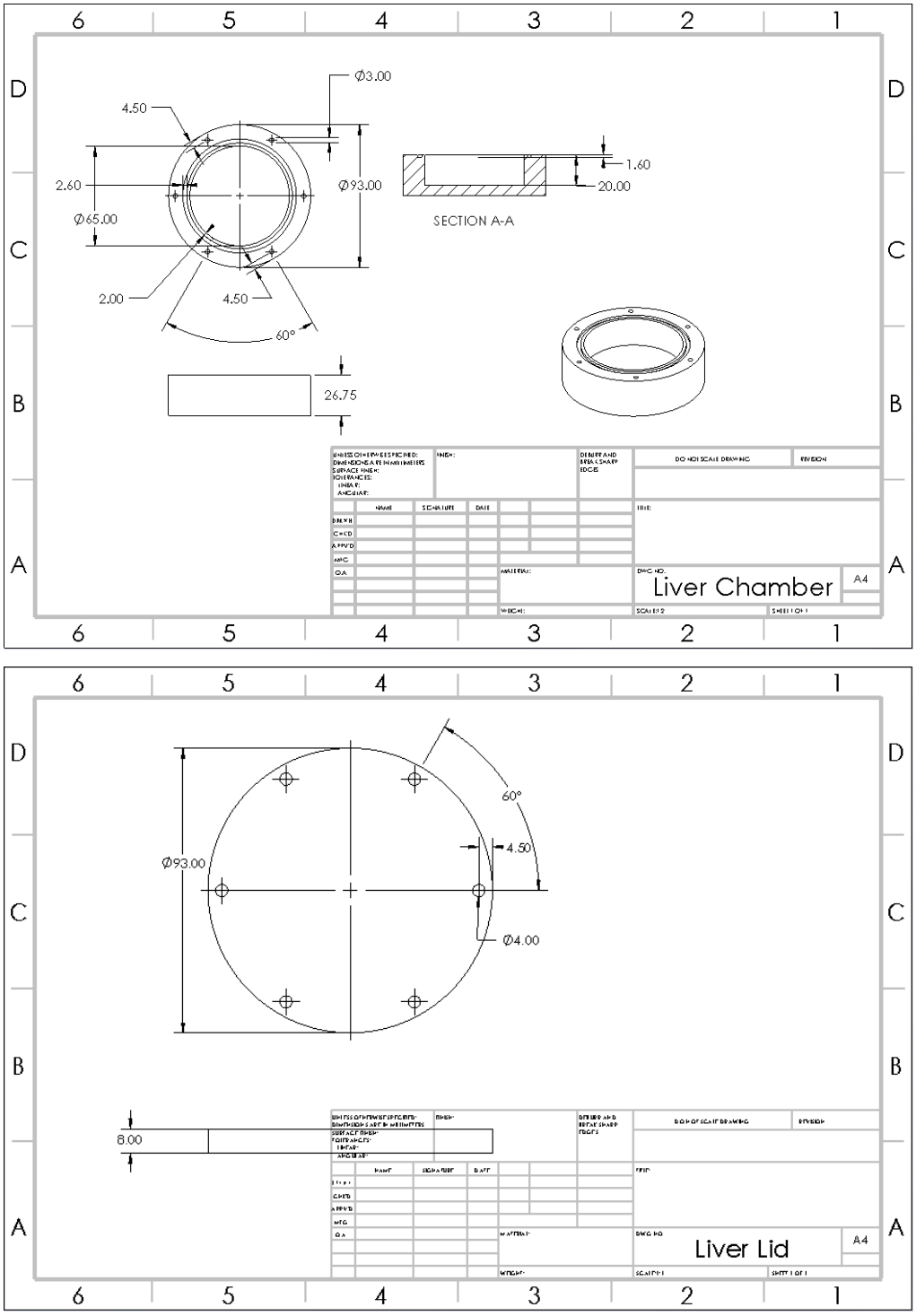


Figure 30: CAD drawings of the chamber and lid parts of the bioreactor realized in SolidWorks®

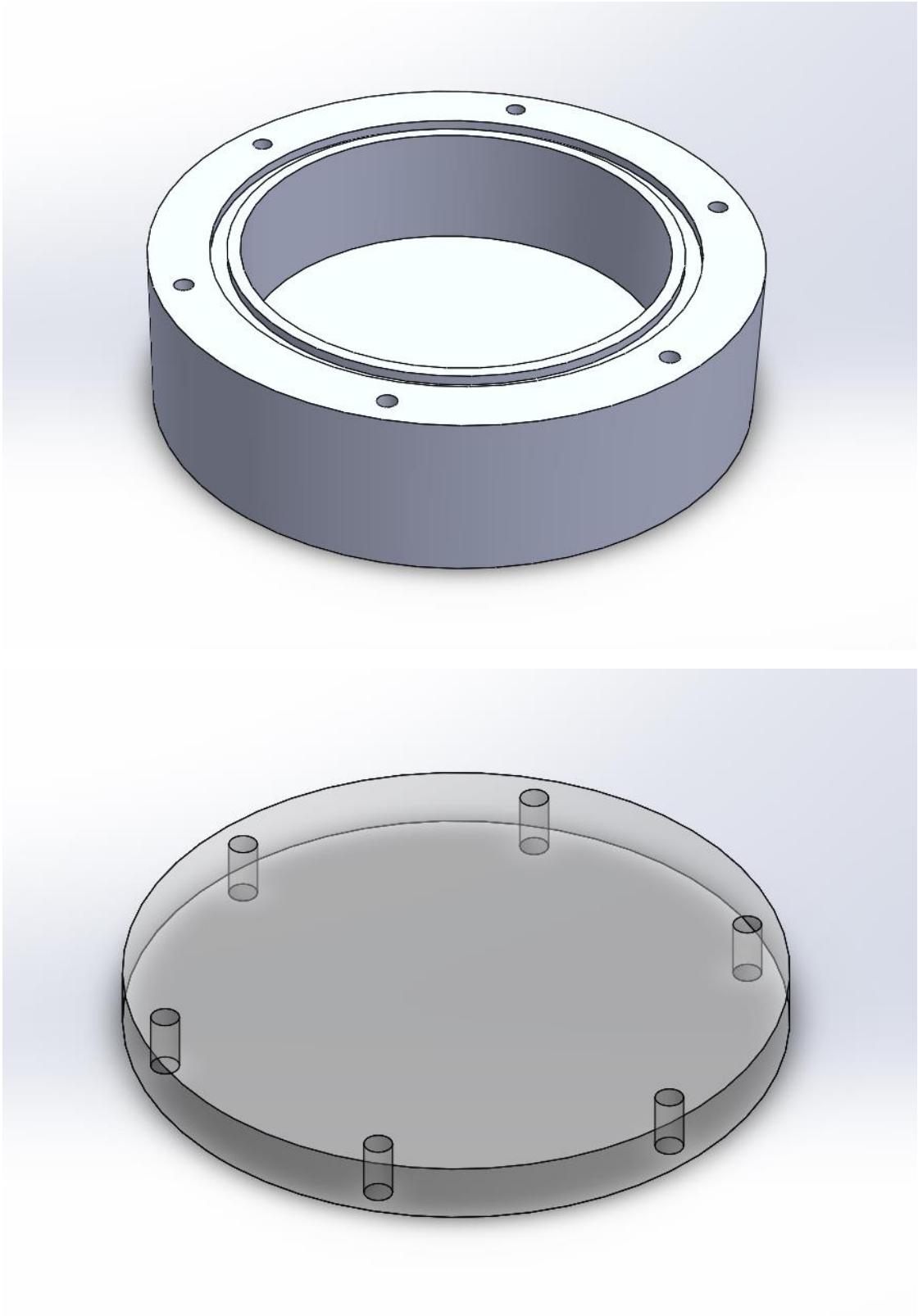


Figure 31: 3D model of the chamber and lid of the bioreactor realized in SolidWorks®.

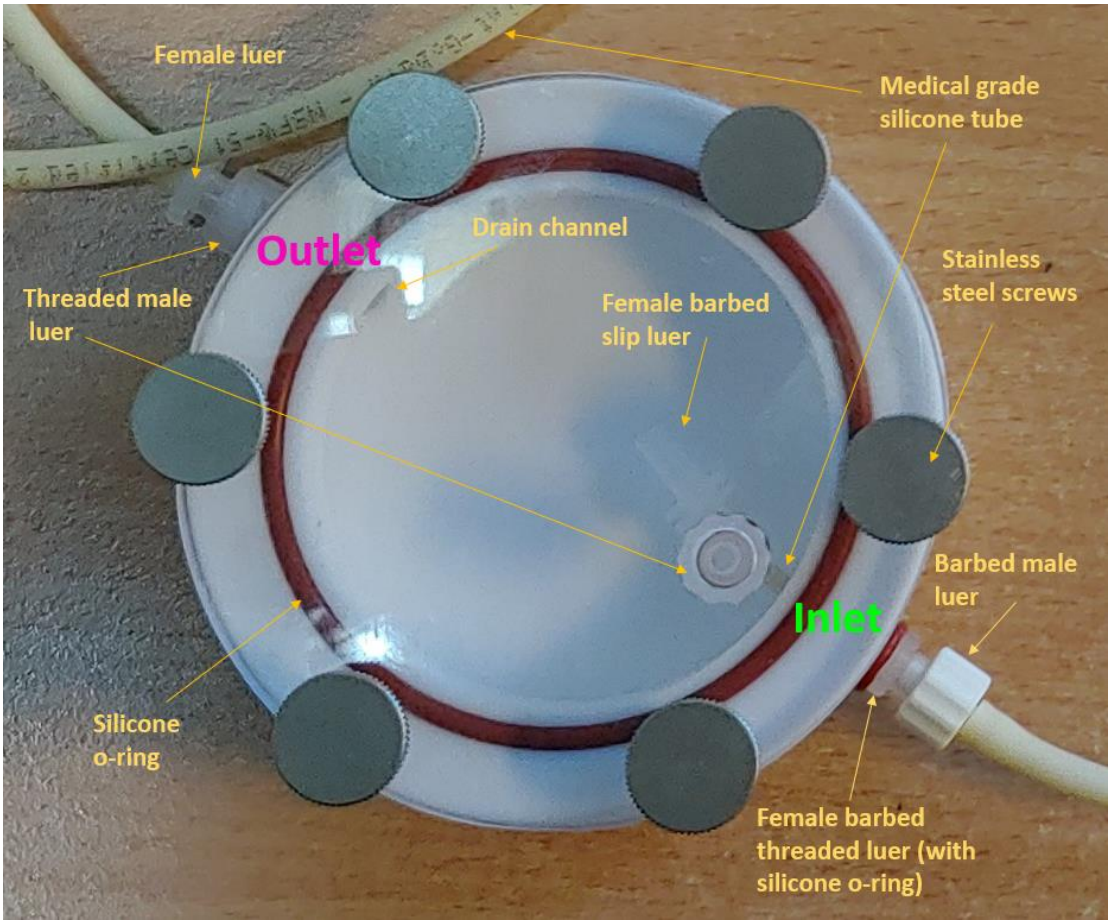


Figure 32: Bioreactor prototype

Perfusion system

The main aim of this additional work was the CAD design and physical construction of a bioreactor chamber suitable for parenchymatous organs. For this reason, a complete perfusion system, comprehensive of a reservoir of culture medium and the presence of a seeded decellularized organ was not implemented. The work was limited to the design, construction and assessment of the hydraulic seal of the structure build. The hydraulic seal was tested as described in Material & Methods, connecting together the bioreactor, a MilliQ water container, a peristaltic pump and a transfer bag. The test was executed at room temperature and the water flow was set at 10 ml/min. The water was kept flowing for 3 days, denoting at first a slight leakage from a precise spot located on the superior edge of the cylindrical PTFE chamber. Such problem was successively solved replacing the groove o-

ring with one of a larger cross section. The test was repeated and this time no leakage was detected.

Chapter 5
Discussion

Injuries and congenital diseases can be particularly challenging to address in a correct functional way. When it comes to gastrointestinal disorders, oesophageal atresia is the most common [67]. Oesophageal atresia is a congenital interruption of the tubular lumen of the organ and is associated with diffused disorders present in various other parts of the body [67]. Surgical repair to reconnect the two ends of the organ is an immediate an effective treatment but is limited to short length gaps and however it is not always possible [69]. To date, gastric pull-up, colon transposition and jejunum transposition constitute the most common approach in treating long-gap oesophageal atresia [71], with gastric pull-up being preferred [72]. These solutions are all afflicted by common complications, that include leaking in the site of closure of the oesophagus, strictures and tight spots, leading to later life complications in operated children. Besides, dysmotility also occurs in 75-100% of patients. These complications have brought the attention of scientists on tissue engineering field, attracted by the possibility of reconstruction and, in most applications, the absence of need of life-long immunosuppression.

Tissue engineering approaches are mainly based on the adoption of acellular or cellular scaffolds, both with the aim of hosting cells deputed to grow forming a functional tissue. Acellular scaffolds are implanted in the body and consent a migration of cells from surrounding tissue, populating the structure. In the context of oesophageal tissue engineering, synthetic scaffold could be appropriately designed to mimic the multi-layered structure of the oesophagus and preloaded with growth factors capable to both allow cell proliferation and differentiation. Such manufacturing could avoid the procedure of cell seeding prior to transplantation, making it a cheaper and safer alternative to decellularized scaffolds [123]. However, in various experiments they leaded to poor results in animal model as they did not show to allow consistent cellular migration [75-79], and they are still limited to small scale [127]. Collagen scaffolds showed greater higher repopulating cells densities, but its application is limited to short surgical created defects [86]. Decellularized matrices are derived from human and animal organs and tissue and they underwent a process of

decellularization to remove cells and immunogenic material, while retaining the macro and micro architecture of the original tissue, as well as the molecular components of its natural extracellular matrix [91]. A significant heterogeneity of results is present in relation to unseeded decellularized matrices used in oesophageal engineering. However, stenosis, strictures and lack of function with poor or mixed clinical outcome represent the main drawbacks of unseeded decellularized scaffolds orthotopically implanted [100,106,107,153,154], despite reporting positive impacts on oesophageal healing process in canine and porcine models [154, 104] and in humans [101,155].

Cellular scaffolds are seeded with cells prior to transplantation, leading to a pre-formation of a functional tissue *in vitro* using classical 2D cultures or bioreactor-based 3D cultures. Many different experiments were performed, varying the kind of scaffold and choosing scaffold coming from various organisms. Multiple types of cells were selected, alone or in combination between each other. [110-121]. The process of seeding cells prior to implantation led to more viable scaffolds with a diminished inflammatory response and increased growth of epithelial and smooth muscle cells [109,115,156,157]. Other studies made use of stem cells, deriving epithelial cells, muscle cells and adipose cells, combining them with non-tissue specific ECM, obtaining cell adhesion and migration the external and luminal side of decellularized porcine oesophagi. However, the seeded stem cells contribution *in* vivo was unclear [115,153,154,156]. It was shown that the use of bioreactors in dynamic cultures was able to address the main challenges of organ tissue engineering, like the maturation of multiple layers in tubular structures, adequate repopulation and tissue growth in constructs before transplantation and cryopreservation of bioengineered organs [122,123]. Another important aspect to consider is that seeded scaffolds need a well-structured vascularization to maintain viability, and this was so far mainly addressed wrapping the construct in the omentum, but the developed vascular tree obtained lacked the perfect architecture required for a full functional tissue [121].

The main aim of the work exposed in this thesis was to provide a contribution to the oesophageal tissue engineering development, after examining and considering the previous attempts in literature. This was done by implementing a bioreactor that could be highly suitable for the growing of a decellularized piglet oesophagus construct after *in vitro* seeding of cells.

The bioreactor consisted in a PTFE main chamber closed with a lid made in Polycarbonate,

using stainless steel screws. PTFE was chosen due to its suitability for biological applications. This material is inert, chemically resistant, and does not undergo corrosion, so this avoids the risk of harmful byproducts release in culture medium that could be toxic to cells. PTFE can be sterilized multiple times by ethanol, autoclave and UV radiation. The chamber was autoclaved in different times because of its use in hydraulic sealing and sterility tests, and in the dynamic culture of the seeded oesophagus. A considerable problem in autoclaving is the eventual dilation of the material thus affecting its mechanical properties and performance. After all the times the PTFE chamber was autoclaved, particular attention was put on if the overall sealing with chamber full of liquid and all the fittings and tubes plugged was maintained as the previous times. No change was observed after all the trials, thus proving the good stability of the material chosen. The perfusion system chosen for the presented work consisted in a circuit comprising the bioreactor, a peristaltic pump and a reservoir bottle. The dynamic recirculation of the medium for the nourishment of the organ was chosen in order to stimulate the activity and viability cells acting mechanical shear stress on them. The nature of oesophagus is of an organ deputed the allow the passage of solid or liquid matter, thus enduring shear stresses of various intensity along the walls of its cavity. The peristaltic pump was driving culture medium from the reservoir bottle inside the chamber in correspondence of the organ fixed to the structure, thus perfusing directly and stimulating the luminal part. Medical grades tubes were used for the medium carriage, being permeable to oxygen and autoclavable. A catheter tube had the double function to allow medium flowing and to hold the oesophagus by one sutured end. The tube is thus adjustable, on the base of the organ length, since a longer or shorter portion of it can be pulled inside the chamber. The tube passes indeed through a wall channel which has a shorter diameter than that of the tube, thus preventing leakages and avoiding the need of other intermediate structures such as adapters. The liquid could flow out the structure from the catheter tube back to the reservoir. A second output channel, in lower position with respect to the catheter tube, presented a 3-way stopcock that can easily allow the changing of medium during culture, even consenting to fill the chamber and nourish the external side of the organ with a different medium with respect to the one used for the luminal side.

Regarding the cells, the use of a mix of hMABs and hFBs to seed the scaffold *in vitro* prior to dynamic culturing was performed on the model of the previous work of Urbani and collegues in the host laboratory [123]. The cells were cultured following established

protocols [151]. hMABs were selected in combination with hFBs for construct seeding following the promising results obtained in the previous work in the department, because hMABs and hFBs together were shown to improve cell migration within the scaffold, engraftment and distribution [123].

Decellularized oesophagus was obtained following a previously established protocol [124,125]. The organ was fixed to the bioreactor chamber. The use of detergent-enzymatic treatment (DET) aimed to remove cellular components, preserving the major extracellular matrix components, while avoiding immune reaction. The preservation of ECM components is crucial for the correct settlement of seeded cells and for the overall mechanical behaviour of the tissue. In the context of biomechanics, the preservation of the original native organ structures is essential to ensure performances such as strength, distensibility and stiffness. The decellularized scaffold was seeded with hMABs and hFBs via multiple micro-injections. Using this technique allowed the cells to reach a better distribution and engraftment in all layers, which constitutes a fundamental requirement for the repopulation of the scaffold. In human oesophagus, most of the muscularis externa is composed of smooth muscle whereas striated muscle predominates in the upper third portion. Some studies where a seeded decellularized scaffold was involved focused on the use of smooth muscle cells or mesenchymal stem cells [28,95,109,153].

Seeding smooth muscle cells in the scaffold prior to implantation showed less inflammatory response, a higher muscular regeneration and lower risk of strictures *in vivo* when compared to unseeded scaffolds [109]. The main problem of using smooth muscle cells is that they are difficult to expand while mesoangioblasts isolated from skeletal muscle can easily proliferate in culture and are able to undergo both spontaneous skeletal and TGF-beta-induced smooth muscle differentiation [158-160]. MABs have been successfully used in combination with synthetic scaffolds to promote regeneration of vascular grafts and skeletal muscle [161,162] and they have already been transplanted in patients [163]. However, hMABs were not seeded alone, but coupled with human fibroblasts since studies were conducted showing that fibroblasts promote local stem cell recruitment and secrete ECM proteins, such as collagen VI and fibronectin, and trophic factors during tissue regeneration [98,164,165]. Fibroblasts were shown to help the distribution of hMABs in the scaffold, but without increasing the engraftment, suggesting that their effect could be related to ECM remodelling and secretion of pro-migratory cytokines rather than proliferative ones [165,126].

Dynamic culture is able to provide intraluminal pressure and improved diffusion of media and growth factors maximising cell growth and differentiation. Ghionzoli and collegues showed that, in static culture hMABs were able to differentiate in smooth muscle cells only on the external surface of the scaffolds, with minimal change in their metabolic profile compared to dynamic conditions where cells showed more oxidative metabolism [129]. The dynamic culture was carried out for 11 days after which tissue sections were cut and analysed with H&E, MT and immunofluorescence. The tissue showed to have been repopulated of cells, although not distributed equally all over the section (Savvidis et al., in preaparation). Immunofluorescence analysis also revealed the presence of Calponin and SM22, typical biomarkers of smooth muscle cells, confirming that a smooth muscle cells differentiation during dynamic culture did occur.

For this thesis, another kind of bioreactor was realized, considering its possible application for parenchymatous organs,such as liver and kidney. Alternatives to organ transplant like bioartificial devices such as BAL and BAK have been used to address end-stage diseased like ESLD and ESKD and acute organ failures (ALF and AKF). However, criticalities related to the devices (such as difficulty of maintaining cells viability) demonstrated that no BAL system improved the survival in patients with ALF and there was no evidence of a more favourable risk/benefit ration compared to liver transplant [132]. Similarly, BAK showed important limitations in terms of morbidity, mortality and cost [136]. Tissue engineering solutions comprised the use of synthetic scaffolds where however it was not possible to accurately represent the vascular and structural native organ architecture [139]. In decellularized livers the challenge is similar, although not to recreate the structure but to maintain the original one [139]. The use of cells for tissue repopulation such as mature hepatocytes and cancer-derived cell lines showed limitations in cells availability, expandability and phenotypic and functional instability [140,141], moving the attention more on induced pluripotent stem cells (iPSCs) [142]. The use of 3D organoids has been explored, in order to obtain a rapid cell growth useful for clinical needs, and they were shown to allow long-term high-density culture conditions to support maturation and longevity of hepatocytes-like cells [131,143]. On kidney tissue engineering, cultures of cells derived from the undifferentiated metanephric mesenchyme (MM) and from unbranched ureteral bud (UB) were attempted, in order to assess the possible *in vitro* reconstitution of tissues. In these cultures, an origination of branched structures and polarized tubules occurred, although the

structures functional capacity was not tested [145]. A novel proposal suggested the use of the ureteral bud as a scaffold to act as conductor of inductive signals present in normal renal growth [149]. The use of a bioreactor was witnessed in a study made to obtain kidney organoids from iPSCs differentiation [150].

Like the bioreactor build for the oesophagus, also the one for parenchymatous organs was manufactured using a CNC machine. The bioreactor comprises a main cylindrical chamber build in PTFE and a lid in polycarbonate, and the two pieces are sealed together using stainless steel bolts. On the external surface area of the Teflon cylinder, two holes were drilled, to function as inlet and outlet channel. At the inlet internal surface, a medical grade tube portion supports a luer fitting where the organ can be cannulated and perfused. So far, the bioreactor was only tested for hydraulic sealing, giving positive results. There was not however the occasion to evaluate its response to the possibility of contamination through a sterility trial, nor to use it in a proper culture, where it could conceivably be involved in a dynamic culture with the use of a syringe or peristaltic pump. It could be used both for decellularization and for recellularization. For instance, in a recellularization process of decellularized liver, it could be possible to use cells derived from iPSCs called hepatocyte-like cells (HLCs), injecting them directly in the parenchyma of the organ. Through a liver portal vein cannula endothelial cells could be infused, to promote re-endothelialisation of blood vasculature.

Regarding future suggestions for the implementation of the oesophagus bioreactor, the perfusion system could be made more complex adding devices such as a bubble trap, to avoid the formation of air bubbles as they can hinder cell attachment, or a gas control, such as an oxygenator to increase the O_2 delivered to cells, necessary for their viability. Moreover, a modification in the flow trend used to perfuse the organ may be tried. In a study of Sibilio et al. conducted in 2019 [169], the effect of a peristaltic-like movement in a bioengineered intestinal tube was examined. In this study they made use of a bioreactor containing a 3D tubular stroma seeded with epithelial cells, and the bioreactor was connected to a peristaltic pump. In order to reproduce the peristaltic-like motion wave, they set the peristaltic pump with an interval pause of 0.5 seconds every 2 seconds of normal dispensing of medium, obtaining a square wave. Reproducing such a perfusion strategy, it could be somehow possible to mimic the movement that the food bolus conducts when travelling along the GI structures, thus improving the seeded and expanded cells functionality. It would also be

interesting to carry out the culture of the seeded scaffold for a longer time than 11 days, addressing the need for vascularization of the tissue, like in the work of Urbani et al. [123], where they transplanted a recellularized rat oesophageal scaffold into the rat omentum to induce growth of vessels. Still on the model of Urbani et al. study [123], as well as hMABs and hFBs other types of cells could be added for the use, like neural crest cells (NCCs), with the aim of induce innervation, and endothelial cells, apt to coat the luminal surface of the oesophagus.

The bioreactor kept the oesophagus to straight, holding both the ends of the organ to the chamber, with the advantage of the possibility to adjust the fixation on the base of the organ length. The use of the so developed bioreactor could be ultimately extended to all luminal organs (i.e. intesting, stomach, trachea), which could luminally perfused and accommodated in the chamber keeping them in a straight and extended fashion, making it possible for infused cells to better migrate, distribute and engraft.

Additionally, future tests will be conducted to assess the functionality of the side project bioreactor designed for parenchymatous organs. First test could be performed on a decellularized liver, and assess the stability, suitability and perfusion capacity of the bioreactor, prior to further extended uses on other parenchymatous organs.

References

[1] Mao AS, Mooney DJ. Regenerative medicine: Current therapies and future directions. *Proc Natl Acad Sci U S A.* 2015;112(47):14452-14459

[2] Heidary Rouchi A, Mahdavi-Mazdeh M. Regenerative Medicine in Organ and Tissue Transplantation: Shortly and Practically Achievable?. *Int J Organ Transplant Med*. 2015; 6(3):93-98.

[3] Bajaj P, Schweller RM, Khademhosseini A, West JL, Bashir R. 3D biofabrication strategies for tissue engineering and regenerative medicine. *Annu Rev Biomed Eng*. 2014;16:247-276

[4] Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*. 2006;22:287-309.

[5] Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization strategies for tissue engineering. *Tissue Eng Part B Rev*. 2009;15(3):353-370.

[6] Forbes SJ, Rosenthal N. Preparing the ground for tissue regeneration: from mechanism to therapy. *Nat Med*. 2014;20(8):857-869.

[7] https://www.nibib.nih.gov/science-education/science-topics/tissue-engineering-and-regenerative-medicine

[8] Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet*. 2003;361(9351):47-49

[9] B. S. Conklin, E. R. Richter, K. L. Kreutziger, D.-S. Zhong, and C. Chen, "Development and evaluation of a novel decellularized vascular xenograft," *Medical Engineering and Physics*, vol. 24, no. 3, pp. 173–183, 2002.

[10] J. Petersen, P Ueblacker, C Goepfert, P Adamietz, K Baumbach, A Stork, J. Rueger, R Poertner, M Amling, and N. Meenen "Long term results after implantation

of tissue engineered cartilage for the treatment of osteochondral lesions in a minipig model", *Journal of Materials Science: Materials in Medicine*, vol. 19, no. 5, pp. 2029-2038, 2008.

[11] Griffith LG, Naughton G. Tissue engineering--current challenges and expanding opportunities. *Science*. 2002;295(5557):1009-1014

[12] Suuronen EJ, McLaughlin CR, Stys PK, Nakamura M, Munger R, Griffith M. Functional innervation in tissue engineered models for in vitro study and testing purposes. *Toxicol Sci*. 2004;82(2):525-533

[13] McAteer H, Cosh E, Freeman G, Pandit A, Wood P, Lilford R. Cost-effectiveness analysis at the development phase of a potential health technology: examples based on tissue engineering of bladder and urethra. *J Tissue Eng Regen Med*. 2007;1(5):343-349

[14] G. Khang, S. J. Lee, M. S. Kim, and H. B. Lee "Biomaterials: Tissue engineering and scaffolds", *Encyclopedia of Medical devices and instrumentation*, 2006.

[15] A. Smith "A glossary for stem-cell biology", *Nature*, vol. 441, no. 7097, p. 1060, 2006.

[16] Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861-872.

[17] J. Yu, M. A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J. L. Frane, S. Tian, J. Nie, G. A. Jonsdottir, V. Ruotti, R. Stewart, et al. "Induced pluripotent stem cell lines derived from human somatic cells", *Science*, vol. 318, no. 5858, pp. 1917-1920, 2007.

[18] Raimondi M.T, Slide of the lecture "Dispense_Raimondi_TRM_2017_2018_Cap_da_01_a_05", Milano, Politecnico di Milano, 2017-2018

[19] P. Weiss, "In vitro experiments on the factors determining the course of the outgrowing nerve fiber", *Journal of Experimental Zoology*, vol. 68, no. 3, pp. 393 - 448, 1934.

[20] S. Farè, Slides of the lecture: "Biomateriali", Milano, Politecnico di Milano, 2015-2016.

[21] Ho ST, Hutmacher DW. A comparison of micro CT with other techniques used in the characterization of scaffolds. *Biomaterials*. 2006;27(8):1362-1376.

[22] S. Farè, Slides of the lecture "Strutture bioartificiali e biomimetiche", Milano, Politecnico di Milano, 2016-2017.

[23] Zinger O, Anselme K, Denzer A, et al. Time-dependent morphology and adhesion of osteoblastic cells on titanium model surfaces featuring scale-resolved topography. *Biomaterials*. 2004;25(14):2695-2711

[24]Lüthen F, Lange R, Becker P, Rychly J, Beck U, Nebe JG. The influence of surface roughness of titanium on beta1- and beta3-integrin adhesion and the organization of fibronectin in human osteoblastic cells. *Biomaterials*. 2005;26(15):2423-2440.

[25] A. Signoriello, "Superfici osteoconduttive in titanio nell'implantologia orale", Tesi di laurea triennale in Ingegneria Biomedica, Università degli Studi di Padova, 2010-2011.

[26] Sachlos E, Czernuszka JT. Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *Eur Cell Mater*. 2003;5:29-40. Published 2003 Jun 30

[27] Gilpin, A., & Yang, Y. (2017). Decellularization Strategies for Regenerative Medicine: From Processing Techniques to Applications. BioMed research international, 2017, 9831534.

[28] Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. *Biomaterials*. 2006;27(19):3675-3683

[29] Maghsoudlou P, Georgiades F, Smith H, et al. Optimization of Liver Decellularization Maintains Extracellular Matrix Micro-Architecture and Composition Predisposing to Effective Cell Seeding. *PLoS One*. 2016;11(5):e0155324. Published 2016 May 9

[30] Tapias LF, Ott HC. Decellularized scaffolds as a platform for bioengineered organs. *Curr Opin Organ Transplant*. 2014;19(2):145-152.

[31] Park KM, Shin YM, Kim K, Shin H. Tissue Engineering and Regenerative Medicine 2017: A Year in Review. *Tissue Eng Part B Rev*. 2018;24(5):327-344.

[32] Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. *Trends Biotechnol*. 2004;22(2):80-86.

[33] Janssen FW, Oostra J, Oorschot Av, van Blitterswijk CA. A perfusion bioreactor system capable of producing clinically relevant volumes of tissue-engineered bone: in vivo bone formation showing proof of concept. *Biomaterials*. 2006;27(3):315-323.

[34] Langer R, Vacanti JP. Tissue engineering. *Science*. 1993;260(5110):920-926.

[35] Verbeek R. Generation of mesenchymal stem cells as a medicinal product in organ transplantation. *Curr Opin Organ Transplant*. 2013;18(1):65-70.

[36] Vaes B, Van't Hof W, Deans R, Pinxteren J. Application of MultiStem(®) Allogeneic Cells for Immunomodulatory Therapy: Clinical Progress and Pre-Clinical Challenges in Prophylaxis for Graft Versus Host Disease. *Front Immunol*. 2012;3:345. Published 2012 Nov 27.

[37] B. Hampson, J. Rowley, and N. Venturi, "Manufacturing patient-speci_c cell therapy products", *BioProcess Int*, vol. 6, no. 8, pp. 60-72, 2008.

[38] Martin I, Smith T, Wendt D. Bioreactor-based roadmap for the translation of tissue engineering strategies into clinical products. *Trends Biotechnol*. 2009;27(9):495-502.
[39] Haycock JW. 3D cell culture: a review of current approaches and techniques. *Methods Mol Biol*. 2011;695:1-15.

[40] Dvir T, Benishti N, Shachar M, Cohen S. A novel perfusion bioreactor providing a homogenous milieu for tissue regeneration. *Tissue Eng*. 2006;12(10):2843-2852.

[41] Fassnacht D, Pörtner R. Experimental and theoretical considerations on oxygen supply for animal cell growth in fixed-bed reactors. *J Biotechnol*. 1999;72(3):169-184

[42] Bancroft GN, Sikavitsas VI, van den Dolder J, et al. Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. *Proc Natl Acad Sci U S A*. 2002;99(20):12600-12605.

[43] Radisic M, Yang L, Boublik J, et al. Medium perfusion enables engineering of compact and contractile cardiac tissue. *Am J Physiol Heart Circ Physiol*. 2004;286(2):H507-H516.

[44] Thompson CA, Colon-Hernandez P, Pomerantseva I, et al. A novel pulsatile, laminar flow bioreactor for the development of tissue-engineered vascular structures. *Tissue Eng*. 2002;8(6):1083-1088.

[45] Bijonowski BM, Miller WM, Wertheim JA. Bioreactor design for perfusion-based, highly-vascularized organ regeneration. *Curr Opin Chem Eng*. 2013;2(1):32-40.

[46] C. Weinand, J. W. Xu, G. M. Peretti, L. J. Bonassar, and T. J. Gill, "Conditions a_ecting cell seeding onto three-dimensional sca_olds for cellular-based biodegradable implants", *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 91, no. 1, pp. 80-87, 2009.

[47] H. C. Ott, B. Clippinger, C. Conrad, C. Schuetz, I. Pomerantseva, L. Ikonomou, D. Kotton, and J. P. Vacanti, "Regeneration and orthotopic transplantation of a bioarti_cial lung", *Nat Med*, vol. 16, no. 8, p. 927, 2010.

[48] Ott HC, Matthiesen TS, Goh SK, et al. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008;14(2):213-221.

[49] Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. *Hepatology*. 2011;53(2):604-617.

[50] Soto-Gutierrez A, Zhang L, Medberry C, et al. A whole-organ regenerative medicine approach for liver replacement. *Tissue Eng Part C Methods*. 2011;17(6):677-686

[51] Uygun BE, Soto-Gutierrez A, Yagi H, et al. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med*. 2010;16(7):814-820. doi:10.1038/nm.2170

[52] Moran EC, Baptista PM, Evans DW, Soker S, Sparks JL. Evaluation of parenchymal fluid pressure in native and decellularized liver tissue. *Biomed Sci Instrum*. 2012;48:303-309.

[53] Xia Y, Prawirasatya M, Heng BC, Boey F, Venkatraman SS. Seeding density matters: extensive intercellular contact masks the surface dependence of endothelial cell-biomaterial interactions. *J Mater Sci Mater Med*. 2011;22(2):389-396.

[54] R. I. Issa, B. Engebretson, L. Rustom, P. S. McFetridge, and V. I. Sikavitsas, "The effect of cell seeding density on the cellular and mechanical properties of a mechanostimulated tissue-engineered tendon", *Tissue Engineering* Part A, vol. 17, no. 11-12, pp. 1479{1487, 2011.

[55] M. E. Scarritt, N. C. Pashos, and B. A. Bunnell, "A review of cellularization strategies for tissue engineering of whole organs", Frontiers in bioengineering and biotechnology, vol. 3, p. 43, 2015.

[56] Conconi MT, De Coppi P, Bellini S, et al. Homologous muscle acellular matrix seeded with autologous myoblasts as a tissue-engineering approach to abdominal wall-defect repair. *Biomaterials*. 2005;26(15):2567-2574.

[57] Cebotari S, Lichtenberg A, Tudorache I, et al. Clinical application of tissue engineered human heart valves using autologous progenitor cells. *Circulation*. 2006;114(1 Suppl):I132-I137.

[58] P.P. Pairolero, V.F. Trastek, W.S. Payne "Esophagus and diaphragmatic hernias" Schwartz S.I., Shires G.T., Spencer F.C. (Eds.), Principles of Surgery, McGraw-Hill, New York (1989)

[59] G.G. Jamieson, C.J. Martin "Antireflux surgery: The anatomy of the distal esophagus and associated structures", Jamieson G.G. (Ed.), Anatomy of General Surgical Operations, Churchill Livingstone, New York (1992)

[60] C.A. Pellegrini, L.W. Way Esophagus and diaphragm Way L.W. (Ed.), “Current Surgical Diagnosis and Treatment”, Appleton & Lange, Stamford, CT (1994)

[61] K.P. Pearl “Anatomy of the esophagus and the posterior mediastinum” Nyhus L.M., Baker R.J. (Eds.), Mastery of Surgery, Little, Brown & Company, Boston (1992)

[62] D. Lieberman-Meffert, M. Allgower, P. Schmidt, et al. “Muscular equivalent of the lower esophageal sphincter” *Gastroenterology*, 76 (1979), p. 31

[63] G.G. Jamieson, A.C. Duranceau “Oesophagectomy; oesophagomyotomy; oesophageal bypass: The anatomy of the thoracic oesophagus and mediastinum”,Jamieson G.G. (Ed.), Anatomy of General Surgical Operations, Churchill Livingstone, New York (1992)

[64] Cuschieri A. Endoscopic vagotomy for duodenal ulcer disease: procedures and appraisal. *Adv Surg*. 1996;29:291-302.

[65] C.A. Pellegrini, L.W. Way “Esophagus and diaphragm” Way L.W. (Ed.), Current Surgical Diagnosis and Treatment, Appleton & Lange, Stamford, CT (1994)

[66] Spitz L. Oesophageal atresia. *Orphanet J Rare Dis*. 2007;2:24. Published 2007 May 11.

[67] https://www.msdmanuals.com/en-gb/professional/pediatrics/congenital-gastrointestinal-anomalies/esophageal-atresia

[68] Clatworthy H.W. (1986) Robert E. Gross. In: Rickham P.P. (eds) Historical Aspects of Pediatric Surgery. Progress in Pediatric Surgery, vol 20. Springer, Berlin, Heidelberg

[69] Kunisaki, S. M.; Foker, J. E. (2012). "Surgical Advances in the Fetus and Neonate". *Clinics in Perinatology*. 39 (2): 349–361.

[70] Esophageal atresia - symptoms, tests, Foker treatment". Children’s Hospital Boston. Retrieved 1 June 2012.

[71] "Esophageal Atresia Treatment Program". Children's Hospital Boston. Retrieved 1 June 2012.

[72] Hirschl RB, Yardeni D, Oldham K, et al. Gastric transposition for esophageal replacement in children: experience with 41 consecutive cases with special emphasis on esophageal atresia. *Ann Surg*. 2002;236(4):531-541.

[73] Zani A, Pierro A, Elvassore N, De Coppi P. Tissue engineering: an option for esophageal replacement?. *Semin Pediatr Surg*. 2009;18(1):57-62.

[74] Totonelli, G., Maghsoudlou, P., Fishman, J. M., Orlando, G., Ansari, T., Sibbons, P., Birchall, M. A., Pierro, A., Eaton, S., & De Coppi, P. (2012). Esophageal tissue engineering: A new approach for esophageal replacement. *World Journal of Gastroenterology*, *18*(47), 6900-6907

[75] 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: 84-91

[76] Berman EF. The experimental replacement of portions of the esophagus by a plastic tube. *Ann Surg* 1952; 135: 337-343

[77] 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: 812-824

[78] 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.

[79] Watanabe K, Mark JB. Segmental replacement of the thoracic esophagus with a Silastic prosthesis. *Am J Surg* 1971; 121: 238-240

[80] 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: 104-111

[81] Shinhar D, Finaly R, Niska A, Mares AJ. The use of collagencoated vicryl mesh for reconstruction of the canine cervical esophagus. *Pediatr Surg Int* 1998; 13: 84-87

[82] Jungebluth P, Alici E, Baiguera S, …. & Macchiarini P. Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. *Lancet* 2011; 378: 1997-2004

[83] Natsume T, Ike O, Okada T, Takimoto N, Shimizu Y, Ikada Y. Porous collagen sponge for esophageal replacement. *J Biomed Mater Res* 1993; 27: 867-875

[84] 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: M736-M739

[85] Takimoto Y, Nakamura T, Teramachi M, Kiyotani T, Shimizu Y. Replacement of long segments of the esophagus with a collagen-silicone composite tube. *ASAIO J* 1995; 41: M605-M608

[86] Takimoto Y, Nakamura T, Yamamoto Y, Kiyotani T, Teramachi M, Shimizu Y. The experimental replacement of a cervical esophageal segment with an artificial prosthesis with the use of collagen matrix and a silicone stent. *J Thorac Cardiovasc Surg* 1998; 116: 98-106

[87] Yamamoto Y, Nakamura T, Shimizu Y, Matsumoto K, Takimoto Y, Kiyotani T, Sekine T, Ueda H, Liu Y, Tamura N. Intrathoracic esophageal replacement in the dog with the use of an artificial esophagus composed of a collagen sponge with a double-layered silicone tube. *J Thorac Cardiovasc Surg* 1999; 118: 276-286

[88] 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.

[89] Teramachi M, Okumura N, Nakamura T, Yamamoto Y, Kiyotani T, Takimoto Y, Matsuda S, Ikada Y, Shimizu Y. Intrathoracic tracheal reconstruction with a collagen-conjugated prosthesis: evaluation of the efficacy of omental wrapping. *J Thorac Cardiovasc Surg 1997*; 113: 701-711

[90] Elliott MJ, De Coppi P, Speggiorin S, Roebuck D, Butler CR, Samuel E, Crowley C, McLaren C, Fierens A, Vondrys D, Cochrane L, Jephson C, Janes S, Beaumont NJ, Cogan T, Bader A, Seifalian AM, Hsuan JJ, Lowdell MW, Birchall MA. Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study. *Lancet* 2012; 380: 994-1000

[91] Badylak SF, Weiss DJ, Caplan A, Macchiarini P. Engineered whole organs and complex tissues. *Lancet* 2012; 379: 943-952

[192] Baiguera S, Jungebluth P, Burns A, Mavilia C, Haag J, De Coppi P, Macchiarini P. Tissue engineered human tracheas for in vivo implantation. *Biomaterials* 2010; 31: 8931-8938

[93] Fishman JM, Ansari T, Sibbons P, De Coppi P, Birchall MA. Decellularized rabbit cricoarytenoid dorsalis muscle for laryngeal regeneration. *Ann Otol Rhinol Laryngol* 2012; 121: 129-138

[94] Totonelli G, Maghsoudlou P, Garriboli M, Riegler J, Orlando G, Burns AJ, Sebire NJ, Smith VV, Fishman JM, Ghionzoli M, Turmaine M, Birchall MA, Atala A, Soker S, Lythgoe MF, Seifalian A, Pierro A, Eaton S, De Coppi P. A rat decellularized small bowel scaffold that preserves villus-crypt architecture for intestinal regeneration. *Biomaterials* 2012; 33: 3401-3410

[95] Tan JY, Chua CK, Leong KF, Chian KS, Leong WS, Tan LP. Esophageal tissue engineering: an in-depth review on scaffold design. *Biotechnol Bioeng* 2012; 109: 1-15

[96] 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: 84-91

[97] Mertsching H, Schanz J, Steger V, Schandar M, Schenk M, Hansmann J, Dally I, Friedel G, Walles T. Generation and transplantation of an autologous vascularized bioartificial human tissue. *Transplantation* 2009; 88: 203-210

[98] Urciuolo A, Quarta M, Morbidoni V, et al. Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat Commun.* 2013;4:1964

[99] Kajitani M, Wadia Y, Hinds MT, Teach J, Swartz KR, Gregory KW. Successful repair of esophageal injury using an elastin based biomaterial patch. *ASAIO J* 2001; 47: 342-345

[100] Badylak S, Meurling S, Chen M, Spievack A, Simmons-Byrd A. Resorbable bioscaffold for esophageal repair in a dog model. *J Pediatr Surg* 2000; 35: 1097-1103

[101] Badylak SF, Hoppo T, Nieponice A, Gilbert TW, Davison JM, Jobe BA. Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold. *Tissue Eng Part A*. 2011;17(11-12):1643-1650.

[102] Clough A, Ball J, Smith GS, Leibman S. Porcine small intestine submucosa matrix (Surgisis) for esophageal perforation. *Ann Thorac Surg* 2011; 91: e15-e16

[103] Freud E, Greif M, Rozner M, Finaly R, Efrati I, Kidron D, Odes M, Mares AJ. Bridging of esophageal defects with lyophilized dura mater: an experimental study. *J Pediatr Surg* 1993; 28: 986-989

[104] Nieponice A, Gilbert TW, Badylak SF. Reinforcement of esophageal anastomoses with an extracellular matrix scaffold in a canine model. *Ann Thorac Surg* 2006; 82: 2050-2058

[105] Urita Y, Komuro H, Chen G, Shinya M, Kaneko S, Kaneko M, Ushida T. Regeneration of the esophagus using gastric acellular matrix: an experimental study in a rat model. *Pediatr Surg Int* 2007; 23: 21-26

[106] Lopes MF, Cabrita A, Ilharco J, Pessa P, Paiva-Carvalho J, Pires A, Patrício J. Esophageal replacement in rat using porcine intestinal submucosa as a patch or a tube-shaped graft. *Dis Esophagus* 2006; 19: 254-259

[107] Doede T, Bondartschuk M, Joerck C, Schulze E, Goernig M. Unsuccessful alloplastic esophageal replacement with porcine small intestinal submucosa. *Artif Organs* 2009; 33: 328-333

[108] Lopes MF, Cabrita A, Ilharco J, Pessa P, Patrício J. Grafts of porcine intestinal submucosa for repair of cervical and abdominal esophageal defects in the rat. *J Invest Surg* 2006; 19: 105-111

[109] Badylak SF, Vorp DA, Spievack AR, Simmons-Byrd A, Hanke J, Freytes DO, Thapa A, Gilbert TW, Nieponice A. Esophageal reconstruction with ECM and muscle tissue in a dog model. *J Surg Res* 2005; 128: 87-97

[110] Green N, Huang Q, Khan L, Battaglia G, Corfe B, MacNeil S, Bury JP. The development and characterization of an organotypic tissue-engineered human esophageal mucosal model. *Tissue Eng Part A* 2010; 16: 1053-1064

[111] Saxena AK, Ainoedhofer H, Höllwarth ME. Esophagus tissue engineering: in vitro generation of esophageal epithelial cell sheets and viability on scaffold. *J Pediatr Surg* 2009; 44: 896-901

[112] Saxena AK, Ainoedhofer H, Höllwarth ME. Culture of ovine esophageal epithelial cells and in vitro esophagus tissue engineering. *Tissue Eng Part C Methods* 2010; 16: 109-114

[113] Kofler K, Ainoedhofer H, Höllwarth ME, Saxena AK. Fluorescence-activated cell sorting of PCK-26 antigen-positive cells enables selection of ovine esophageal epithelial cells with improved viability on scaffolds for esophagus tissue engineering. *Pediatr Surg Int* 2010; 26: 97-104

[114] Ohki T, Yamato M, Murakami D, Takagi R, Yang J, Namiki H, Okano T, Takasaki K. Treatment of oesophageal ulcerations using endoscopic transplantation of tissue-

engineered autologous oral mucosal epithelial cell sheets in a canine model. *Gut* 2006; 55: 1704-1710

[115] Wei RQ, Tan B, Tan MY, Luo JC, Deng L, Chen XH, Li XQ, Zuo X, Zhi W, Yang P, Xie HQ, Yang ZM. Grafts of porcine small intestinal submucosa with cultured autologous oral mucosal epithelial cells for esophageal repair in a canine model. *Exp Biol Med* (Maywood) 2009; 234: 453-461

[116] Grikscheit T, Ochoa ER, Srinivasan A, Gaissert H, Vacanti JP. Tissue-engineered esophagus: experimental substitution by onlay patch or interposition. *J Thorac Cardiovasc Surg 2003*; 126: 537-544

[117] Sato M, Ando N, Ozawa S, Miki H, Kitajima M. An artificial esophagus consisting of cultured human esophageal epithelial cells, polyglycolic acid mesh, and collagen. *ASAIO J* 1994; 40: M389-M392

[118] Sato M, Ando N, Ozawa S, Nagashima A, Kitajima M. A hybrid artificial esophagus using cultured human esophageal epithelial cells. *ASAIO J* 1993; 39: M554-M557

[119] Miki H, Ando N, Ozawa S, Sato M, Hayashi K, Kitajima M. An artificial esophagus constructed of cultured human esophageal epithelial cells, fibroblasts, polyglycolic acid mesh, and collagen. *ASAIO J* 1999; 45: 502-508

[120] Hayashi K, Ando N, Ozawa S, Kitagawa Y, Miki H, Sato M, Kitajima M. A neo-esophagus reconstructed by cultured human esophageal epithelial cells, smooth muscle cells, fibroblasts, and collagen. *ASAIO J* 2004; 50: 261-266

[121] Nakase Y, Nakamura T, Kin S, Nakashima S, Yoshikawa T, Kuriu Y, Sakakura C, Yamagishi H, Hamuro J, Ikada Y, Otsuji E, Hagiwara A. Intrathoracic esophageal replacement by in situ tissue-engineered esophagus. *J Thorac Cardiovasc Surg* 2008; 136: 850-859

[122] Ritchie AC, Wijaya S, Ong WF, Zhong SP, Chian KS. Dependence of alignment direction on magnitude of strain in esophageal smooth muscle cells. *Biotechnol Bioeng* 2009; 102: 1703-1711

[123] Urbani L, Camilli C, Phylactopoulos DE, et al. Multi-stage bioengineering of a layered oesophagus with in vitro expanded muscle and epithelial adult progenitors. *Nat Commun*. 2018;9(1):4286. Published 2018 Oct 16.

[124] Maghsoudlou P, Georgiades F, Smith H, et al. Optimization of liver decellularization maintains extracellular matrix micro-architecture and composition predisposing to effective Cell Seeding. *PLoS One*. 2016;11(5):e0155324. Published 2016 May 9.

[125] 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.

[126] Jank BJ, Xiong L, Moser PT, et al. Engineered composite tissue as a bioartificial limb graft. *Biomaterials*. 2015;61:246-256.

[127]. Maghsoudlou, P. et al. Preservation of micro-architecture and angiogenic potential in a pulmonary acellular matrix obtained using intermittent intratracheal flow of detergent enzymatic treatment. *Biomaterials* 34, 6638–6648 (2013)

[128] https://histology.leeds.ac.uk/what-is-histology/H_and_E.php

[129] Ghionzoli M, Repele A, Sartiani L, et al. Human amniotic fluid stem cell differentiation along smooth muscle lineage. *FASEB J.* 2013;27(12):badyab4853-4865.

[130] Feeback D.L. (1987) Organs and Systems. In: Histology. Oklahoma Notes. Springer, New York, NY

[131] M. C.T. Nicolas, R. D. Hickey, H. S. Chen, S. A. Mao and and S. L. N. L. Higuita, Y. Wang, “Liver Regenerative Medicine: From Hepatocyte Transplantation to Bioartificial Livers and Bioengineered Grafts,” vol. 25, no. 5, pp. 1032–1057, 2017.

[132] W. Bernal, W. M. Lee, J. Wendon, F. S. Larsen, and R. Williams, “Acute liver failure: A curable disease by 2024?,” *J. Hepatol.*, vol. 62, no. S1, pp. S112–S120, 2015.

[133] Song, J. H., & Humes, H. D. (2009). The bioartificial kidney in the treatment of acute kidney injury. *Current drug targets*, *10*(12), 1227–1234.

[134] Humes HD, MacKay SM, Funke AJ, Buffington DA. Tissue engineering of a bioartificial renal tubule assist device: in vitro transport and metabolic characteristics. *Kidney Int*. 1999;55(6):2502-2514.

[135] Fissell WH, Lou L, Abrishami S, Buffington DA, Humes HD. Bioartificial kidney ameliorates gram-negative bacteria-induced septic shock in uremic animals. *J Am Soc Nephrol*. 2003;14(2):454-461.

[136] US Renal Data System. USRDS 2002 Annual Data Report: Atlas of End-Stage Renal Disease in the United States. Bethesda, MD: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, 2002.

[137] Kottamasu P, Herman I. Engineering a microcirculation for perfusion control of ex vivo-assembled organ systems: challenges and opportunities. *J Tissue Eng*. 2018;9:2041731418772949.

[138] Croce, S., Peloso, A., Zoro, T., Avanzini, M. A., & Cobianchi, L. (2019). A Hepatic Scaffold from Decellularized Liver Tissue: Food for Thought. Biomolecules, 9(12), 813.

[139] S. Holgersson, "Assessing Rat Liver-Derived Biomatrix for Hepatic Tissue Engineering with Human Fetal Liver Stem Cells," *Adv. Tissue Eng. Regen. Med. Open Access*, vol. 1, no. 3, 2016

[140] Navarro-Tableros, V., Herrera Sanchez, M. B., Figliolini, F., Romagnoli, R., Tetta, C., & Camussi, G. (2015). Recellularization of rat liver scaffolds by human liver stem cells. *Tissue engineering. Part A*, *21*(11-12), 1929–1939.

[141] D. Szkolnicka and D. C. Hay, "Concise Review: Advances in Generating Hepatocytes from Pluripotent Stem Cells for Translational Medicine," *Stem Cells*, vol. 34, no. 6, pp. 1421–1426, 2016.

[142] Baxter M, Withey S, Harrison S, et al. Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. *J Hepatol*. 2015;62(3):581-589.

[143] Hu H, Gehart H, Artegiani B, et al. Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. *Cell*. 2018;175(6):1591-1606.e19.

[144] Butter A, Aliyev K, Hillebrandt KH, et al. Evolution of graft morphology and function after recellularization of decellularized rat livers. *J Tissue Eng Regen Med*. 2018;12(2):e807-e816.

[145] Sakurai H, Barros EJ, Tsukamoto T, Barasch J, and Nigam SK. An in vitro tubulogenesis system using cell lines derived from the embryonic kidney shows dependence on multiple soluble growth factors. *Proc Natl Acad Sci USA* 94: 6279-6284, 1997.

[146] Bush KT, Sakurai H, Tsukamoto T, and Nigam S. Acute renal failure: cellular features of injury and repair. In: *Atlas of Diseases of the Kidney*, edited by Berl T, Bonventre JV, and Schrier RW. Oxford, UK: Blackwell Science, 1999.

[147] Steer DL, Bush KT, Meyer TN, Schwesinger C, and Nigam SK. A strategy for in vitro propagation of rat nephrons. *Kidney Int* 62: 1958-1965, 2002.

[148] Dekel B, Burakova T, Arditti FD, Reich-Zeliger S, Milstein O, Aviel-Ronen S, Rechavi G, Friedman N, Kaminski N, Passwell JH, and Reisner Y. Human and porcine early kidney precursors as a new source for transplantation. *Nat Med* 9: 53-60, 2003.

[149] Steer DL, Nigam SK. Developmental approaches to kidney tissue engineering. *Am J Physiol Renal Physiol*. 2004;286(1):F1-F7.

[150] Przepiorski, A., Sander, V., Tran, T., Hollywood, J. A., Sorrenson, B., Shih, J. H., Wolvetang, E. J., McMahon, A. P., Holm, T. M., & Davidson, A. J. (2018). A Simple Bioreactor-Based Method to Generate Kidney Organoids from Pluripotent Stem Cells. *Stem cell reports*, *11*(2), 470–484.

[151] Tonlorenzi R, Dellavalle A, Schnapp E, Cossu G, Sampaolesi M. Isolation and

characterization of mesoangioblasts from mouse, dog, and human tissues. *Curr Protoc Stem Cell Biol*. 2007;Chapter 2

[152] Jensen T, Blanchette A, Vadasz S, et al. Biomimetic and synthetic esophageal tissue engineering. *Biomaterials*. 2015;57:133-141.

[153] Poghosyan T, Sfeir R, Michaud L, et al. Circumferential esophageal replacement using a tube-shaped tissue-engineered substitute: An experimental study in minipigs. *Surgery*. 2015;158(1):266-277.

[154] Luc G, Charles G, Gronnier C, et al. Decellularized and matured esophageal scaffold for circumferential esophagus replacement: Proof of concept in a pig model. *Biomaterials*. 2018;175:1-18.

[155] Nieponice A, Ciotola FF, Nachman F, et al. Patch esophagoplasty: esophageal reconstruction using biologic scaffolds. *Ann Thorac Surg*. 2014;97(1):283-288.

[156] 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.

[157] Tan B, Wei RQ, Tan MY, et al. Tissue engineered esophagus by mesenchymal stem cell seeding for esophageal repair in a canine model. *J Surg Res*. 2013;182(1):40-48.

[158] Minasi MG, Riminucci M, De Angelis L, et al. The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. *Development*. 2002;129(11):2773-2783.

[159] Brunelli S, Tagliafico E, De Angelis FG, et al. Msx2 and necdin combined activities are required for smooth muscle differentiation in mesoangioblast stem cells. *Circ Res*. 2004;94(12):1571-1578.

[160] Tagliafico E, Brunelli S, Bergamaschi A, et al. TGFbeta/BMP activate the smooth muscle/bone differentiation programs in mesoangioblasts. *J Cell Sci*. 2004;117(Pt 19):4377-4388.

[161] Fuoco, C., Sangalli, E., Vono, R., Testa, S., Sacchetti, B., Latronico, M. V., Bernardini, S., Madeddu, P., Cesareni, G., Seliktar, D., Rizzi, R., Bearzi, C., Cannata, S. M., Spinetti, G., & Gargioli, C. (2014). 3D hydrogel environment rejuvenates aged pericytes for skeletal muscle tissue engineering. *Frontiers in physiology*, *5*, 203

[162] He W, Nieponice A, Soletti L, et al. Pericyte-based human tissue engineered vascular grafts. *Biomaterials*. 2010;31(32):8235-8244.

[163] Cossu G, Previtali SC, Napolitano S, et al. Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy [published correction appears in EMBO Mol Med. 2016 Dec 1;8(12):1470-1471]. *EMBO Mol Med*. 2015;7(12):1513-1528.

[164] Bentzinger CF, Wang YX, von Maltzahn J, Soleimani VD, Yin H, Rudnicki MA. Fibronectin regulates Wnt7a signaling and satellite cell expansion. *Cell Stem Cell*. 2013;12(1):75-87.

[165] Costa-Almeida R, Granja PL, Soares R, Guerreiro SG. Cellular strategies to promote vascularisation in tissue engineering applications. *Eur Cell Mater*. 2014;28:51-67. Published 2014 Jul 22.

[166] Urciuolo A, Quarta M, Morbidoni V, et al. Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat Commun.* 2013;4:1964

[167] Jank BJ, Xiong L, Moser PT, et al. Engineered composite tissue as a bioartificial limb graft. *Biomaterials*. 2015;61:246-256.

[168] Ghionzoli M, Repele A, Sartiani L, et al. Human amniotic fluid stem cell differentiation along smooth muscle lineage. *FASEB J.* 2013;27(12):badyab4853-4865.

[169] Sibilio S, De Gregorio V, Urciuolo F, Netti PA, Imparato G. Effect of peristaltic-like movement on bioengineered intestinal tube. *Mater Today Bio*. 2019;4:100027. Published 2019 Sep 19.