FACULTY OF MEDICINE AND HEALTH SCIENCES

Tissue Engineering

The road to the clinic

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Medicine in Medicine

2023 – 2024

Acknowledgments

Writing this thesis has been a significant learning journey for me. This experience not only deepened my understanding of the subject, but also honed my academic writing and critical thinking expertise.

However, it would not have been possible without the help and support of certain individuals. First, I would like to thank professor Dr Phillip Blondeel for providing this topic and making this thesis possible.

I would like to express my deep appreciation to my co-promotor, Dr Arne Peirsman, for his guidance and support during my thesis. His expertise and insights have been invaluable.

Next, I am grateful to my family for their encouragement during this journey. Lastly, I would like to thank my friends for their understanding and moral support during this process. In particular, Ward Devenyn and Willem Nauwynck.

Social outreach:

In today's world, numerous health conditions exist where the current treatments have minimal impact. This is detrimental to people's health all across the world. These include arthrosis, urethral strictures, bladder pathologies, and end-stage renal disease, which are prevalent across all levels of society. As a result, researchers across the globe attempt to develop tissue and organ substitutes to restore tissue and organ functions, to address these healthcare challenges. Subsequently, engineered tissues are made from a combination of cell sources, biomaterials and a variety of techniques. Over the past decades, tissue engineering has gained a significant momentum, with advancements opening up new approaches to replicate tissues and organs. Despite the high technological level in tissue engineering, it is important to make tissue engineering products accessible to the general public.

Contents

Abstract

English abstract:

Tissue engineering is a multidisciplinary field, were researchers attempt to develop functional tissue and organ substitutes. Notably, to maintain, restore or enhance functions of their damaged or diseased counterparts. The current treatments for organ failure due to injury or disease, such as organ transplantation and kidney dialysis, are considered imperfect solutions and a major healthcare challenge. As a result, the field of tissue engineering tries to address these challenges using a combination of different tissue and cell sources, biomaterials and techniques. However, limiting factors, such as a lack of appropriate biomaterials and an inability to generate large vascularized tissues, hinder the progress of tissue engineering technology towards clinical translation. Over the past decades, tissue engineering has gained a significant momentum, with advancements opening up new approaches to replicate the native tissue microenvironment. Although, challenges remain when addressing more complex solid organs such as kidneys.

In this pragmatic review, we will address the current state of tissue engineering and the road towards the clinic. We review four tissue structures (i.e. cartilage, urethra, bladder and kidney tissue), from simple tissues to complex organs to paint a picture of the current trends in tissue engineering. We searched scientific databases such as PubMed, Google Scholar, Embase and the Web of Science, to establish a frame of reference. Next, we are going to use the FDA's drug development process classification to assess the progression of the engineered tissue research.

First, the least complex tissue structure, cartilage. There are already cartilage engineered products freely available on the market for articular cartilage and menisci treatments. Second, the urethra, a tubular organ structure. Urethra engineering research consists mainly of preclinical in vivo trials for the treatment of urethral strictures. Third, the bladder, a hollow nontubular organ, is mainly undergoing preclinical trials in animal models. Fourth, kidney engineering is still in the preliminary stages of research, due to its complexity. However, researchers have found creative ways to tackle this challenge, such as cell therapy, auxiliary kidney organoids and xenotransplantation.

Although, more future research is necessary, tissue engineering remains a promising field of study, with advances offering new opportunities to address the various challenges.

Dutch abstract:

Tissue engineering is een multidisciplinair vakgebied waar onderzoekers functionele weefsel- en orgaansubstituten proberen te ontwikkelen. Met name om de functies van hun beschadigde of zieke tegenhangers te herstellen of te verbeteren. De huidige behandelingen voor orgaan falen door verwonding of ziekte, zoals orgaantransplantatie en nierdialyse, worden beschouwd als onvolmaakte oplossingen en een grote uitdaging voor de gezondheidszorg. Daarom probeert *tissue engineering* deze uitdagingen aan te gaan met een combinatie van verschillende weefsel- en cel bronnen, biomaterialen en technieken. Beperkende factoren, zoals een gebrek aan geschikte biomaterialen en een gebrek aan mogelijkheden om grote gevasculariseerde weefsels te ontwikkelen, belemmeren echter de voortgang van de *tissue engineering* technologie naar klinische toepassing. In de afgelopen decennia heeft *tissue engineering* een significant momentum gekregen, met ontwikkelingen die nieuwe mogelijkheden bieden om het micromilieu van het natieve weefsel na te bootsen. Er blijven echter uitdagingen bestaan bij de fabriceren van complexe organen zoals nieren.

In deze pragmatische review bespreken we de huidige stand van zaken op het gebied van *tissue engineering* en de progressie naar de kliniek. We bespreken vier weefselstructuren (*cartilage, urethra, bladder and kidney*), van eenvoudige weefsels tot complexe organen, om zo een beeld te schetsen van de huidige trends in *tissue engineering* onderzoek. We voeren een pragmatisch literatuuronderzoek uit met behulp van wetenschappelijke databases zoals *PubMed*, *Google Scholar*, *Embase* en *the Web of Science*. Vervolgens gebruiken we de classificatie van *the FDA's drug development process* om de voortgang van *tissue engineering* onderzoek te beoordelen.

Eerst de minst complexe weefselstructuur, kraakbeen. Er zijn al *cartilage engineered* producten vrij verkrijgbaar op de markt voor de behandeling van gewrichtskraakbeen- en menisci letsels. Ten tweede de urethra, een buisvormige orgaanstructuur. Onderzoek naar *urethra engineering* bestaat voornamelijk uit preklinische in vivo trials voor de behandeling van urethrale stricturen. Ten derde is er de blaas, een hol niet-buisvormig orgaan, waar voornamelijk preklinisch onderzoek in diermodellen plaatsvindt. Ten vierde bevindt de engineering van nieren zich nog in een vroeg onderzoek stadium vanwege de hoge complexiteit. Onderzoekers vinden echter creatieve manieren om deze uitdaging aan te gaan, zoals celtherapie, *auxiliary kidney organoids* en xenotransplantatie.

Hoewel er in de toekomst meer onderzoek nodig is, blijft *tissue engineering* een veelbelovend onderzoeksgebied, waarbij de vooruitgang nieuwe mogelijkheden biedt om de verschillende uitdagingen aan te pakken.

Introduction

Context

Tissue engineering (TE) refers to attempting to develop functional tissue and organ substitutes. In order, to maintain, restore or enhance functions of their damaged or diseased in vivo counterparts (1, 2). TE is one component in the field of regenerative medicine. Regenerative medicine leverages the innate potential of the human body to efficiently repair and regenerate damaged tissues using engineered biomaterials (3). As a branch of regenerative medicine, TE is an interdisciplinary field that aims to be a cure, not a treatment. The current treatments for tissue or organ failure due to injury or disease (e.g. organ transplantation and kidney dialysis) are considered imperfected solutions and a major healthcare challenge (4, 5).

The idea of replicating living tissues in vitro is far from being new. A study dates back to 1911, when they try to cultivate cell cultures in vitro. However, with the limited knowledge and understanding at that time, they did not succeed and only observed necrobiosis of the tissues and survival of a few cells (6). In 1954 the first organ, a kidney was transplanted between identical twins by Joseph Murray. Five years later, Murray pioneered once again by performing the world's first successful organ transplant between nonidentical individuals. As a result of Murray's work many people's lives have been saved with organ transplantation since (7). Organ transplantation continues to be a crucial treatment for patients with severe organ function impairment. However, the demand for this treatment far surpasses the available donor organ supply. This disparity is expected to increase as the global population ages.

This leads to the emergence of the concept known as 'tissue engineering', which first was introduced three decades ago. In 1993, Robert Langer and Joseph P. Vacanti (1) introduced the new field: 'tissue engineering' to the world. The article discusses the foundations and challenges of TE and its attempts to provide solutions to tissue creation and repair (1). Over the past 20 years, several achievements in the construction of functional tissues and organs have helped to improve the quality of life for many patients (8). TE has become a promising field with multiple techniques for repairing damaged tissues to overcome the hurdles associated with conventional organ donation (i.e. graft-versus-host disease and long waiting lists) and has become an alternative due to increasing demand for organ transplants (9). In summary, TE has gained significant momentum as a promising field for research, with advancements opening new possibilities in addressing various health challenges. For example, 2022 was a milestone in xenotransplantation research. For the first time, kidneys and a heart from genetically modified pigs were transplanted into human

recipients. Xenotransplantation has the potential to address the most significant unmet need in transplantation by providing an unlimited, renewable source of organs that can save lives (10-12). Additionally, what was learned from the first human xenotransplants and what the future of xenotransplantation may hold, was reflected in a Nature Q&A by Aschheim et al. (13).

Tissue engineering

The process of TE is a multistep process and involves the engineering of different components that will be combined to generate the desired neo-tissue or organ. The following paragraphs will lay out the different components (e.g. tissue and cell sources, and the different techniques) that are involved in TE.

Tissue and cell sources

Grafts

There are four types of biological cell grafts researchers use autografts, allografts, xenografts, and isografts, shown in **figure 1**. Autografts are built from the patient's own cells, which have the major advantage of histocompatibility and have no ethical issues related to their use. Allografts consist of donated tissue by another individual and xenografts are tissues obtained from other species. The transplantation of allografts and xenografts are less suitable due to histoincompatibility and possible transmission of infectious diseases (14). Lastly isografts are a type of graft where the tissue is transplanted between genetically identical individuals of the same species (e.g. identical twins) (15).

The need for finding suitable cells for TE has commenced the research of different types of donor cells. In the search of those cells a balance must be struck between ethical issues, safety issues and efficacy (1).

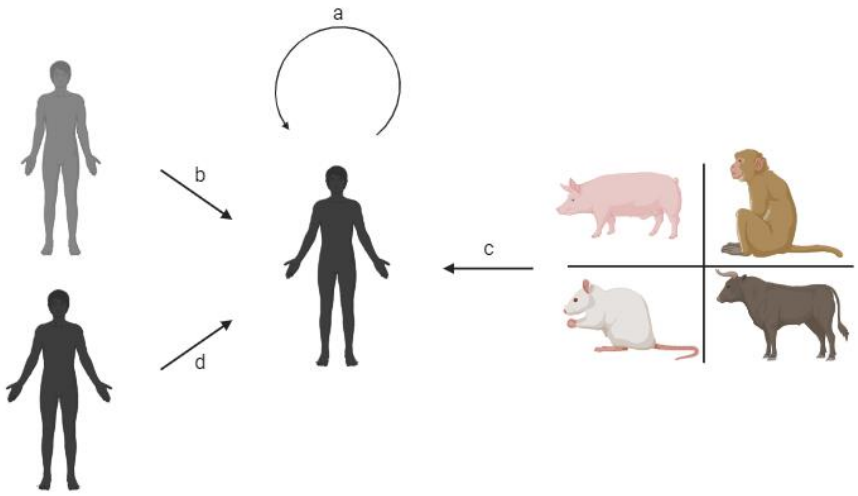


Figure 1. The different types of grafts. Autografts **(a)** are built from a patient's own cells. Allografts **(b)** consist of donated tissue from another individual. Xenografts **(c)** are grafts obtained from other species (e.g. bovine, porcine, mice or nonhuman primate). Isograft **(d)** is a type of graft where the tissue is transplanted between genetically identical individuals of the same species (e.g. identical twins).

Human mesenchymal stem cells

The bone marrow stroma contains several cell populations, including mesenchymal stem cells (MSCs). MSCs are not only found in bone marrow, but also in skeletal muscle tissue, adipose tissue, synovial membranes, saphenous veins, dental pulp, periodontal ligaments, cervical tissue, umbilical cords, umbilical cord blood, amniotic fluid, placenta, lung tissue, liver tissue, and dermal tissue (16). MSCs are responsible for the regeneration of mesenchymal tissues, such as bone, cartilage, muscle, ligament, adipose tissue and stroma. Research on Mesenchymal Stem Cells (MSCs) has shown their ability to differentiate into various cell types such as adipocytes, chondrocytes, myoblasts, and osteoblasts (17, 18). These cells are highly attractive in the scientific community due to their multilineage differentiation potential, availability, extensive in vitro expansion capacity, paracrine effects crucial for cell survival and proliferation, and immunoregulatory properties. Furthermore, the intrinsic ability of MSCs to differentiate into chondrocytes makes them a suitable cell source for the treatment of cartilage disorders (e.g. osteoarthritis) (16).

Human embryonic stem cells

Human embryonic stem cells (ESCs) are pluripotent cells that are derived from the inner cell mass of human embryos. ESCs can differentiate in most cell types (i.e. of all three germ layers). However, due to immune rejection after transplantation, teratoma formation and ethical issues concerning

the usage of human embryo's, ESCs are becoming less relevant (19). Consequently, the usage of autografts would be ideal because of their histocompatibility but the ability of most somatic cells to multiply in vitro is limited. Adult stem cells are the precursors of adult somatic cells and they can differentiate in a variety of cell types of their native tissue, which makes them a useful donor cell source but they are less potent in multiplying then ESCs (20).

Induced pluripotent stem cells

In 2006, Takahashi et al. achieved a significant technological advancement with the development of induced pluripotent stem cells (iPSCs). iPSCs have a similar development potential as ESCs but are derived from the patient's somatic cells (e.g. fibroblasts), using the Yamanaka factors (i.e. OCT4, SOX2, KLF4 and MYC), a cocktail of four transcription factors (19).

Since its discovery, iPSCs emerged as an exciting alternative to ESCs as a renewable resource for TE (2). Human iPSC technology has evolved rapidly since 2006 and major progress has been made in stem cell biology and regenerative medicine. Furthermore, human iPSCs have been widely used for disease modelling, drug discovery and cell therapy development. In particular, the combination of human iPSC technology with recent developments in gene editing and 3D organoids makes iPSC-based platforms even more powerful in each area of their application, including precision medicine, reviewed by Shi et al. (21).

Cell therapy

Cell therapy involves utilizing stem cells to stimulate the body's own regenerative processes or to substitute damaged tissues following a cellular transplant (21). Donor cells must be modulated by biomaterials and signal molecules in a precisely emulated environment to differentiate into correct tissue. We now enter the domain of biomaterials in TE. Living tissues are essentially materials engineered by nature itself to have a specific structure that affects cell properties and which drives all consequent biological events. There are many different strategies to engineer cell environments. Biomaterials are capable to actively modulate cellular behaviours: adhesion, proliferation, migration, differentiation and maturation. Furthermore, new advancements have led to the CRISPR-Cas9 technology. This enables scientist to modify the DNA of the donor cell as desired (2, 22).

Techniques

There are two general strategies in TE, shown in **figure 2.** The traditional top-down method and the bottom-up method also known as the modular method. Traditional TE strategies tend to use a top-down approach. This involves planting cells on a biodegradable scaffold, the cells are expected to populate the scaffold and create the appropriate ECM and microarchitecture with the help of perfusion, growth factors and/or mechanical stimulation. Despite advances in this area, such as cell-seeding scaffolds or decellularized ECM templates, the top-down approach struggles to recreate complex microstructural properties of tissues (23, 24).

On the other hand, the bottom-up method strives to overcome this obstacle by creating biomimetic structures using modular tissues. The basis of the modular method lies in the fact that many tissues are built up by repeating functional components (e.g. the lobules of the liver). The aim is to create microscale modular tissues that are used as building blocks to construct a larger tissue. These modules can be made through various techniques such as 3D cell clusters (e.g. spheroids and organoids), cell sheets, cell-laden hydrogels, 3D printing or Microfluidics and organ-on-a-chip. Once made, the modules can be assembled into larger tissues by various methods such as random packing, stacking of layers or directed assembly (23).

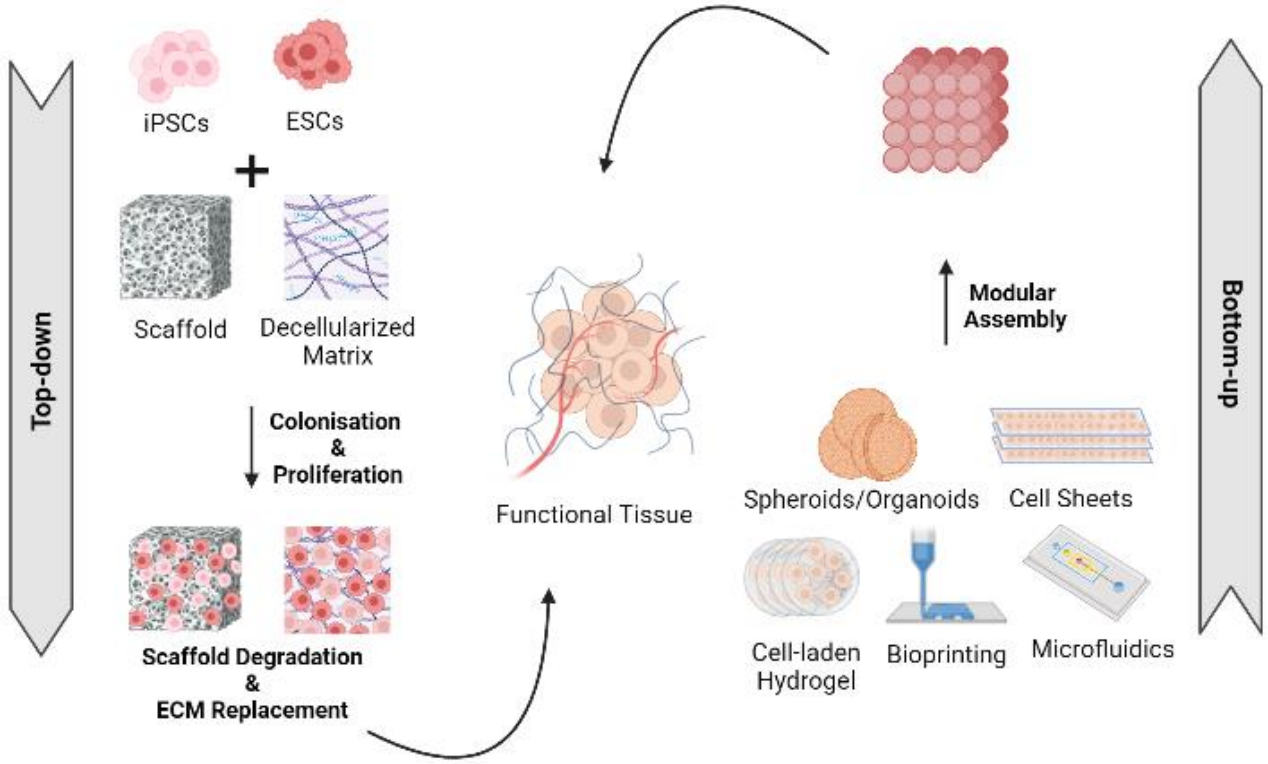


Figure 2. Top-down tissue engineering versus bottom-up tissue engineering. Top-down strategy: Cells (e.g. iPSCs or ESCs) are seeded on a natural/synthetic scaffold or a decellularized matrix. The cells proliferate,

migrate and colonize the construct, gradually replacing the extracellular matrix (ECM) and transforming in functional tissue. Bottom-up strategy: spheroids, organoids, cell sheets, cell-laden hydrogels, bioprinting or microfluidics as building blocks of the modular approach.

Top-down

Cell-seeding scaffolds

As described above, the top-down method consists of a triad of cells, signals and scaffolds. The aim is to mimic the microenvironment of an ECM. A diverse selection of stem cells constitutes the cell source. Cytokines and growth factors allow the cells to differentiate and proliferate into the appropriate cell lines. The scaffolds are required to have certain characteristics such as proper surface properties that induce adhesion, proliferation and differentiation of stem cells; low toxicity and immunogenicity; high porosity; an interconnected porous network for transport of nutrients and metabolic waste; suitable biodegradability of the scaffold based on the specific tissue (25-27).

Generally, cell-seeding scaffolds are classified into natural and synthetic-based scaffolds. Synthetic scaffolds have the advantage that the chemical and physical properties (e.g. porosity and degradation process) can be perfectly optimized for a particular application. In contrast, natural scaffolds are seen as an equivalent alternative. They are composed of components from either native ECM or components extracted from other biological systems (26). It has been shown that natural biomaterials behave similarly to the ECM and have innate biological functions. The scaffolds are biocompatible and biodegradable allowing them to be used in a range of applications in TE (28-31).

Many techniques have been developed for TE applications; one technique is called electrospinning. Electrospinning is a technique that creates ultra-thin fibres using a high-voltage electrostatic field. For example, Li et al. (32) produced a poly(D,L-lactide-co-glycolide) (PLGA) scaffold by an electrospinning process. The structure of the PLGA scaffold is similar to that of natural ECM and is characterized by a broad range of pore sizes, high porosity, and a high surface area-to-volume ratio. These characteristics make them ideal for cell attachment, growth, and proliferation. The electrospun structure offers effective mechanical properties that are suitable for soft tissues (e.g. skin or cartilage).

Decellularized matrices

Decellularized matrices are ECM scaffolds made by removing the cellular portion of tissues. After the cellularization process, all essential components of cell preservation and homeostasis are retained. The architecture of the original tissue is likely preserved including the primitive

microvascular system. Generally, there are 3 decellularization techniques physical, chemical and enzymatic (33, 34).

Bottom-up

Spheroids

Spheroids are composed of a 3D structure of cells formed by exposing cells to a non-adhesive environment. This causes the cells to adhere together and produce their own ECM and form 3D aggregates, which allows for tight cell-cell/cell-ECM interactions. An optimal 3D structure enables cell growth by providing each cell in this microenvironment equivalent transport of nutrients, moisture, gasses (e.g. oxygen), growth factors and removing degradation products (35, 36). Spheroids are widely used in cell therapy, models for drug screening, disease models and organoids. This is thoroughly discussed in a review by Jiang et al. (37).

Organoids

Organoids are mini-cell clusters grown in vitro, they are self-organizing and can differentiate into functional cell types. They mimic the function and structure of in vivo organs on a microscale hence the name “mini organs”. These organoids can be cultured from embryonic stem cells (ESCs), (induced) pluripotent stem cells (iPSCs) or adult stem cells (38). Lancaster et al. (2014) define organoids as: “A collection of organ-specific cell types that develops from stem cells or organ progenitors and self-organizes through cell sorting and spatially restricted lineage commitment in a manner similar to in vivo” (39). These in vitro organoids offer a simplified and accessible model (e.g. drug testing model and disease models), that mimic specific 3D structures, composition of cell types, and the function of organs (40). For example, Cruz et al. (41) produced a polycystic kidney disease organoid, to study the genetic kidney disorder with high efficiency and specificity.

Cell sheets

According to De Pieri et al. (2021), recent research in the field of cell sheets has focused on the scaffold-free bottom-up approach. Cell sheets are grown on a thermoresponsive surface. The bottom consists of a polymer, usually poly(N-isopropyl acrylamide) (pNIPAM) although different polymers have been developed with various properties. pNIPAM undergoes a transition from hydrophobic to hydrophilic at a certain temperature (i.e. 32°C). At the normal culture temperature of 37°C, pNIPAM is hydrophobic causing the cells to adhere to the pNIPAM surface layer. As the cell-sheets grow, the cells deposit ECM proteins and form a tissue-like network. Upon lowering the

temperature below 32°C, the pNIPAM surface layer becomes hydrophilic. The pNIPAM molecules become hydrated, causing the cultured cells to spontaneously detach as a continuous cell sheet. Remarkably, the cell sheets maintain their surface proteins and cell-cell junctions alongside with the ECM (42). Cells sheets completely preserve cell-cell junctions, cell surface proteins and ECM. Therefore, 3D tissues can be fabricated by stacking the cell sheets, without using scaffolds. In addition, the 3D layered cell sheets provide a stronger tissue function, more complete tissue regeneration and more therapeutic effects in comparison to a single cell sheet (43). For example, Haraguchi et al. (43) developed a protocol for the fabrication of functional 3D tissues by stacking cell sheets. Using this protocol, it is possible to create 3D tissue with a capillary-like pre-vascular network by inserting endothelial cells between the cell sheets.

Cell-laden hydrogels

Cell-laden hydrogels are hydrogel structures that contain cells. A hydrogel is defined as a multicomponent system consisting of a crosslinked network of polymers and water located between the polymers. Depending on the polymer, the hydrogel has different properties. For example, the amount of water the hydrogel can absorb, biodegradation capacity and mechanical strength of the hydrogel (44). However, hydrogels have a limited mechanical strength and are susceptible to permanent fractures of the polymer network. As a result, researchers addressed these challenges by engineering the mechanics of hydrogels to become stiff and tough (45). In addition, self-healing hydrogels were produced by Phadke et al. (46). They fabricated the self-healing properties through hydrogen bonding, by adding side groups (e.g. amine or carboxyl groups) into the polymer backbone of the hydrogel network. Next, the hydrogels can be combined with 3D printing, by using the hydrogel as a ink (i.e. the prepolymer of the hydrogel). Allowing, the manufacturing of reproducible 3D tissue constructs (47).

3D printing

The basic principle of 3D printing (or additive manufacturing) is similar for almost all its applications (48, 49). It involves constructing a 3D model layer by layer, the 3D model can be designed by computer aided design software (CAD). Alternative, the 3D model can be scanned via computed tomography (CT) or magnetic resonance imaging (MRI). Bioprinting is a subset of 3D printing with the aim of printing a 3D model with desired properties as an application of TE. Bioprinting is used in both top-down and bottom-up methods. In the top-down method, the focus is on printing the suitable scaffolds. In the bottom-up method, bioprinting is used to print the various nano- and

microscale modules. The recent review by Pantermehl et al. (2021) describes the advantages of this process as follows. Desired growth factors can be directly imprinted. This process allows more control over the formation of the final structure and enables a much higher cell density compared with the conventional top-down approach (49). Cells are sensitive to biochemical and mechanical properties of the microenvironment, which makes using the correct bioink important in 3D bioprinting. Each bioprinting method has their own bioink properties, in order to achieve the desired 3D structure with accuracy and high embedded cell viability (50). Most common bioinks used in TE are hyaluronic acid (HA), alginate, gelatine, collagen and their analogs, because of their high printability and biocompatibility (51). Also, hydrogels can be used as a ink, as discussed in the section above (47). The properties of bioink have an inherent effect on the bioprinting process. For example, the viscosity of the bioink. Viscosity is determined by temperature, the polymer concentration and its molecular weight. Bioinks with a high viscosity are associated with a higher shear stress during printing, resulting in cell damage. However, bioinks with a low viscosity fall and spread out after printing. In conclusion, a middle ground must be reached in the search for the right bioink viscosity properties (51).

Microfluidics and organ-on-a-chip

Microfluidics is a technique that is defined by engineering and manipulating fluids at the microscale (i.e. micrometer-scale) (52). Thanks to this technique, researchers were able to bring organ-on-a-chip (OOC) devices to fruition. OOC devices are essentially microfluidic devices designed to replicate the specific microenvironment of organs (53). The aim of OOC technology is to establish effective and applicable microphysiological models capable of analyzing the physiological events that define the interaction between organs, the immune system and exogenous factors (e.g. pharmaceutics) in states of health and disease (54).

The road to the clinic

After 30 years of research findings, what is the state of progress of TE towards clinical practice? The aim of this literature study is to review this process from bench-to-bedside. We conducted a pragmatic literature review using scientific databases, such as Pubmed, Google Scholar, Embase and the Web of Science, to establish a frame of reference. Next, we are going to use the FDA's drug development process classification (55) to assess the progression of the engineered tissue research. The FDA’s drug development process includes four levels, shown in **figure 3**. In summary, before safety and efficacy can be tested in humans, thorough toxicological tests must

be conducted in a preclinical setting. Subsequently, after successful clinical trials (i.e. in human testing), the product can enter the market, after a thorough review and approval process. This development process allows us to identify at what level of research the engineered tissue currently is and its progression to the clinic.

Note that, the U.S. Food and Drug Administration (FDA) regulates human cells or tissue intended for implantation, transplantation, infusion, or transfer into a human recipient as a human cell, tissue, and cellular and tissue-based product (HCT/Ps). Hence, depending on the specific characteristics and intended use of the engineered tissue, it could potentially be classified as a drug, device, biological product, or combination thereof (56, 57).

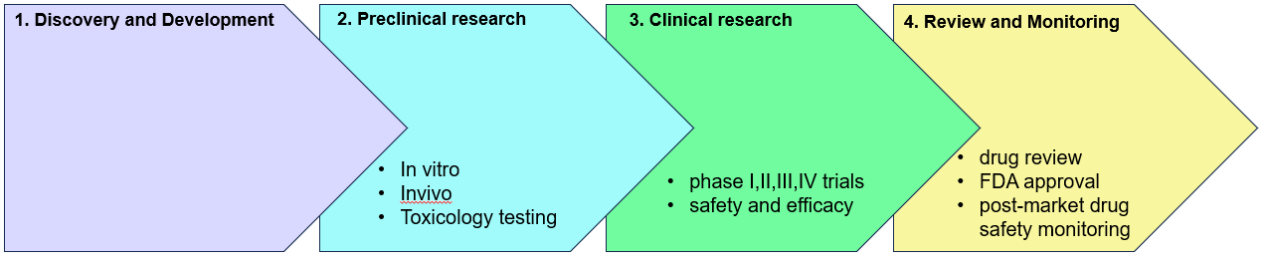


Figure 3. The FDA's drug development process. The FDA's drug development process consists of 4 steps: step 1: discovery and development, step 2: preclinical research, step 3: clinical research (i.e. studies, or trials that are done in people), step 4: review and monitoring.

Results and discussion

In this pragmatic review, we divide the engineered tissues according to the classification of Ashkan Shafiee et al. (5). By establishing this classification, we are going to use four example tissue structures (i.e. cartilage, urethra, bladder and kidney tissue) to represent the road to the clinic. This classification consists of 4 levels, as the level increases the complexity of the tissue will also increase. The least complex level are flat tissues and organs (e.g. the skin and cartilage). Subsequently, one level higher are the tubular organ structures (e.g. blood vessels and tracheas). Then level 3, the hollow nontubular organ structures (e.g. bladder, uterus and vagina). Finally, the most complex structures are the solid organs (e.g. the heart, kidneys, pancreas and liver). The four example tissue structures range from a simple tissue (i.e. cartilage and urethra tissue) to a complex organ (i.e. bladder or kidney tissue). Next, we can study the progress for each tissue structure which will give us an overall picture in the field of TE.

The more complex the tissue, the more challenges arise, such as a reliable cell source, compatible scaffolds and tissue vascularisation. For example, it is possible to supply oxygen and

nutrients and remove waste materials from cells that are within 100–200 μm from capillaries (58). To engineer thicker tissues and organs, vascularization is an essential requirement. In addition, the function of an organ is determined by the performance of its individual cells, both separately and combined, along with the interaction of its different individual cells and their ECM. Accordingly, a significant challenge is providing advanced biomaterials and scaffolds that allow all cell types in an organ to collaborate and construct their own ECM and constitute the proper microenvironment (59). As a result, the fabrication of functional solid organs remains currently out of reach, since they are too complex for our current knowledge and capabilities (5).

For each level of complexity, we delved into the details of one specific tissue structure. We selected cartilage, due to its limited regenerative capacity, rising obesity rates and the ageing of the population. Furthermore, we selected the following structures of the urinary tract: the urethra, bladder and kidneys in order for the distinct levels to be more connected to each other and thus achieve a coherent review.

For each tissue structure, we discussed the physiological tissues based on their histology and function. Next, we discussed a prevalent pathology for each tissue structure based on current epidemiology data and current treatment strategies. Last, we reviewed the relevance of TE in treating these pathologies and discussed the different engineering approaches to treat these pathologies.

Flat tissues and organs: Cartilage

Histology and function of cartilage

Cartilage is a tough, durable type of connective tissue that structurally supports certain soft tissues, notably in the respiratory tract and provides cushioned, low-friction surfaces in joints. The cells of cartilage, chondrocytes, which are embedded within lacunae, produce the ECM that surrounds them. Chondrocytes only make up a small percentage of the tissue's dry weight, which consists mostly of ECM. Yet, chondrocytes are fundamental for tissue homeostasis. Chondrocytes are derived from mesenchymal stem cells (MSCs), which are present in the bone marrow of adults. During embryogenesis, MSCs differentiate into chondrocytes and continue to divide, undergoing several lineages, until they eventually become rounded, mature articular chondrocytes that cannot proliferate. (60, 61).

The ECM is characterised by collagen fibrils (i.e. mostly type II collagen), hyaluronan (i.e. a non-sulphated GAG) and high concentrations of sulphated glycosaminoglycans (GAGs) on densely

packed proteoglycans (i.e. aggrecan), which bind a large amount of water. The high content of bound water allows cartilage to serve as a shock absorber.

Cartilage itself lacks blood vessels, lymphatic vessels and nerves. Chondrocytes receive nutrients via diffusion from capillaries located in the surrounding connective tissue, called the perichondrium. However, articular cartilage lacks perichondrium and is sustained by the diffusion of oxygen and nutrients from the synovial fluid. The limits of such diffusion define the maximum thickness of the cartilage and repair when damaged.

There are three major forms of adult cartilage: hyaline cartilage, elastic cartilage and fibrocartilage. The difference lies in composition of the ECM. Hyaline cartilage is the most common of the three types. In adults, hyaline cartilage is located in the articular surfaces of movable joints, in the walls of the respiratory tract (i.e. nose, larynx, trachea and bronchi), in the ventral ends of ribs, and epiphyseal plates of long bones. In the embryo, the initial skeleton is formed by hyaline cartilage, which then undergoes a process of gradual ossification. The ECM is rich in fibrils of type II collagen and aggrecan complexes with bound water. The synthesis of sulphated GAGs and secretion of proteoglycans are stimulated by many hormones and growth factors. In hyaline cartilage, this is regulated by somatotropin, secreted by the anterior pituitary gland. This hormone acts indirectly, prompting the liver to release insulin-like growth factors (i.e. somatomedins), which directly affect chondrocytes.

Elastic cartilage is in essence similar to hyaline cartilage except that the ECM contains an abundant network of elastic fibers in addition to a meshwork of collagen type II fibrils. Hence, making elastic cartilage more elastic than hyaline cartilage. Elastic cartilage is found in the auricle of the ear, the walls of the external ear canal, Eustachian tubes, the epiglottis and the upper respiratory tract; each including an accompanying perichondrium.

Fibrocartilage essentially contains a combination of hyaline cartilage in small amounts of dens connective tissue. Histologically it consists of chondrocytes in a hyaline matrix, usually layered with larger areas of bundled type I collagen fibrils with scattered fibroblasts. It is found in intervertebral discs, menisci, in attachments of certain ligaments and in the pubic symphysis. Fibrocartilage provides a strong, yet cushioning support (60).

Epidemiology and current strategies of osteoarthritis

Injuries to articular cartilage and menisci can lead to cartilage degeneration that eventually results in arthritis. According to the World Health Organization (WHO) in 2019, about 528 million people worldwide were living with osteoarthritis; an increase of 113% since 1990. As osteoarthritis (OA) is

more prevalent in older people (i.e. about 70% are older than 55), global prevalence is expected to increase with the ageing of populations. Additionally, obesity remains the most important risk factor for the incidence and progression of osteoarthritis (62). Rates of overweight and obesity continue to grow in adults and children. From 1975 to 2016, the prevalence of overweight or obese children and adolescents aged 5-19 years increased from 4% to 18% globally, stated by the WHO (63).

The current treatment and management rests on tertiary prevention (i.e. slow down the disease and optimize function), joint replacement surgery and analgesics (e.g. NSAIDs and opioids) (64). Current surgical approaches have a limited capacity for tissue regeneration, providing only short-term relief of symptoms (65).

Cartilage engineering

New TE approaches are emerging as alternatives to current surgical methods for cartilage and meniscus repair. A tissue engineered product could potentially provide more consistent clinical results in the formation of hyaline repair tissue and in filling the entire defect compared with existing treatments, such as microfracture or implantation of autologous chondrocytes (65, 66).

Regenerative medicine is a broad field with various approaches, often overlapping with each other. As a result, there are different cartilage regeneration approaches (i.e. TE and cell therapy approach). Multiple cell therapy and TE products are undergoing clinical trials for cartilage lesions and meniscus tears, this is summarised in the review by Kwon et al. (65). In summary, current surgical repair techniques for articular cartilage and meniscus pathologies are insufficient to halt the development and progression of OA. Consequently, accelerating the development of alternative TE strategies. Additionally, challenges for clinical translation are discussed. Such as, obtaining enough number of autologous cells. Next, the production of high-quality autologous neo-tissue is difficult to achieve due to the different biological variability of donors. Furthermore, pursuing biomimicry of native cartilage, further research is needed to improve the tribological properties and longevity of neocartilage and neo-menisci. Also, for both animals and humans, the use of new tissue-engineered implants may require new surgical procedures that protect the designed implants and prevent implant displacement. Finally, dealing with the pro-inflammatory environment of the injured or diseased joint (e.g. chronic joint inflammation in OA).

Published in 2019, Kwon et al. (65) provide an overview in clinical studies and tissue-engineered products for articular cartilage and meniscus repair, from 2006 to August 2018. Subsequently, this review will focus on tissue-engineered products and studies, since the period of August 2018 and will examine the evolution towards clinical translation that Kwon et al. highlighted.

Additionally, several cell therapy products for articular cartilage repair have already been developed and are available on the market, reviewed by Negoro et al. (67). These cell therapy approaches fall outside the scope of this review, since the main focus remains TE.

In the following two clinical studies, researchers used different approaches for the treatment of pathologies in knee joint (i.e. osteoarthritis and large cartilage defects of the knee).

First, a recent study by Hamahashi et al. (68) revealed that the use of autologous chondrocyte cell-sheets in conjunction with open-wedge high tibial osteotomy (OWHTO) led to the repair of hyaline cartilage in humans. Following this, they conducted a clinical trial (68) using polydactyly-derived allogenic chondrocyte cell-sheets (PD sheets) and temperature-responsive culture inserts, along with OWHTO shown in **figure 4A**. The goal was to effectively repair cartilage and treat osteoarthritis (OA) of the knee. In this trial, ten patients suffering from OA of the knee were treated. Consequently, a histological analysis was performed on arthroscopic biopsies, 12 months post-transplantation. In addition, gene expression was analysed to evaluate the PD sheets. In conclusion, the transplantation of PD sheets proved to be an effective treatment for OA of the knee. The histological quality of PD sheets was excellent, through the International Cartilage Repair Society (ICRS II), a score of 80.8 (64-96, 0: fibrous tissue, 100: hyaline cartilage) was achieved. They pinpointed several potential sets of gene markers that could predict the results of PD sheet transplantation, which could be instrumental in determining the allogeneic cell source for the creation of cell sheets before they are clinically utilized. In conclusion, they were able to verify that the transplantation of PD sheets in conjunction with OWHTO led to the regeneration of hyaline cartilage. These observations indicate that the combined treatment could potentially offer long-term therapeutic benefits and enhance the alignment of the lower limb.

Secondly, in the prospective, multicenter, single-arm, phase III clinical trial, by Niemeyer et al. (69), they assessed the clinical outcome of using a hydrogel-based autologous chondrocyte implantation (ACI) to treat large articular defects in the knee joint, after a two-year period. The primary outcome measure was the responder rate at 2 years using the Knee Injury and Osteoarthritis Outcome Score (KOOS). In short, 93% of patients were KOOS responders having improved by ≥10 points compared with their pre-operative level. Consequently, the study successfully achieved its primary endpoint, showing that the KOOS response rate significantly exceeded 40%. The lower 95% confidence interval (CI) was recorded at 86.1, which is more than double the predetermined no-effect level. The improvement in KOOS (least squares mean) was noted to be 42.0 ± 1.8 points, with a 95% CI ranging from 38.4 to 45.7. As shown in **figure 4B**, significant improvements were observed from the third month (i.e. the first measurement) to the

24th month, in both the overall KOOS and all five KOOS subscores, with changes from the baseline ($P < 0.0001$). After 24 months, the average Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score reached 80.0 points (with a 95% confidence interval of 70.0-90.0 points), and for lesions that were ≤ 5 cm2, the score was 92.1 points. In conclusion, the use of hydrogel-based ACI has shown to be an effective treatment choice for patients with large cartilage defects in the knee. This is demonstrated by the significant and clinically meaningful improvements observed after only two years of follow-up, as shown in **figure 4B**. Additionally, MRI analyses indicated an increase in maturation, re-organization, and integration of the repair tissue.

Next, in the following three preclinical trials, researchers made scaffolds by different approaches to regenerate a chondral defect in animal models (e.g. rat, goat or porcine model).

First, Browe et al. (70) used biodegradable scaffolds, derived from the ECM of solubilized articular cartilage (AC-ECM) with the aim of repairing chondral lesions. These scaffolds were securely anchored to the joint surface using a 3D-printed fixation device shown in **figure 4C**, eliminating the need for sutures or adhesives. MSCs were cultured on these scaffolds in vitro, which induced chondrogenesis. This was demonstrated by the increased expression of type II collagen and the production of GAGs , in contrast to MSCs without AC-ECM scaffolds. When the AC-ECM scaffolds were complemented with TGFβ3, this led to increased recruitment of MSCs. In this preclinical trial, skeletally mature goats were used. After 6 months, the AC-ECM treated defects showed high quality cartilage repair, in comparison with microfracture alone. Loading these scaffolds with TGFβ3 further increased these repair effects. These data suggest the potency of an accessible and clinically relevant scaffold for the regeneration of articular cartilage.

Second, Steele et al. (71), created a porous zonal micro-structured scaffold from a biocompatible polymer (i.e. poly [ϵ-caprolactone]) to better replicate the intricate structure of articular cartilage. Little studies exist devoted to the repair of osteochondral defects; most efforts to date have focused on the development of scaffolds for the repair of chondral defects (e.g. hydrogels). Thanks to the multiple fabrication strategies, they produced a stiffness gradient throughout the scaffold similar to native cartilage. Over four weeks in vitro, the chondrocyte-seeded scaffolds accumulated ECM, including GAGs and collagen type II. Next, they tested the scaffold in a preclinical in vivo trial (i.e. skeletally mature porcine model with osteochondral defects). During the 6-month follow-up shown in **figure 4D**, the acellular and chondrocyte-seeded scaffolds remained intact, with in ECM deposits throughout the scaffolds in all the repaired lesions. The more prevalent type II collagen was not detected in the scaffolds, only type I collagen localised in the subchondral bone and repaired tissue. Steele et al. (71) provided compelling evidence

demonstrating the osteointegration of the micro-structured scaffolds, as shown in **figure 4D**. However, based on histological scoring, the quality of repair provided by the micro-structured scaffolds was not superior to that of the control. In summary, these findings indicate consistent retention and osteointegration of the micro-structured scaffold, along with extended degradation. They suggest that these properties may contribute beneficial to the long-term repair of osteochondral defects.

Last, the study by Liu et al. (51), 3D bioprinting was used to create a bone marrow-derived mesenchymal stem cell (BMSC)-laden biomimetic multiphasic scaffolds with an anti-inflammatory strategy. First, they addressed advancements in bioprinting, explaining that previous research shows that the 3D bioprinting method is superior in cartilage regeneration efficiency compared to other cell-seeded scaffold methods. Notably, through 3D bioprinting cells, biomolecules and mechanical structures can be assembled in a highly controlled manner. Which allows the assembly of a multilayered tissue that mimics the natural physiological tissue in terms of structure. Also, 3D bioprinting provides a more uniform distribution of cells compared to conventional post seeding approaches. Furthermore, by using 3D bioprinting a more precise microenvironment is created to guide cell behaviour, which leads to a better biomimetic tissue. Additionally, few cartilage tissue engineering studies have been extended to cartilage regeneration under inflammatory conditions. Inflammatory conditions can lead to a reduction in chondrogenesis, a decrease in the motility of MSCs and triggers a cartilage matrix-degrading enzyme release cascade that results in cartilage matrix degeneration. Cartilage regeneration and suppression of OA development must be effectively coupled and therefore form an effective microenvironment. They addressed these challenges by fabricating a custom polycaprolactone (PCL) and methacrylated hyaluronic acid (MeHA)-based biomimetic multiphasic composite biodegradable scaffold with three different functional layer domains to mimic the osteochondral structure, shown in **figure 4E**. Along with MeHA, the photocrosslinkable hydrogel that was used as bioink, thanks to a good biocompatibility with increased mechanical stiffness and long-term stability. Furthermore, this design provides an OA inflammatory management by using a diclofenac sodium (DC)-loaded matrix metalloproteinase (MMP)-sensitive functional drug delivery hydrogel (MMP-MeHA(DC)) coating on the top layer of the scaffold facing the joint cavity, shown in **figure 4E**. They aim to establish a microenvironment with effective cartilage repair and OA progression management, using a rat model. In conclusion, this study provides evidence through joint function and gait analysis, that the strategy of using BMSC-laden bioprinting is beneficial in enhancing the recuperation of joint function, strength and gait/speed following a joint injury. It has been documented that MMP-MeHA hydrogels augment chondrogenesis, facilitate the deposition of cartilage matrix, inhibit MSC hypertrophy and reduce

cell calcification in comparison to nondegradable hydrogels after an extended duration of cultivation. Next, the MMP-MeHA(DC) hydrogel demonstrated efficacy in mitigating the inflammatory and nociceptive effects associated with osteochondral lesions and exhibited compatibility with BMSC function, thereby further impeding the progression of OA.

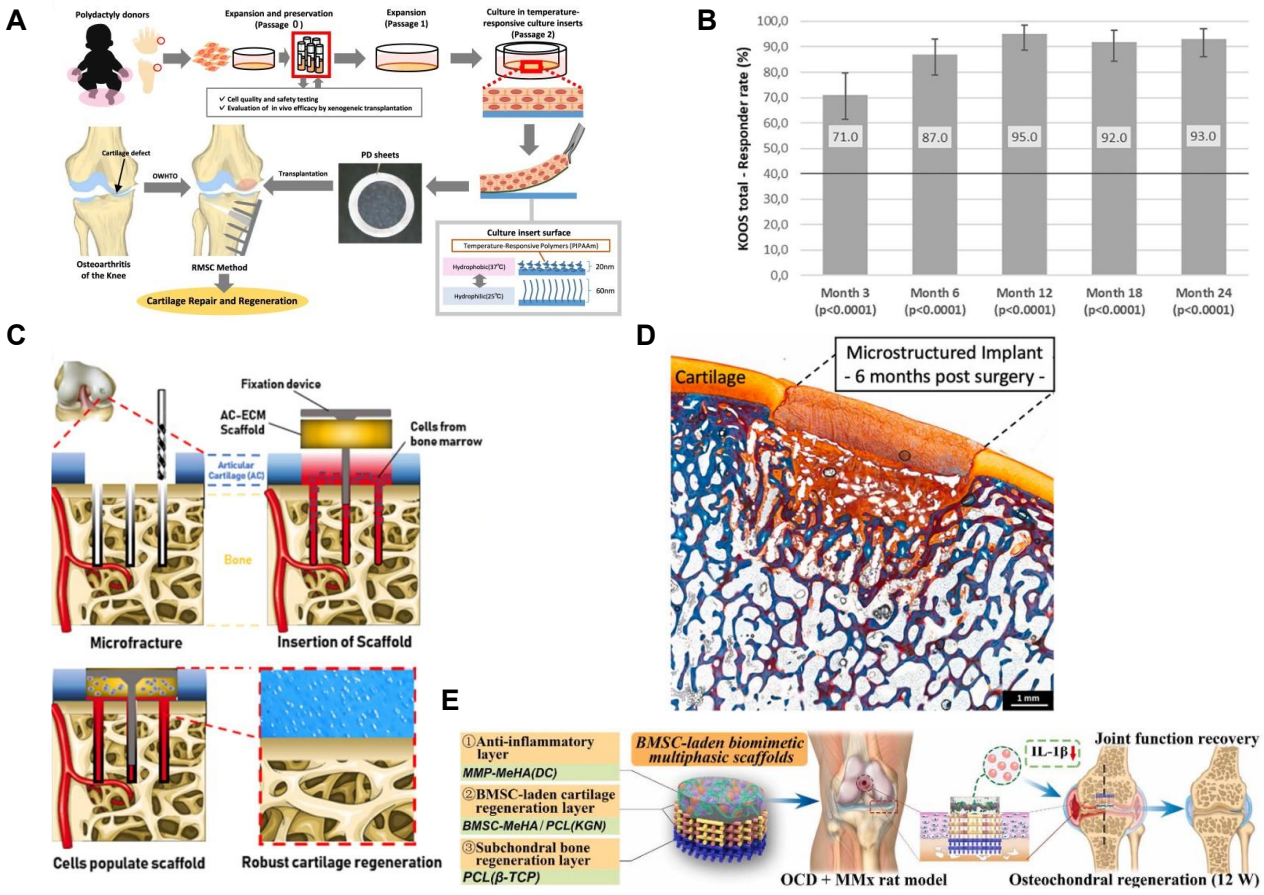


Fig 4. The different approaches of cartilage engineering. **(A)** The production of chondrocyte sheets. PD sheets were fabricated from cartilage tissue obtained from polydactyly surgery. cells were seeded on temperature-responsive culture inserts. The PD sheets were further cultured for 14-16 days and transplanted. OA of the knee patients were first treated with conventional surgical treatments OWHTO, followed by the removal of unhealthy tissue, marrow stimulation, and chondrocyte sheet transplantation (RMSC method), reproduced with permission from (68). **(B)** KOOS responder rates over time through Month 24. Vertical error bars indicate the exact 95% confidence intervals according to Clopper and Pearson. P values are derived from the 1-sided exact binomial test of hypotheses H0: Rate ≤ 40% versus H1: Rate >40%, reproduced with permission from (69). **(C)** Development of fixation system used to anchor the AC-ECM scaffold, reproduced with permission from (70). **(D)** Goldner's trichrome stained section of the repair site at 6 months, to visualize tissue morphology and mineralization. Goldner's trichrome stains mineralized tissue blue/green and collagen orange, reproduced with permission from (71). **(E)** BMSC-laden biomimetic multiphasic scaffolds were fabricated by 3D bioprinting to efficiently facilitate osteochondral regeneration, and scaffold treatment improved the function of injured knee joints in a medial meniscectomy-induced osteoarthritic rat model through chondroprotection and inflammatory modulation, reproduced with permission from (51).

Tubular organ structures: Urethra

Histology and function of the urinary tract

Filtrate is delivered from the collecting ducts into the renal calyces, from this point the filtrate will undergo no further change and is called urine. Urine is transported by the ureters from the renal pelvis to the bladder where it is stored until emptying by micturition. The bladder will be addressed in the section on tubular nonhollow organs.

The walls of the ureters are similar to those of the renal calyces and renal pelvis, with mucosal, muscular and adventitia layers that gradually thicken closer to the bladder. The mucosal surface of these organs is lined by the uniquely stratified urothelium or transitional epithelium. Cells of urothelium are organised as three layers: a single layer of small basal cells resting on the basal membrane, an intermediate region with one to several layers of cuboidal or low columnar cells, and a superficial layer of large globular or elliptical umbrella cells.

Umbrella cells have extensive intercellular junctional complexes (e.g. tight junctions) and apical membranes with dense plaques of uroplakin proteins, that protect the cytoplasm. Consequently, the abundant membranous plaques together with tight junctions serve as an osmotic barrier enabling them to withstand the hypertonic effects of urine and protect underlying cells from this toxic solution. The thick muscularis of the ureters moves urine toward the bladder using peristaltic contractions. When relaxed, the muscularis provides prominent mucosal folds in the empty lumen.

The urethra drains the bladder and in both genders is lined initially by urothelium. However, the male urethra is longer and divided in three regions: the prostatic urethra, the membranous urethra and the spongy urethra. The prostatic urethra is lined by urothelium. Next, the membranous urethra is lined by stratified columnar and pseudostratified columnar epithelium. Lastly, the spongy urethra is lined by stratified columnar and pseudostratified columnar epithelium, but distally with stratified squamous epithelium. In women, the urethra is lined initially with urothelium which then transitions to nonkeratinized stratified squamous epithelium, continuous with that of labia minora (60).

Epidemiology and current strategies of urethral strictures

Several pathological conditions can affect the urethra, and these often result from congenital malformations, the natural aging process, or injuries. These conditions are more common in men than in women because of the longer length of the male urethra, making it more susceptible to injury (72).

Urethral stricture, a pathological narrowing of the urethra restricting fluid transport, is a common disease in men with an associated prevalence of 229-627 per 100,000 males, or 0.6% in susceptible patient groups, who are typically older men (73). Several factors such as trauma, infection, inflammation, congenital malformation, cancer, and iatrogenic injury can lead to the formation of scar tissue, resulting in urethral stricture or even complete blockage (74). Consequently, urethral strictures can significantly impact a patient's quality of life and pose a considerable financial burden on the health-care system (72, 73, 75).

The typical management of anterior urethral stricture often starts with an endoscopic urethrotomy or dilatation. If the stricture recurs, definitive surgical reconstruction is usually the next step. For patients with extensive strictures, especially those in the penile urethra, surgical reconstruction is often the preferred treatment. This is due to the lower success rates observed with procedures such as urethrotomy and dilation. In urethral reconstruction, augmentation urethroplasties with tissue grafts and flaps are usually best suited for longer bulbar and penile strictures. Oral mucosa grafts are considered the gold standard in these procedures. On the other hand, anastomotic urethroplasty is usually the solution for short strictures in the bulbar part of the urethra (76). Despite all the efforts, urethroplasty remains a complex surgical procedure in urology. A consensus has yet to be reached and there are no widely accepted guidelines in the field (77-79).

Urethra engineering

TE presents a promising alternative for tissue repair and replacement of tissues. Urethral engineering strategies primarily involve two components: a scaffold that provides structure, and cells to provide a barrier from transported fluids. Additionally, growth factors can be utilized to guide cell migration, facilitate graft remodelling, and promote vascularization (72). Enabling urethral stricture to be better treated or avoided.

In the following five preclinical studies (i.e. rabbit model or in vitro), researchers used different scaffold approaches using different biomaterials and seeded the scaffolds with several types of cells sources (e.g. epithelial cells, dermal fibroblasts, vesical fibroblasts).

First, in the study by Zhang et al. (80), a functional electrospun collagen/P(LLA-CL) nanofiber scaffold was created by integrating co-axial electrospinning technology with the Wnt pathway inhibitor ICG-001. Next, the anti-fibrosis effect was assessed both in vitro and in a rabbit urethral defect model. This was done to establish preliminary evidence and lay the groundwork for

future large animal studies and potential clinical applications. Twelve male New Zealand white rabbits were split into two groups. The first group (i.e. group 1), consisting of six rabbits, was treated with a non-drug scaffold seeded with epithelial cells. The second group (i.e. group 2) was treated with a scaffold delivering ICG-001, also seeded with epithelial cells. All twelve rabbits underwent urethroplasty and managed to survive for a period of three months. In the first group, five out of six rabbits developed narrow urethral lumens as a result of the urethroplasty, and one rabbit developed a fistula at the penile skin. All the rabbits in the second group exhibited unrestricted lumens, and no sign of penile skin fistulas were observed. The results demonstrated that epithelial cell seeded Col/P(LLA-CL) scaffolds were successfully used in the reconstruction of 2 cm urethral defects in rabbit models, shown in **figure 5A**. Histological results showed, a discontinuous epithelial layer on the lumen surface according to H&E staining and AE1/AE3 immunohistology image, in group 1. Also, the urethral tissue in this group contained a large amount of collagen and less smooth muscle cells, indicated by the Masson staining image. In contrast, group 2 exhibited a multi-layered epithelium. Additionally, the tissue in the submucosa developed more smooth muscle and less collagen in the Masson staining image. Quantitative analysis using Image J revealed significant differences in collagen, smooth muscle, and epithelium between the two groups.

Second, Liu et al. (74) described the efficiency of gelatine-functionalized, tubular nanofibrous scaffolds of poly(L-lactic acid) (PLLA) in regulating the phenotypic expression of epithelial cells (ECs) and smooth muscle cells (SMCs) for urethral reconstruction. This was assessed using an in vitro and rabbit model. The flexible PLLA/gelatine tubular nanofibrous scaffolds with hierarchical architecture were fabricated by electrospinning, with doping of gelatine into the composite fibres at different weight ratios (i.e. 100:0, 75:25, 50:50 PLLA/gelatine). They found that the interface of the PLLA/gelatine (75:25) nanofibrous scaffold promoted adhesion, elongation and proliferation of ECs and SMCs. They employed immunofluorescence staining to determine the expression of epithelial cytokeratin (AE1/AE3, an important membrane surface protein marker of ECs) and actin-smooth muscle contractile protein (α-SMA, an important protein marker of SMCs). ECM protein, elastin, for two cell types were immunostained and analysed to verify whether these nanofibrous scaffolds can guide ECs and SMCs to form anisotropic tissue. Subsequently, the expression levels of keratin, actin, and elastin on the PLLA/gelatine (75:25) nanofibrous are higher than those on the PLLA/gelatine (50:50) nanofibrous scaffold and significantly higher than those on the pure PLLA nanofibrous scaffold. The findings suggest that the nanofibrous scaffold composed of PLLA/gelatine (75:25) can notably enhance the expression of keratin in endothelial cells (ECs) and actin in smooth muscle cells (SMCs), as well as stimulate the production of corresponding elastin, in vitro. A rabbit urethral replacement model was used to

evaluate the urethral tissue reconstruction behaviour through three prepared groups: tissue-engineered autologous PLLA/gelatine (75:25) scaffold, autograft, and tissue-engineered autologous PLLA scaffold. These have shown that engineered cellularized tubular PLLA/gelatine (75:25) grafts can simultaneously promote lumen epithelialization and urethral smooth muscle cell remodelling and capillary formation. They found that as the scaffold degraded, urethral smooth muscle cells grew in an orderly fashion along the tubular PLLA/gelatine nanofibrous scaffold. Additionally, the lumen of the newly formed urethral tissue was lined with 2–3 layers of thin urethral epithelial cells, forming a complete urinary tract epithelial tissue. Masson's trichrome staining of the mid-section of the regenerated urethras revealed that new layers of urethral smooth muscle tissue and collagen layers are distinctly visible and evenly distributed in the transverse section of the neourethra, which closely resembles the distribution found in the autograft and resulted in no strictures, shown in **figure 5B**. In contrast, in the PLLA group due to the hydrophobic nature and poor biocompatibility, H&E staining revealed an uneven distribution of urethral smooth muscle cells throughout the cross-section of the regenerated urethra. Additionally, the urethral epithelium in the lumen was found to be extremely thin. Masson's trichrome staining further confirmed that both the smooth muscle tissue layer and the collagen layer were irregularly distributed in the PLLA-based tissue-engineered urethra. As a result, this led to urethral stricture shown in **figure 5B**.

Next, the third study by Wang et al. (81), developed a three-dimensional (3D) biomimetic, urethra-inspired bacterial cellulose/bladder acellular matrix (BC/BAM) scaffold to promote angiogenesis and accelerate urethral regeneration, in a rabbit model. The spongiosum of penile urethra was decellularized and freeze-dried. After the process of decellularization and solubilization, ECM proteins and GAGs of native tissue were preserved, proved by the immunofluorescent and Alcian blue staining. The preservation of the bioactive components provides biochemical signals for tissue repair, proving to promote angiogenesis and endothelial proliferation, which was demonstrated in previous research (82). Additionally, it was indicated that VEGF was preserved after solubilization of BAM. The functional development of the human umbilical vein endothelial cells (HUVECs) on the scaffolds were observed by the expression of functional proteins of ECs, CD31 (i.e. is a membrane protein that mediates cell-to-cell adhesion and preserves endothelium integrity) and vWF using immunofluorescent staining. Accordingly, the vWF and CD31 showed the highest expression in BC/BAM, which were 1.7 and 2.0 times higher than the BC group and higher than the BAM group. This indicates that the biomimetic scaffold may have a positive effect on angiogenesis in vivo. All eighteen New Zealand white rabbits survived until sacrifice. At one month, shown in **figure 5C**, urethral stricture to some extend was observed in BC group by retrograde urethrography. Similarly, in the BAM group, stricture was also observed,

although of lower degree than in the BC group. In contrast, the BC/BAM group demonstrated no stricture. At three months, urethral fistula was observed in the BC group. Next, in the BAM group urethral stricture still existed and a rough urethral lumen was observed, which always leads to scar formation, shown in **figure 5C**. In contrast, all rabbits in the BC/BAM group could normally urinate and showed a normal appearing urethra. The percentage of urethral strictures in the separate groups (i.e. BC, BAM and BC/BAM group) were respectively 100%, 67% and 0%, which indicates an obvious difference in repair rate among each group.

Next, in the fourth study by Jiao et al. (83) developed a fibrinogen-poly(L-lactide-co-caprolactone) copolymer (Fib-PLCL) nanofiber scaffold seeded with epithelial cells on the surface, with the aim to achieve effective urethral tissue regeneration. In vitro, the scaffold made of Fib-PLCL was found to enhance the adhesion and survival of epithelial cells on its surface. Compared to the PLCL scaffold, the Fib-PLCL scaffold exhibited higher expression levels of cytokeratin and actin filaments. In vivo, using a urethral defect model of New Zealand rabbit, the repairing potential of Fib-PLCL scaffold was evaluated and is shown in **figure 5D(b)**. The urethral defect was replaced by either the Fib-PLCL, PLCL scaffold or an autograft. Next, in the Fib-PLCL group no significant strictures were identified. Subsequently, the Fib-PLCL had induced luminal epithelialization, remodelling of urethral SMCs and development of capillaries. Also, histological analysis revealed that the urothelial integrity in the Fib-PLCL group had advanced to match that of a native urothelium, with enhanced urethral tissue development. As a result, the Fib-PLCL scaffold is more appropriate for urethral defect reconstruction than the latter (i.e. PLCL scaffold) and similar to the autograft group. This is shown in **figure 5D(a)**, illustrating the relative urine flow range after scaffold transplantation, at different time periods.

Finally, the fifth preclinical study by Caneparo et al. (84) made an endogenous ECM scaffold, using a self-assembly technique in vitro. They used mesenchymal cells, such as fibroblasts, to secrete and assemble endogenous ECM, allowing the production of tissues devoid of exogenous biomaterial. In contrast, the other preclinical trials (74, 80, 81, 83) used exogenous biomaterials. Caneparo et al. (84) aimed to engineer a 3D substitute tissue, featuring optimal structural and functional properties by mixing a small proportion of dermal fibroblasts (DF), which improve mechanical resistance, and vesical fibroblasts (VF), which support a better epithelium. They used a the functional properties of the produced substitutes were determined using DF or VF alone or VF:DF mixes in ratios of 70%:30%, 80%:20% and 90%:10%. The addition of DF improved the mechanical strength of the flat urethral tissues. Tissues produced using VF/DF mixes showed increased tissue thickness compared to the condition where VF alone was used. Maximal strength, UTS and failure strain of the flat urethral tissues linearly increased ($R2 \geq 0.97$) with decreasing

VF/DF ratios. All substitutes containing DF had maximal force values superior to those reported for native human bladder. However, VF-only or 10% DF tissues had a higher elasticity than those produced with 100%, 30% or 20% DF. All tissues showed an elevated level of urothelial organization with obvious pseudo-stratification. The basal urothelial cells were better organized with a higher ratio of VF used for stroma production. PAS coloration highlighting the presence of polysaccharides showed a thick dark purple layer at the apical region of the urothelium. Next, mucous secreting cells could be observed in all substitutes, which contained polysaccharides. The mucous secreting cells seemed to correlate positively to the percentage of VF used for stroma production. The barrier function was assessed, shown in **figure 5E**, through permeability assays of the substitutes, using stroma without urothelium as a negative control. Tissues made only with DF showed reduced impermeability 50% of the initial urea found in the receiving chamber at eight hours, whereas this quantity ranged from 10 to 20% for the 90% and 100% VF substitutes, respectively. The 100% and 90% VF tissues had the highest barrier function, respectively. Using scanning and transmission electron microscopy, the uroplakin presence and maturation were assessed. Umbrella cells were absent in some locations for the DF substitutes, replaced with intermediate cells with a spherical shape. Also, these images allowed observation of tight junctions between the umbrella cells. Subsequently, all substitutes were observed to contain discoid and fusiform vesicles, which are the least and most mature vesicles respectively, responsible for transporting uroplakins to the plasma membrane. These results correlated with the permeability results, explaining the lower functionality of the substitutes produced using only DF. The molecular characterization of the substitutes was primarily conducted using immunofluorescence labelling against unique markers of the basement membrane and urothelium layers. The chosen markers were: laminin 5 to stain the basement membrane, P63 a transcription factor expressed by urothelial progenitors in the basal layer of the urothelium, Ki67 present in the nucleus of proliferative cells and expressed in the urothelium's basal layer, Keratin 14 (K14) a proliferative marker in urothelium, Claudins form tight junctions between epithelial cells, ZO-1 present at the zonula occludens and UPK-2 antibodies to target uroplakins. Except for K14, all the markers demonstrated a similar localization and intensity across the substitutes reconstructed at different VF/DF ratios. Because K14 localization and intensity increased by adding DF.

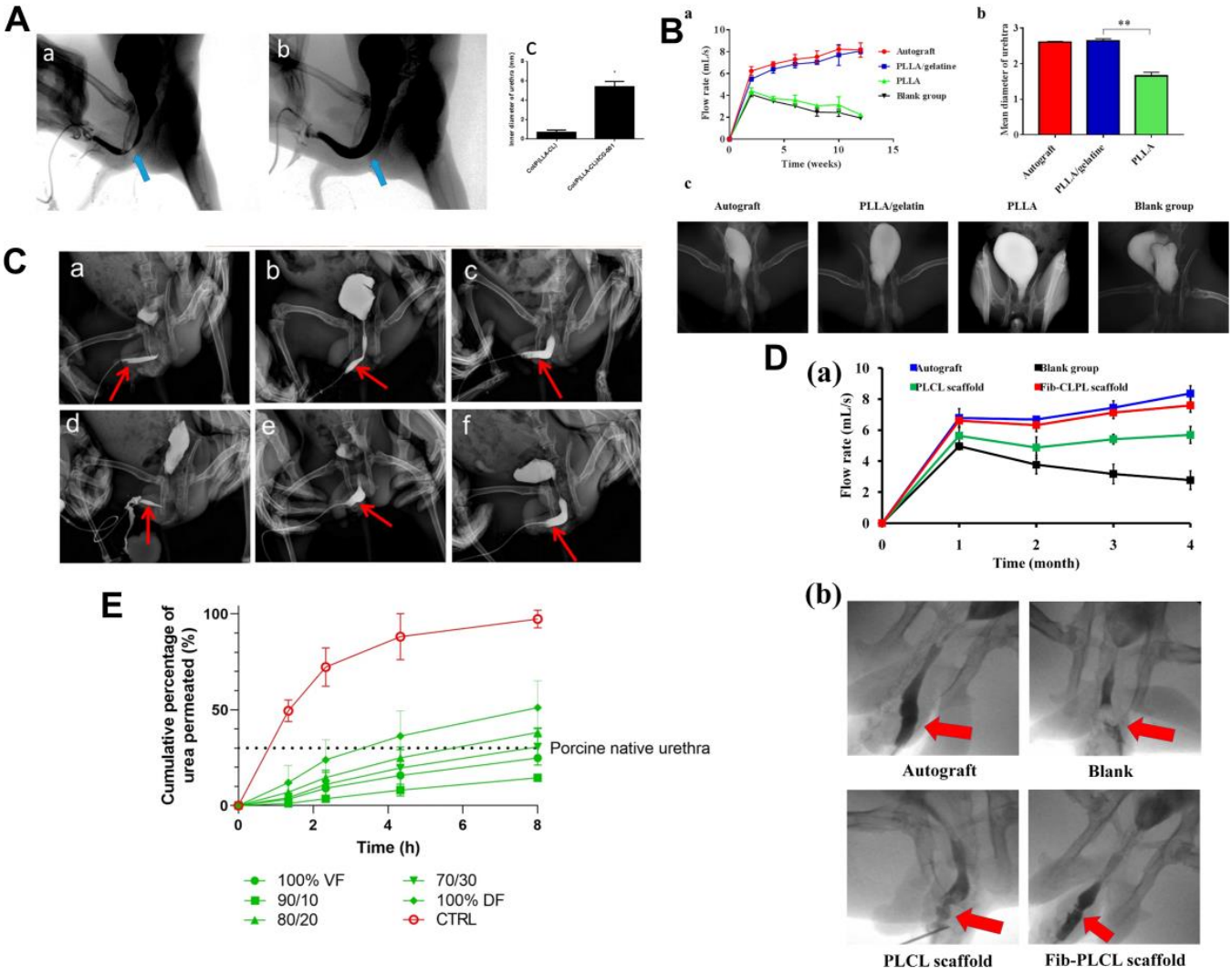


Figure 5. Urethra engineering. **(A)** The representative image of retrograde urethrography after rabbit urethroplasty with the non-drug scaffold (a); and ICG-001 delivering scaffold (b); the lumen diameter of urethras is shown in (c). The blue arrow indicates the surgery position (* $p < 0.05$), reproduced with permission from (80). **(B)** Surgical outcome of cellularized tubular autologous scaffolds. The urinary flow analyses of rabbits at 2, 4, 6, 8, 10, and 12 weeks after graft implantation (a). The average diameter of the neourethra of the rabbits in each group at 3 months after surgery ($n = 3$, $p < 0.01$) (b). Voiding cystourethrograms of each group 3 months after surgery (c), reproduced from (74). **(C)** Urethrography images of BC (a, d), BAM (b, e) and BC/BAM (c, f) groups. The red arrows represent the urethrography site of the urethra, (a, b, c) at 1 month and (d, e, f) at 3 months, reproduced from (81). **(D)** Surgical outcomes of the implantation of cellularized Fib-PLCL and PLCL nanofiber scaffolds for urethral injury treatment in rabbit model. (a) Graph representing the relative urinary flow range of rabbits after scaffold graft transplantation at different time interval. (b) Retrograde urethrography images illustrate the urethral tissue development in the animals grafted with autograft, PLCL scaffold, and Fib-PLCL scaffold or blank group after 4 months, reproduced from (83). **(E)** Barrier function of the urethral substitutes. Cumulated percentage of urea permeated (%) measured using a custom-made system. CTRL is the negative control group. VF is for the condition where the stroma of the urethra substitute was reconstructed using only VF. The 90/10 is for a mix of 90% VF and 10% DF, 80/20 is for a mix of 80% VF and 20% DF, 70/30 is for a mix of 70% VF and 30% DF, and 100% DF is for the condition where the stroma of the urethra

substitute was reconstructed using only DF. The dotted line is the mean value obtained at eight hours for porcine native urethra, reproduced from (84).

Hollow nontubular organ structures: bladder

Histology and function of the bladder

Two main functions of the urinary bladder are urine storage and emptying. The capacity of the bladder in an average healthy adult is about 400-600 ml. As described above, the bladder consists of the following four layers: urothelium, lamina propria, muscularis propria, serosa/adventitia. The urothelium, the muscularis and adventitial layer become gradually thicker towards the bladder. Additionally, the apical umbrella cells are especially well developed in the bladder.

The urothelium has five to seven structural layers when the urinary bladder is relaxed. However, in the filled bladder the urothelium reorganises into two or three layers to accommodate the increased volume, this happens without structural damage. Due to this transitional function of the urothelium, it is also called transitional epithelium.

The lamina propria is a heterogenous network of structural proteins and cells, composed of an ECM with elastic fibers, capillaries, lymphatics, immune cells, afferent and efferent nerve endings, fibroblasts, myofibroblasts, adipocytes, interstitial cells of Cajal (i.e. signal transducers to the bladder's smooth muscle cells) and the muscularis mucosae.

Muscularis propria, known as the detrusor muscle, is composed of three sublayers: inner longitudinal, middle circular and outer longitudinal. These layers are particularly well defined around the neck of the urinary bladder, but less defined around the rest of the bladder wall.

The serosa is an extension of the peritoneal membrane and contains additional blood vessels. Furthermore, the adventitia a loose connective tissue layer that servers as the bladder's outer layer in places where there is no serosa (60, 85).

Current strategies of reconstructive bladder surgery

Congenital disorders, cancer, trauma, infection, inflammation, iatrogenic injuries, or other conditions of the genitourinary system can lead to bladder damage and loss of function. Often requiring reconstructive surgeries. In standard clinical practice, the intestine is utilized as the tissue source for reconstructive bladder surgery (i.e. enterocystoplasty), since no other autogenous or heterogeneous material, natural or artificial, has shown better results. However, the existing methods may lead to several complications. These complications range from those related to intestinal resection, to those resulting from the constant exposure of urine to tissues that are not

naturally equipped for this contact. An engineered bladder construct that mimics the structural and functional characteristics of native bladder is a promising alternative option for bladder substitution (86, 87).

Bladder engineering

Advancements in bladder tissue engineering, were thoroughly reviewed by Pokrywczynska et al. (88) in 2014, which suggested that in vitro engineered bladder wall substitutes could see clinical use soon. However, before these methods can be optimized for clinical use, they must first undergo preclinical testing on large animal models with bladder defects.

In 2006 the first short-term clinical trial related to the field of bladder engineering was conducted by Atala et al. (89). Seven patients (i.e. 4-19 years old) suffering from neurogenic bladder (i.e. myelomeningocele), were treated with biodegradable bladder-shaped scaffolds made of homologous decellularized bladder submucosa (i.e. four patients) and composite scaffolds made of collagen and polyglycolic acid (PGA) (i.e. three patients), seeded with autologous urothelial cells (UCs) and smooth muscle cells (SMCs), shown in **figure 6A**.

The mean follow-up period was 46 months, all cases showed good histological outcomes, with the identification of urothelium and smooth muscle in the newly formed bladder wall. However, only two patients (both treated with the collagen and PGA compound) demonstrated a functional increase in bladder size. The remaining five patients did not show good compliance or bladder capacity and required intermittent catheterisation. Also, it was noted that patients in whom the seeded composite scaffold of collagen and PGA wrapped with omentum to support vascularity, showed promising results. Prior to surgery, all seven patients were subjected to intermittent catheterisation and showed abnormal bladder innervation, due to their neurogenic bladder. Consequently, there was no working neuronal network in de bladder wall even preoperatively.

The following preclinical trial (i.e. in vitro model) addressed this challenge, which is the restoration of the neuronal network within the neobladder wall (90). Adamowicz et al. (90) developed a new biocomposite biomaterial scaffold derived from the amniotic membrane (Am) covered with a graphene layer, seeded with SMCs derived from porcine detrusor and porcine urothelial cells (UC) to evaluate the properties of the developed biomaterial. Graphene creates an interface between cells and external stimuli, taking over the role of the neural network in the bladder. A 3D printed chamber equipped, shown in **figure 6B**, with 3D printed graphene-based

electrodes was fabricated to deliver electrical field stimulation and record pressure changes caused by contracting SMCs seeded biocomposite. As a result, the presence of the graphene layer significantly increased the electrical conductivity of the biocomposite. UCs and SMCs showed an organized growth pattern on the graphene-coated surfaces and contractile responses of SMCs were observed when electrically stimulated.

In the following three preclinical studies, researchers are using decellularized ECM scaffolds, each with a different approach.

First, Pokrywczynska et al. (91) aimed to evaluate the utility of bladder acellular matrix (BAM) for reconstruction of clinically significant large urinary bladder wall defects in a long-term porcine model (i.e. shown in **figure 6C**). Six out of ten animals survived the six-month follow-up period. Four died due to the mechanical failure of the scaffold, the separation of the scaffold from the native bladder tissue or a blocked catheter. The engineered bladders had a normal function, no signs of postvoid residual urine in the bladder or upper urinary tracts. However, macroscopic graft shrinkage was observed. Next, the urothelium completely covered the luminal surface of the graft, but SMC regeneration was observed in the peripheral region of the graft and gradually decreasing toward the centre. Also, the levels of markers for urothelial cells, smooth muscle, blood vessels, and nerves were lower in the reconstructed bladder wall compared to the native bladder. In summary, this study has established that BAM is a biocompatible biomaterial with the capacity to regenerate substantial, clinically relevant defects in the urinary bladder wall. However, additional research is needed to improve urinary bladder regeneration using cell-seeded BAM.

Subsequently, Xiao et al. (92) constructed a bladder patch with BAM, seeded with adipose derived stem cells (ASCs) to promote bladder tissue regeneration after bladder reconstruction in a rat model of bladder augmentation cystoplasty. When the tissue-engineered graft constructed with scaffold material and seeding cells are transplanted into the body, its surrounding environment changes from a simple in vitro medium to the complex tissue environment in vivo. To address this challenge, early and rapid vascular network formation is crucial to maintain the graft's vitality and long-term survival. As a result, Xiao et al. used a self-designed perfusion system and four different perfusion decellularization schemes to prepare the BAM. In addition, before the augmentation cystoplasty the tissue engineered bladder patch was incubated with the omentum to promote the neovascularization. The preparation process of the whole bladder scaffold is shown in **figure 6D**. To conclude, it was shown that the bladder patch composed of the BAM and seeded ASCs regenerated the bladder wall structure better than the bladder patch composed of BAM alone.

Lastly, Sabetkish et al. (93) attempted to introduce a viable alternative to synthetic biomaterials and other natural scaffolds to support the augmented bladder wall during augmentation cystoplasty, using a novel hourglass technique with whole decellularized bladder scaffolds in a rabbit model. The hourglass technique, shown in **figure 6E**, involves attaching the base of the acellular scaffold to the base of the original bladder through the serosa layer, to prevent bladder exposure. The control group underwent resection of the muscle and mucosa of the vesical dome. Then, the acellular scaffold was sutured directly to the bladder wall for bladder augmentation. Mechanical testing showed that decellularized rabbit bladder samples were highly similar to their natural counterpart. After one, three and nine months of follow-up, macroscopically no significant shrinkage, infections and reactive changes were observed in the hourglass technique group, compared with the natural bladder tissue. In contrast, the control group showed a noticeable shrinkage, in some animals there was rejection and separation from the bladder wall. According to histopathological evaluation of the hourglass technique group, no fibrosis and inflammatory changes and the successful cell seeding with urothelial lining were observed in all follow-up stages. Next, there was no significant difference in the histological improvement of biopsies obtained at three months compared to those obtained nine months postoperatively. However, the histopathological evaluation of the control group revealed a significantly higher grade of fibrosis in the last follow-up. These results confirmed that decellularized bladder promoted muscle and epithelium regeneration without urinary leakage.

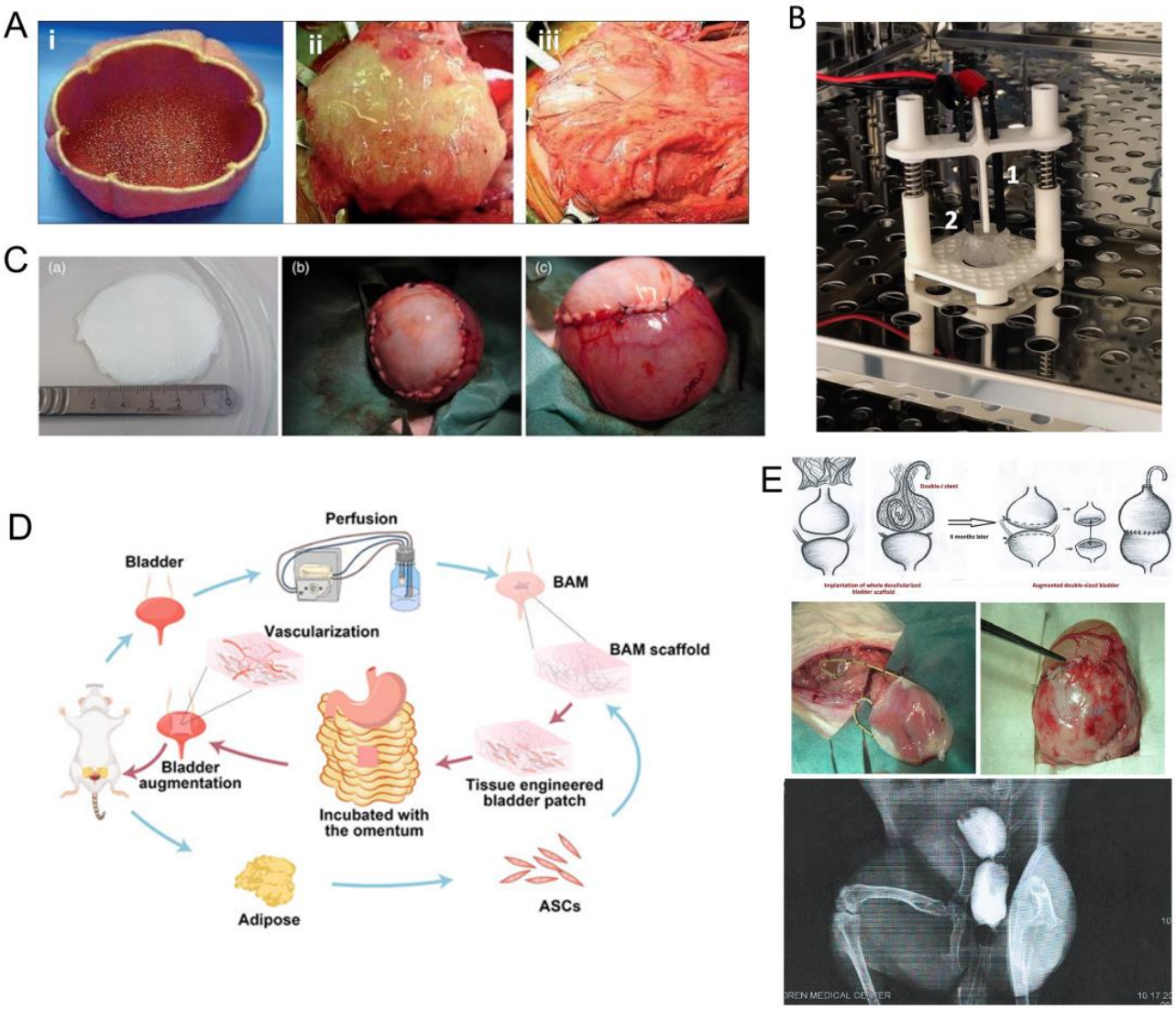


Figure 6. Bladder TE. **(A)** Construction of engineered bladder scaffold seeded with cells (i), and engineered bladder anastomosed to native bladder (ii). Implant covered with fibrin glue and omentum (iii), reproduced from (89). **(B)** A 3D printed stimulator in CO2 incubator. (1) Graphene based electrodes, (2) Biocomposite fixed in cellcrown, reproduced from (90) and all rights reserved. **(C)** Bladder acellular matrix graft used for urinary bladder reconstruction (a). Urinary bladder augmented with bladder acellular matrix (b). Graft margins marked with nonabsorbable sutures (c), reproduced from (91). **(D)** Schematic illustration on the preparation of the whole bladder scaffold by the self-designed perfusion system and the tissue engineered bladder patches incubated with the omentum for bladder augmentation, reproduced with permission from (92). **(E)** Steps of surgical technique to achieve a double-sized bladder and the schematic view of inverted hourglass technique for blabber augmentation accompanied with the voiding cystourethrography results after the surgery, reproduced from (93).

Solid organs: kidney

Histology and function of kidneys

Kidneys are a complex organ, containing more than 20 types of specialized cells that play a crucial role in maintaining the body's homeostasis (94). Kidneys are responsible for several vital functions such as eliminating waste (specifically ammonia), managing fluid/electrolyte balance and maintaining the metabolic blood acid-base balance. Additionally, they produce/modify hormones that regulate blood pressure, manage calcium/potassium levels and stimulate the production of red blood cells. The renal corpuscle, which includes the glomerulus and the surrounding Bowman's capsule, serves as the filtration unit of the kidney. The tubules of the kidney are responsible for reabsorption and excretion. Together, these components carry out most of the kidney's functions (95).

In mammals, the formation of new nephrons, or nephrogenesis, stops during foetal development or shortly after birth. Although the tubular cells of the kidney have an impressive ability to regenerate in the context of acute kidney injury (AKI), humans lack the ability to produce new nephrons after birth (96).

Epidemiology and current strategies of kidney disease

AKI may lead to chronic kidney disease (CKD) due to the failure to replace damaged renal cells with functional tubular cells which results in tubulo-interstitial fibrosis and scarring, causing CKD (97). CKD is a progressive condition and affects more than 10% of the world's population, which amounts to more than 800 million people (98). CDK is more prevalent in older individuals, women, racial minorities, and in people experiencing diabetes mellitus and hypertension. Additionally, CKD represents an especially large burden in low- and middle-income countries, resulting in millions of people dying every year because they do not have access to affordable and effective care. This led to CKD having one of the highest mortality rates worldwide (97, 98).

CKD often leads to end-stage renal disease (ESRD), where patients either depend on haemodialysis or a kidney transplant to survive due to a loss of kidney function. It is estimated that in 2030, 5.439 million people worldwide will need kidney replacement therapy because of aging of the global population, and an increase in diabetes, cardiovascular diseases and obesity (99, 100). Both renal replacement therapies (i.e. haemodialysis and kidney transplantation) have their limitations: dialysis can only replace a small portion of kidney function and is unable to restore the affected endocrine functions of the kidney. Transplantation, although useful, is limited by the shortage of donor organs and the need for lifelong immunosuppressive therapy after the procedure

(101). As mentioned in the introduction, the demand for organ transplantation far exceeds the available donor pool, shown in **figure 7**. National data from the U.S. organ procurement and transplantation network (OPTN) showed that there are 88,864 candidates as of November 9 (2023) on the current waiting list. However, the number of kidneys transplanted in 2022 were 20,092 (102). Similar data from the Eurotransplant International Foundation (Eurotransplant). Eurotransplant is a service organization for collaborating transplant centers, laboratories and donor hospitals in eight countries (i.e. Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia), representing a combined population of 137 million people. The Eurotransplant data showed that there were 10,083 candidates waiting for a kidney, since October 2023, and only 450 kidneys transplanted in 2022 (103). These data shows that there is a high demand for kidney transplantation but an insufficient supply of kidney donors.

In summary, the current kidney replacement therapies have their limitations. These limitations push the field of TE to develop new kidney replacement therapies to restore kidney function.

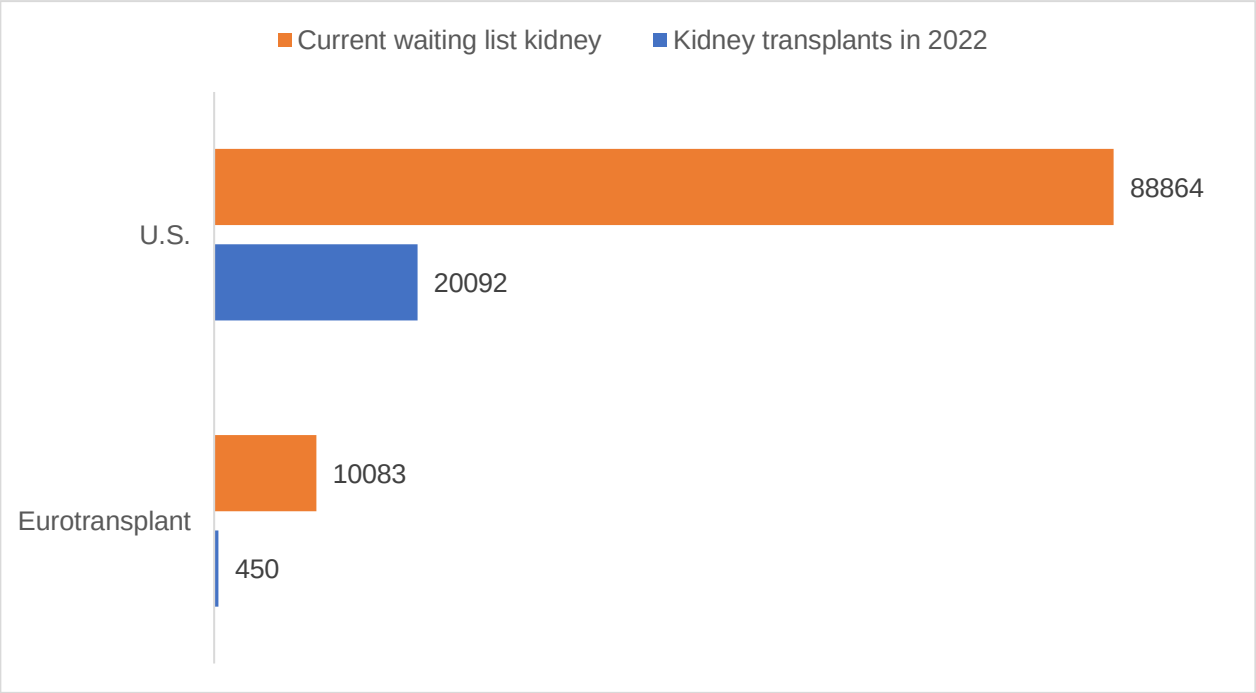


Figure 7. Kidney transplantation versus the current waiting list in the United States and Eurotransplant countries. The Eurotransplant active waiting list since October 2023. The current U.S. waiting list as of November 9, 2023. The data also includes the number of transplantations performed in 2022 in the U.S. and the Eurotransplant countries (102, 103).

Kidney engineering

To address this issue, cell therapy approaches have been developed. For example, cells have been isolated in vitro that produce erythropoietin. These cells could eventually be used to treat anaemia caused by end-stage renal failure in precision medicine (104). Other more ambitious approaches aim to replace the entire kidney function. Although, isolated renal cells can retain their phenotypic and functional characteristics in vitro, transplantation of these cells in vivo may not result in structural remodelling (59).

Additionally, the need to achieve sufficient vascularisation is a universal challenge in many TE applications but is particularly important in renal TE, because adequate vasculature is central to the functionality of renal tissue, despite its role in nutrient and metabolite exchange. Furthermore, all vascular components of the kidney must withstand a large amount of flow and pressure, as the two kidneys together receive 20%-25% of the cardiac output. This is crucial for two reasons: firstly, it facilitates the proper maturation of the renal vasculature. Secondly, it ensures the proper function of an engineered kidney (105).

Many different tissue engineering approaches have been introduced to address the challenge of engineering a vascular network, thoroughly reviewed by Lim et al. (105). They addressed the recent focus on developing bioprinting techniques, that offer a higher level of fabrication control using biocompatible cell-laden bioinks, which is believed to be necessary to recapitulate the complex architecture of the renal vascular network. Because, there are 25-30 different cell types with functional roles that all need to be anatomically placed in the right position for the organ to work.

Also, advances in bioprinting approaches, which have successfully produced stand-alone larger diameter vessels and microvessels, now focus on establishing a superior biomimetic network of microvessels. Additionally, they highlighted the advances in decellularization, recellularization, vascular casting, and organoid development, which demonstrated how vascular networks may be better integrated into renal tissue.

Garreta et al. (106) describe the generation of organoids as one of the biggest scientific advances in regenerative medicine. In 2013, Taguchi et al. (107) achieved one of the early milestones, demonstrating kidney organogenesis in vitro. Next, Takasato et al. (108) developed a differentiation protocol that simultaneously induces all four renal progenitors (nephron progenitors, ureteric bud progenitors, renal interstitial progenitors, and endothelial progenitors) from human iPSCs to generate what are referred to as kidney organoids. Today, various protocols and approaches (i.e. are being applied to produce increasingly more functional organoids that are

proving useful in modelling kidney development and disease. These advancements and challenges are well described in a Nature article (2023) by Eric Bender (109).

A challenge they discussed is the enormous gap between the current organoid research and the clinical needs of patients. Therefore, researchers are attempting to develop auxiliary kidneys. These small functional kidneys could stabilize and maintain the health of patients with kidney failure. Haemodialysis delivers an average of 10% of normal glomerular filtration and clearance rate, which amounts to an average of 50,000 glomeruli. Consequently, to replace haemodialysis treatment, at least 50,000 functional glomeruli are needed, Wiersma et al. (110) argued. In addition, current organoids are small (i.e. ranging up to 0.13 cm^2) and contain a low number of nephrons. Consequently, the current kidney organoids have little function. In order to take kidney organoids towards clinical applications, it is essential to demonstrate that the culture of these human iPSC-derived kidney organoids can be scaled to contain meaningful numbers of nephrons.

To address this challenge, they developed a protocol to create large-scale (i.e. 2.5 up to 12.6 cm^2 , shown in **figure 8.1A**), human iPSC-derived nephron sheets. The nephron sheets can further be developed similarly as a regular organoid (i.e. they can be transplanted and become functional). To investigate the presence of kidney structures in these nephron sheets (i.e. shown in **figure 8.1B-E**), samples were analysed for glomerular structures (NHPS1 and NPHS2), endothelial cells (CD31), proximal tubules (LTL, CUBN), distal tubular structures (ECAD), and stromal cells (MEIS1/2/3 and PDGFRα/β) using confocal microscopy and showed evenly distributed renal structures throughout the sheet. Up to 40,000 glomerular structures were counted in the nephron sheets with a surface area of ~2.5 cm^2, using 3D-imaging techniques. When transplanted into immunodeficient mice, the nephron sheets became vascularized and matured, demonstrating functionality by the reuptake of injected low-molecular mass dextran molecules in the tubular structures. Furthermore, they also developed a protocol for cryopreserving intermediate mesoderm cells during differentiation, which can be successfully defrosted and recovered to make such tissue sheets. Which significantly increases accessibility, since there is no longer a need to maintain a continuous culture of human iPSCs and will allow individual quality control of each batch.

Van Den Berg et al. (111) also worked with organoids and investigated whether organoid maturation increases with longer culturing time. They aimed to examine the degree of glomerular, tubular and vascular maturation across time, between organoids after renal subcapsular transplantation in the absence of any supporting growth factors versus organoids after continued culture in vitro. The human iPSCs were cultured for 7 days in vitro, using a modified version of the

kidney organoid differentiation protocol of Takasato et al. (108). Hence, they used a different culture medium named the feeder-free Essential 8 (E8)-defined medium. The E8 medium improves the efficiency of iPSC derivation and was first introduced by Chen et al. (112). At day 25, the kidney organoids developed completely formed nephron structures, indicated by immunofluorescence staining. It showed the presence of appropriately segmenting nephron structures with glomeruli (NPHS1+), proximal tubule (LTL+) and distal tubule/collecting duct (ECAD+). Also, the organoids contained (NPHS1+) glomerular structures surrounded by (CD31+) endothelial cells and (PDGFR-b+) pericytes. However, formation of the capillary loop inside the glomerular structures was not fully apparent. Scanning (SEM) and transmission electron microscopy (TEM) analysis at day 25 showed a glomerular structure in the organoid that was surrounded by a putative Bowman's capsule. The podocytes inside the Bowman's capsule were aligned and showed small, immature foot processes. In addition, the tubular structures were multilayered epithelial structures with no or a small lumen, with some evidence of apical microvilli forming. However, the multi-layered or pseudo-stratified epithelium, and the lack of a clear tubular basement membrane, suggested incomplete polarization. As suggested by the immunofluorescence, there was no evidence of glomerular vasculature in these organoids. Next, the bisected 25-day old kidney organoids were transplanted under the renal capsule of immunocompromised mice for up to 28 days (i.e. shown in **figure 8.2B**). Toluidine blue staining demonstrated presence of glomerular and tubular structures within the organoid after transplantation (i.e. shown in **figure 8.2C**). SEM analysis suggested the presence of vasculature inside the organoid and the glomerular structures (i.e. shown in **figure 8.2D**). Immunofluorescence staining showed (i.e. shown in **figure 8.2F**) host-derived mouse endothelial cells (MECA-32+) inside the organoid and an invasion of host cells inside glomerular-like structures (NPHS1+ and WT1+). In addition, peritubular vascularization was observed in association with tubular epithelium (i.e. shown in **figure 8.2G**). Furthermore, the combined presence of human CD31+ and mouse MECA-32+ endothelial cells (i.e. shown in **figure 8.2E**) in these organoids at day 14 were observed, showing the contribution of both host and donor to the vascular network. This recapitulates the embryonic development of the kidney vasculature, where both angiogenic hemangioblast precursor cells, as well as vasculogenic endothelial precursors within the organ itself, are required for development of the glomerular microcirculation. In contrast, after 32 days the prolonged culture group observed progressive loss of CD31+ cells. One cause for a lack of vascularization of the glomeruli would be the absence of VEGF production by the podocytes. Therefore, VEGF production by these organoids were evaluated and found increased VEGF levels during time in culture (i.e. shown in **figure 8.2A**).

Next, the kidney organoids matured progressively with time of transplantation. Additionally, after organoid transplantation host-derived peritubular capillaries were observed particularly at 28 days of transplantation, whereas the CD31+ cells that were originally present in the organoids did not show such an organization. This further indicates the progressive development of a renal microcirculatory network.

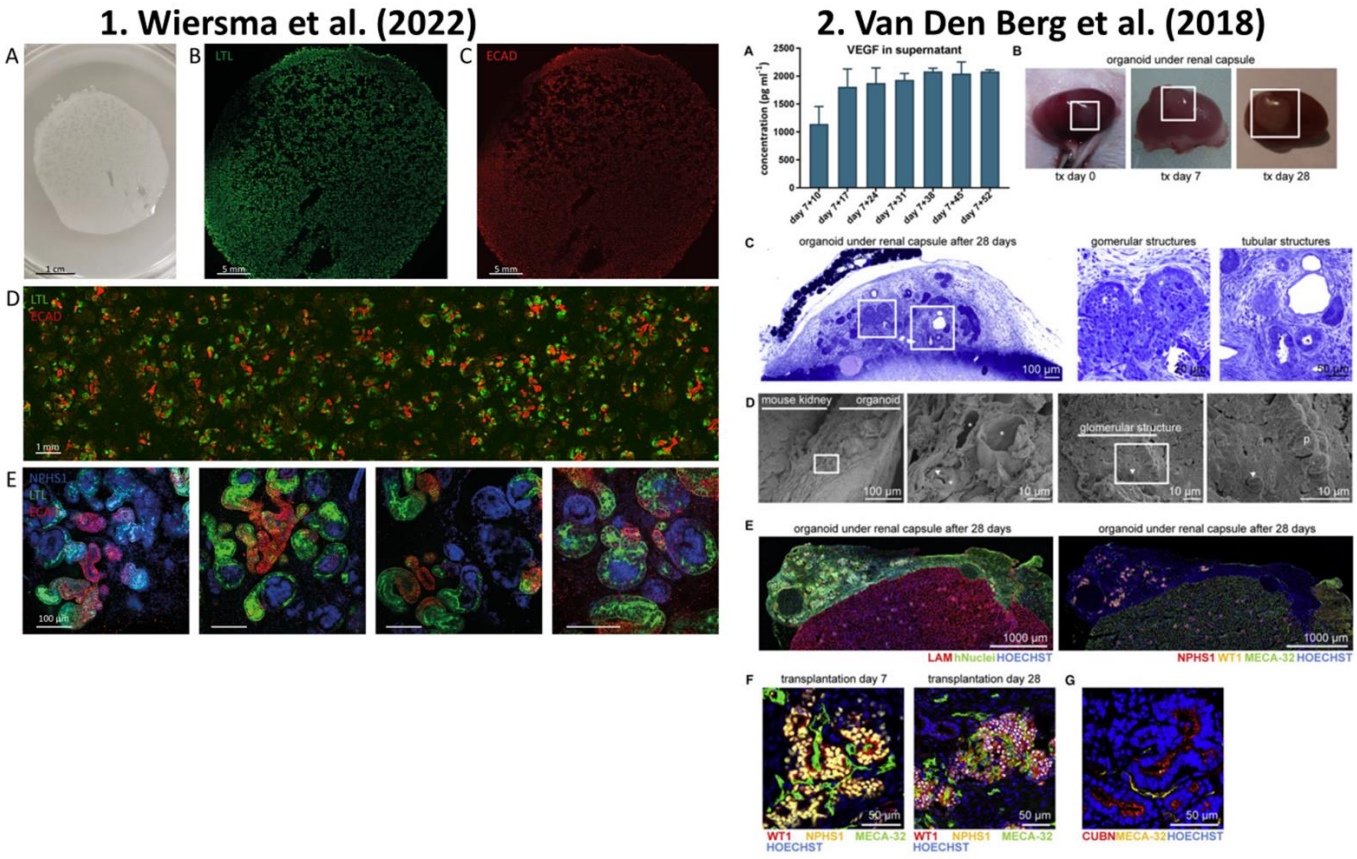


Figure 8. Organoid engineering. **(1)** Upscaling of nephron sheets to a diameter of at least 4 cm. (A) Image of large-scale nephron sheet of 12.6 cm^2 made with a 4 cm diameter cover template (iPSC-MAFB). (B), (C) Immunofluorescence analysis of the entire nephron sheet shows presence and equal distribution of kidney tubular structures stained for proximal (LTL, B) and distal (ECAD, C) markers. (D) Section of the large nephron sheet highlights the distribution of tubular structures stained for LTL and ECAD combined. (E) Detection of nephron structures after immunofluorescence staining for glomerular structures (NPHS1), proximal tubules (LTL), and distal tubules (ECAD), reproduced with permission from (110). **(2)** Kidney organoids become vascularized upon transplantation for 7 and 28 Days. (A) Concentration of VEGF (pg ml^{-1}) determined by Luminex assay in the supernatant of three cultured organoids measured weekly from day 7 + 10 until day 7 + 52. Data are represented as means ± SEM. (B) Transplanted human kidney organoid under renal capsule of mice on the day of transplantation, after 7 and 28 days showing growth upon vascularization. (C) Toluidine blue staining of organoid under the renal capsule after 28 days of transplantation. Boxed areas highlight glomerular and tubular structures displayed on the right. (D) Scanning electron microscopy images suggests blood vessels in the kidney organoid after transplantation and inside a glomerular structure. Close-up views of boxed areas are displayed. (E) Immunofluorescent overview of human nuclei and LAMININ in

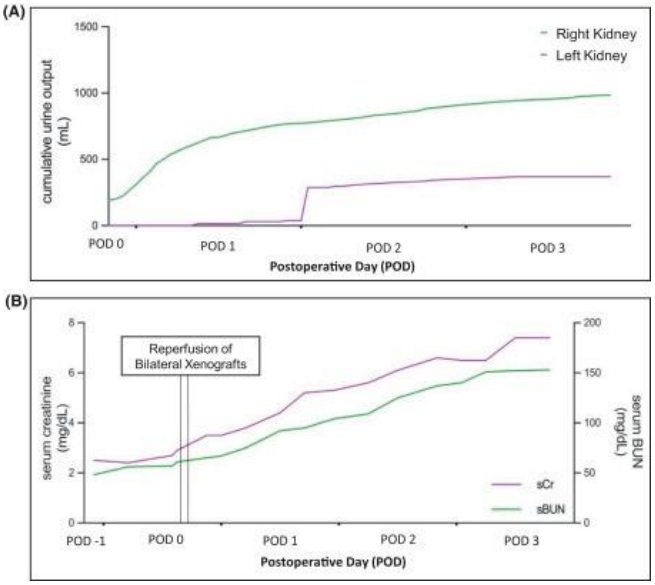
the organoid under the renal capsule of a mouse kidney (left) and integration of mouse endothelial cells (MECA-32+) in the organoid and glomerular structures (right). (F) Mouse endothelial cells (MECA-32+) were observed in association with glomerular structures (NPHS1+, WT1+) in the human kidney organoid after 7 and 28 days of transplantation. (G) Peritubular vascularization observed as MECA-32+ endothelial cells aligning tubular (CUBN+) structures, reproduced with permission from (111).

As mentioned earlier in the introduction, xenotransplantation reached a milestone in 2022 and has the potential to increase the organ donor pool, in an unlimited and renewable fashion. In the two following preclinical trials (11, 12), researchers transplanted genetically modified porcine kidneys into brain-dead human recipients. The genetic modifications were designed to improve transplant outcomes, reduce immune rejection, control organ size and regulate complement, coagulation and inflammation. Hyperacute rejection was not observed, by both studies. Therefore, it was concluded that the elimination of the immunogenic pig protein (i.e. alpha-gal) alone can prevent hyperacute rejection in pig-to-human transplantation. Porrett et al. (12) described that the urine production was initially considerable from the right kidney, but significantly less from the left kidney (i.e. shown in **figure 9.1**). Both left and right kidney did not excrete significant amounts of creatinine into the urine, as a result the serum creatinine did not decrease in the human recipient. The etiology was unclear and likely multifactorial. In contrast, Montgomery et al. (11) observed immediate urine output, doubling of the kinetic eGFR (i.e. from 23 to 62 ml per minute per 1.73 m^2). In both recipients, the creatinine level decreased after implantation of the xenograft, from 1.97 to 0.82 mg/dl in Recipient 1 and from 1.10 to 0.57 mg/dl in Recipient 2 (i.e. shown in **figure 9.2**). Histological findings from Porrett et al. (12), at post-operative day one, were consistent with thrombotic microangiopathy (TMA), with diffuse glomerular capillary congestion, swollen endothelial cells, and near complete obliteration of the peripheral capillary lumina along with the presence of fibrin thrombi. Two days later, there was evidence of progressive tubular injury with extensive acute tubular necrosis, but no additional features of TMA. At both time points: C4d, IgM, IgG, IgA, C1q and C3 were negative. The kidney xenografts were well-perfused and Doppler signals throughout the parenchyma at all time points. However, the study was terminated after day three because of an exsanguinating haemorrhage due to his coagulopathy. After termination, wedge demonstrated no evidence of cortical necrosis or interstitial haemorrhage and glomerular capillary congestion was no longer diffuse. In addition, chimerism was not detected, as evidenced by the absence of the gene expression for a large porcine ribosomal protein (pRPL4).

Histologic and ultrastructural findings by Montgomery et al. (11) after 48 hours showed no interstitial inflammation, tubulitis, arteritis, glomerulitis, peritubular capillaritis, or any C4d deposition in the peritubular capillaries. In addition, wedge biopsies conducted on the removed kidneys (i.e.

54 hours post-reperfusion) did not exhibit any signs indicative of T-cell mediated rejection. Immunofluorescence detected focal C4d deposition at 54 hours in recipient two's xenograft, no signs in recipient one. Furthermore, the glomerular basement membrane maintained its normal thickness and the podocyte foot processes were not affected in both xenografts, showed by EM. In summary, histologic architecture was preserved, both on light microscopy and EM, and the absence of substantial immune-mediated injury.

1. Porret et al. (2022)



2. Montgomery et al. (2022)

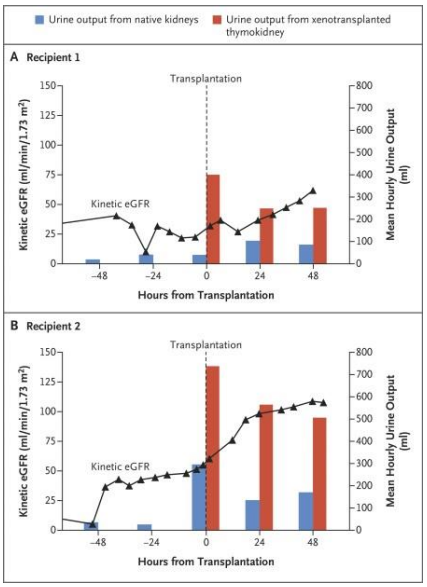


Figure 9. Porcine renal xenotransplant function in the human decedents. **(1)** (A) Cumulative posttransplant urine output from transplantation to study end from right and left xenografts. (B) Creatinine serum levels in the decedent. Results prior to POD 0 reflect function of decedent's native kidneys prior to native nephrectomies, reproduced with permission from (12). **(2)** Shown is the mean hourly urine output from the native bladder and the thymokidney in Recipient 1 (Panel A) and Recipient 2 (Panel B). The kinetic eGFR showed progressive increases after implantation of the kidney, reproduced with permission from (11).

Conclusion

In this section we conclude the results of each engineered tissue structure (i.e. cartilage, urethra, bladder and kidney respectively), shown in **table 1**.

First, the least complex tissue structure cartilage, shown in **figure 10**. There are already cartilage engineered products freely available on the market for articular cartilage and menisci treatments (65). Two out of the five selected trials were clinical trials (68, 69) and the remaining

three were preclinical trials (51, 70, 71) using animal models (i.e. goat, porcine and rat model). The freely available engineered cartilage products and the amount of clinical trials reflects the progress of cartilage engineering research. The two clinical trials (68, 69) use autologous chondrocytes as the cell source. Perhaps because the in vitro culturing of precursor cells (i.e. MSCs) of chondrocytes, makes the methodology more complex. However, MSCs were used as cell source in the preclinical trials, by Browe et al. (70) and Liu et al. (51), in animal models (i.e. goat and rat model respectively). The preferred cell source for cartilage engineering are (autologous) chondrocytes and their precursors (i.e. MSCs). Next, a different engineering technique was used in each trial (i.e. cell-sheets + OWHTO, cell-loaded hydrogel, cell-seeded decellularised matrix, cell-seeded synthetic scaffold, bioprinted scaffold), but the endpoints were the same, to regenerate a cartilage defect. The use of the different techniques in cartilage engineering indicates that no gold standard has yet been found.

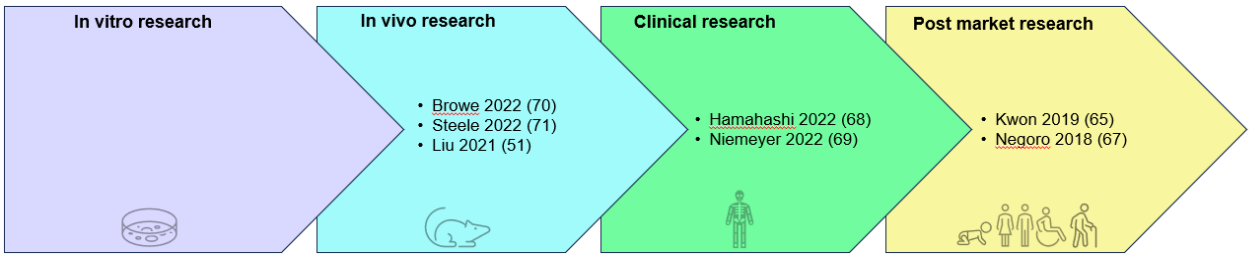


Figure 10. The progress of cartilage engineering.

Second, all the selected urethra engineering studies were preclinical trials, which reflects the progress in urethral engineering research, shown in **figure 11**. The trial by Caneparo et al. (84) was conducted in vitro and the remaining four (74, 80, 81, 83) in vivo (i.e. rabbit model). Notably, Caneparo et al. (84) concluded that their next step forward will be implementing the tissue substitutes (i.e. 90/10 and 80/20 VF/DF mixes) in a rabbit model. The abundance of rabbit models suggests that they are preferred for preclinical in vivo urethra engineering research. The most commonly used cell source is ECs, which are used in all in vivo studies (74, 80, 81, 83). In order to recreate the urethral barrier function. Although, Zhang et al. (80) used a combination of ECs and DFs. Liu et al. (74) used a combination of ECs with SMCs. All four in vivo trials (74, 80, 81, 83) used a cell-seeding scaffold technique (i.e. three synthetic scaffolds and one decellularized matrix scaffold). The in vitro trial by Caneparo et al. (84) used a spheroid-like technique (i.e. a self-assembly technique). In addition, they addressed the limitations of their study. Due to the clinical context the substitute production protocol must be as simple as possible. Therefore, they limited the complexity to only two different cell types (i.e. VFs and DFs), as it allows for obtaining

mechanical resistance and functionality similar to the native urethra. We must also take into account that a thicker tissue is the and leads to ischaemia and graft shrinkage. Urethra engineering consists mainly of techniques using ECs seeded on a scaffold (e.g. synthetic scaffold or decullularised matrix scaffold). Similarly, Pederzoli et al. (72) concluded that urethral engineering strategies primarily involve two components: a scaffold that provides structure, and cells to provide a barrier from transported fluids. Additionally, growth factors can be utilized to guide cell migration, facilitate graft remodelling, and promote vascularization.

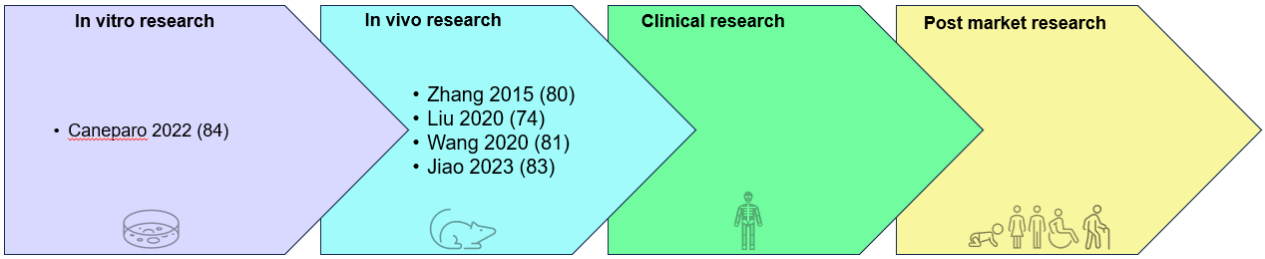


Figure 11. The progress of urethra engineering.

Third, we will discuss the progress of bladder engineering, shown in **figure 12**. One out of the five selected bladder studies was a clinical trial by atala et al. (89). The remaining four were preclinical trials. One in vitro preclinical trial by Adamowicz et al. (90) and three in vivo preclinical trials (91-93). The in vitro trial by Adamowicz et al. (90) developed a new conductive biocomposite scaffold (i.e. Am with a covered graphene layer). However, the introduction of new biomaterials has limitations, the main one being reduced biocompatibility. Thus requiring further testing. Therefore, trials of newer and more unique techniques tend to be at a lower level of the development process (i.e. in vitro trials), due to a lack of available data. The most common used cell source is a combination of UCs and SMCs, to mimic the native bladder tissue. Next, bladder engineering techniques consist of using cell-seeded scaffolds or acellular scaffolds. The following three preclinical trials (91-93) used BAM grafts. The preclinical trial by Pokrywczynska et al. (91) and the preclinical trial by Sabetkish et al. (93) only used BAM grafts to promote bladder tissue regeneration and prevent graft fibrosis. They focused on the recellularization, efficacy and structural function of the BAM grafts. Pokrywczynska et al. (91) noted that the next step forward would be cellularization of BAM scaffolds to prevent graft fibrosis. However, the cellularization can only partially resolve graft fibrosis and further research should be focused on appropriate graft revascularization, which decreases ischemia, necrosis and graft fibrosis. Subsequently, the more recent preclinical trial by Xiao et al. (92) addressed these challenges by seeding the BAM scaffold with ACSs. Furthermore,

to promote vascularization, they used a self-designed perfusion system and four different perfusion decellularization schemes to prepare the BAM grafts. The ASCs planted on the scaffolds could promote SMCs, blood vessels and neurons regeneration. This reflects the evolution of progress in bladder engineering research.

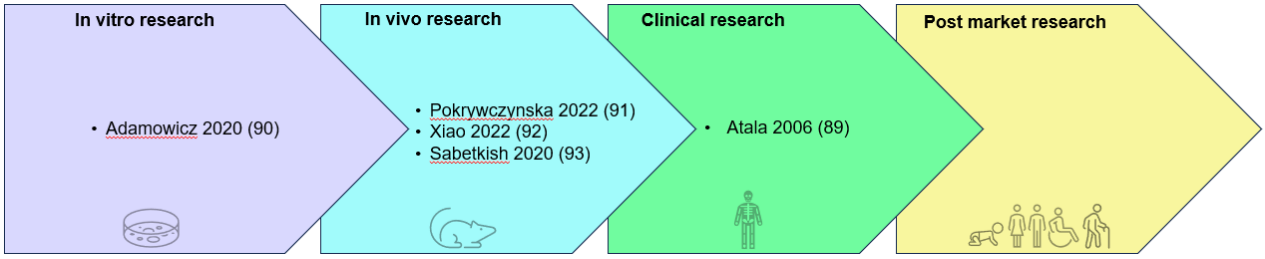


Figure 12. The progress of bladder engineering.

Fourth, whole kidney engineering is still in the preliminary stages of research, due to the complex nature of functional organs as shown in **figure 13**. Although, there is a high demand for alternative kidney replacement therapies. As a result, researchers invent different approaches to mimic kidney function (i.e. cell therapy, kidney organoids, xenotransplantation of genetically modified pig kidneys). Cell therapy approaches have been developed that only replace the endocrine kidney function (i.e. EPO production) (104). Kidney organoids can be used in several ways (e.g. disease model, drug testing models, research model and auxiliary kidney organoids). Auxiliary kidney organoids would not cure patients with kidney failure but would stabilise them. Bender et al. (109) argued, before we can bioengineer a fully functional organ, we must first focus on engineering a tissue that can give a fraction of the function (e.g. 10-20%) to patients. Wiersma et al. (110) produced upscaled a kidney organoid protocol, using human iPSCs-derived nephron sheets in immunodeficient mice, and the ability to cryopreserve the cells during differentiation. These are important steps forward in translating these protocols into future clinical applications, such as auxiliary kidney tissues. Next, Van Den Berg et al. (111) investigated whether organoid maturation increases with longer culturing time due to a lack of long-term kidney organoid protocols. They showed evidence for substantial glomerular, tubular, and vascular maturation only in the presence of a patent functional vasculature, using an immunodeficient mice model. Notably, kidney organoids may provide a suitable technology for kidney treatment (i.e. auxiliary kidney tissue), drug screening, disease modelling and studying kidney regeneration. A different approach is xenotransplantation, which has the potential to expand the donor organ pool in an unrestricted and sustainable fashion. Porrett et al. (12) and Montgomery et al. (11) transplanted genetically

engineered pig kidneys into brain-dead human recipients. The genetic modifications were designed to improve transplant outcomes, reduce immune rejection, control organ size and regulate complement, coagulation and inflammation. The xenotransplantation approach is still very far from the clinic, although this may change in the future. These two preclinical trials (11, 12) showed a proof of concept, with promising results. Although, with limited conclusions, mostly due to the short follow-up.

Figure 13. The progress of kidney engineering.

The application of diverse techniques, cell sources and biomaterials demonstrate that there is no consensus with regard to a gold standard within tissue engineering. Many challenges, such as vascularization, cell maturation and biomimicry, have already been highlighted and how researchers are using a combination of cells, biomaterials and different approaches to replicate the native microenvironment and manage them. This is easier with regard to simple tissue structures (e.g. cartilage). However, complex tissue structures, such as the bladder or kidneys, prove to be more difficult. Although, more future research is necessary, TE remains a promising field of study, with advances offering new opportunities to address the various challenges.

Finally, we address the limitations of this pragmatic review. The first limitation of our study is that we selected relevant and recent articles from scientific databases for this review, this approach may be subject to selection bias. Secondly, this review was not written with the methodology like a systematic review, which could make the reproducing this work difficult. Third, the heterogeneity of the articles makes it difficult to compare things with each other, however it is possible to compare the endpoints of the articles. We selected four tissues for which tissue engineering could provide a solution, in addition to existing treatments. Because of this, other tissues have not been discussed.

	Study	Graft/cell source	Technique	Type of research
Cartilage engineering	Hamahashi et al. (2022)	Autologous chondrocytes	Cell-sheets + OWHTO	Clinical trial
	Niemeyer et al. (2022)	Autologous chondrocytes	Cell-laden hydrogel	Clinical trial
	Browe et al. (2022)	MSCs	Cell-seeded decellularized matrix	Preclinical trial (goat model)
	Steele et al. (2022)	Chondrocytes	Cell-seeding scaffold (synthetic)	Preclinical trial (porcine model)
	Liu et al. (2021)	MSCs	Bioprinted scaffold	Preclinical trial (rat model)
Urethra engineering	Zhang et al. (2015)	ECs and DFs	Cell-seeding scaffold (synthetic)	Preclinical trial (rabbit model)
	Liu et al. (2020)	ECs and SMCs	Cell-seeding scaffold (synthetic)	Preclinical trial (rabbit model)
	Wang et al. (2020)	ECs	Cell-seeded decellularized matrix	Preclinical trial (rabbit model)
	Jiao et al. (2023)	ECs	Cell-seeding scaffold (synthetic)	Preclinical trial (rabbit model)
	Caneparo et al. (2022)	DFs and VFs	self-assembly (spheroid-like)	Preclinical trial (in vitro)
Bladder engineering	Atala et al. (2006)	Autologous UCs and SMCs	Cell-seeded scaffold (biodegradable)	Clinical trial
	Adamowicz et al. (2020)	UCs and SMCs	Cell-seeded scaffold (biocomposite)	preclinical trial (in vitro)
	Pokrywczynska et al. (2022)	BAM graft	Decellularized matrix	Preclinical trial (porcine model)
	Xiao et al. (2022)	ASCs	cell-seeded decellularized matrix	Preclinical trial (rat model)
	Sabetkish et al. (2020)	BAM graft	Decellularized matrix	Preclinical trial (rabbit model)
Kidney engineering	Wiersma et al. (2022)	iPSCs	organoid	Preclinical trial (in immunodeficient mice)
	Van Den Berg et al. (2018)	iPSCs	organoid	Preclinical trial (in immunodeficient mice)
	Porrett et al. (2022)	Genetically engineered porcine kidneys	Xenograft transplantation	Preclinical trial (brain-dead human recipient)
	Montgomery et al. (2022)	Genetically engineered porcine kidney	Xenograft transplantation	Preclinical trial (brain-dead human recipients)

Table 1. A simplified overview of each graft/cell source, technique and type of trial for each tissue structure and study. First, the studies for cartilage engineering (51, 68-71). Second, the studies for urethra engineering (74, 80, 81, 83, 84). Third, the studies for bladder engineering (89-93). Fourth, the studies for kidney engineering (11, 12, 110, 111).

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Appendix

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18 November 2023

Oscar Lemmens Arne Peirsman

UNIVERSITEIT GENT

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

Bijlage 1

Academiejaar 2022 – 2023

Masterproef I: LOGBOEK

A. Persoonlijke gegevens:

Naam: Oscar Lemmens

Promotor(en): Phillip Blondeel

Co-promotor(en): Arne Peirsman

TITEL: Tissue engineering: the road to the clinic.

Vakgroep/Dienst: Plastische heelkunde

B. Contactmomenten

Datum	aanwezigen	onderwerp
15 oktober 2022	Oscar Lemmens, Arne Peirsman	MS Teams gesprek, eerste introductie tot het onderwerp
12 november 2022	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: concreet afspraken maken ivm introductie schrijven
24 november 2022	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: feedback ivm introductie
25 februari 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: update na de examenperiode
1 april 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: concreet afspraken maken
10 april 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
16 april 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
1 mei 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
13 mei 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback

C. Eerste versie Inleiding, Vraagstelling, Methodologie: ingeleverd

Bij: Arne Peirsman

Op: 19 mei 2023

Voor akkoord,
Datum, handtekening (co-)promotor
Arne Peirsman, M.D. Ph.D. Fellow

UNIVERSITEIT GENT

FACULTEIT GENEESKUNDE EN GEZONDHEIDSWETENSCHAPPEN

Bijlage 1

Academiejaar 2023 – 2024

Masterproef II: LOGBOEK

A. Persoonlijke gegevens:

Naam: Oscar Lemmens

Promotor: Phillip Blondeel

Co-promotor: Arne Peirsman

TITEL: Tissue engineering: the road to the clinic

Vakgroep/Dienst: Plastische heelkunde

B. Contactmomenten

Datum	aanwezigen	onderwerp
11 oktober 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
21 oktober 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
26 oktober 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
5 november 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback
11 november 2023	Oscar Lemmens, Arne Peirsman	MS Teams gesprek: vooruitgang bespreken/ feedback

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Figure 4A

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