Towards a tissue engineered heart valve for life

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TOWARDS A TISSUE ENGINEERED
HEART VALVE FOR LIFE

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TOWARDS A TISSUE ENGINEERED HEART VALVE FOR LIFE

PROEFSCHRIFT

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voor Winnie
# CONTENT

| Summary | III |
| Chapter 1 | General Introduction | 1 |
| Chapter 2 | Improved Geometry of Decellularized Tissue Engineered Heart Valves to Prevent Leaflet Retraction | 15 |
| Chapter 3 | The Effects of Scaffold Remnants in Decellularized Tissue Engineered Constructs on the Recruitment of Blood Cells | 33 |
| Chapter 4 | Computational Designed 3D Printed Self-Expandable Polymer Stents with Biodegradable Capacity for Minimally-Invasive Heart Valve Transplantation | 51 |
| Chapter 5 | In Vivo Remodeling of Decellularized Tissue Engineered Heart Valves During One Year Follow Up | 67 |
| Chapter 6 | General Discussion | 85 |
| Appendix | Patent Application | 101 |
| References | 107 |
| Samenvatting | 125 |
| Dankwoord | 127 |
| Curriculum Vitae | 131 |
| Publications | 133 |
SUMMARY

TOWARDS A TISSUE ENGINEERED HEART VALVE FOR LIFE

Annually, 280,000 people worldwide require a heart valve transplantation. Current available prostheses are life-saving devices, but lack growth potential. This is a major drawback for pediatric patients that suffer from congenital heart diseases and need staged interventions to accommodate for growth, leading to increasing risks for morbidity and mortality.

Decellularized tissue engineered heart valves (DTEHVs) have shown great potential to overcome these limitations. From previous long-term in vivo studies, it appeared that these valves have fast regenerative capacity, but also developed severe insufficiency over time. The mode of failure was a gradual decrease in leaflet size, most likely as a result of valve leaflet retraction. Based on computational simulations, this failure mechanism was explained by the valvular geometry, being subjected to tissue compression rather than stretch, under physiological loading conditions.

An improved geometry was suggested to enable tissue stretch and counteract for cell-mediated leaflet retraction, by enlarging the coaptation area and enhancing the belly curvature. In order to impose this improved geometry during tissue culture, a constraining bioreactor insert was developed and patented, whereby human cell based DTEHVs were produced. These valves maintained functional in an in vitro test system up to 16 weeks implantation simulation and revealed a considerable decrease in radial tissue compression.

Currently, these DTEHVs are implanted trans-venously by making use of a metal stent. To accommodate for growth of such stented valves, the feasibility for a self-expandable biodegradable stent was investigated by using 3D printing technology and a flexible thermoplastic copolyester. Computational models were used to design biodegradable polymer stent devices that exert radial forces comparable to metal alternatives, with minor plastic deformation after crimping.

So far, DTEHVs have shown to possess the unique capacity for fast recellularization. These valves still retain scaffold remnants after implantation, which might trigger rapid cell infiltration. Based on in vitro experiments wherein tissues and scaffolds were exposed to
circulating cells under physiological flow conditions, it was shown that peripheral blood cells actively infiltrate into the decellularized tissue engineered constructs and migrate towards these scaffold remnants, where the secretion of inflammatory cytokines was suppressed in the presence of scaffold remnants.

To investigate the effects of the improved DTEHV geometry on mechanical functionality and remodeling, these DTEHVs have been implanted into sheep, where they remained competent up to one year. These results show the importance of geometry on valve functioning, and their effect on cell mediated leaflet retraction. Furthermore, preliminary results show tissue regeneration over time, which observations were explained by computational simulations.

In conclusion, this thesis describes a solution to impose and improve the geometry of DTEHVs and demonstrated that scaffold remnants within these constructs are responsible for active recellularization. Furthermore, it shows the feasibility for developing self-expandable biodegradable polymer stents to enable minimally invasive valve replacements that can accommodate for future growth. Finally, this thesis describes the importance of valvular geometry on trans-venous implanted DTEHVs, which are now fully functional for one year in sheep, showing active tissue remodeling.

These improved DTEHVs are holding a significant promise to represent a tissue engineered heart valve for life.
CHAPTER 1

GENERAL INTRODUCTION
1.1 Heart Valve Diseases

1.1.1 What are Heart Valve Diseases?

Heart valves open and close 40 million times per year. That is 3 billion times over a lifetime of 75 years (1). Unfortunately, proper function of these delicate tissue constructs is not straightforward. Heart valve diseases resulted world-wide in 290,000 patients in 2003, that needed a transplantation, which number is expected to triple to 850,000 by 2050 (2).

Heart valve diseases can result in valvular stenosis (blocking) or regurgitation (leaking) (3). Stenosis is the result of stiffened leaflets that reduces the opening area of the valve and causes a reduction in forward blood flow (Fig. 1.1A). Regurgitation occurs when the leaflets do not close properly, resulting in an inversed blood flow backwards into the heart (Fig 1.1B). Both phenomena can lead to severe heart failure.

**Figure 1.1** | Valve Diseases: Heart valve diseases can lead to stenosis, thereby obstructing the outflow tract (A), or result in increased regurgitation due to inappropriate closing behaviour (B).
1.1.2 How do heart diseases occur?

**Acquired valvular diseases**

Valvular insufficiency can develop during life, having multiple causes. Calcification is a disease that stiffens the leaflets and results in stenosis. Although this disease has been associated with aging, ongoing debate is making it more clear that the pathology behind the disease is not exclusively dedicated to a senile event causing tissue degeneration (4). Current research is investigating the relation of calcification with underlying diseases such as diabetes (5) and kidney failure (6). Other causes leading to infections such as endocarditis and rheumatic fever, cause inflammation and leads to thickening, merging, and even shortening of the leaflets, making these valves prone to regurgitation (7). Continuous high blood pressure can lead to dilatation of the valvular annulus, which will result in a size mismatch of the leaflets and will eventually lead to regurgitation (8).

**Congenital valvular diseases**

Heart valve diseases can also have a congenital origin. 75 per 1000 babies are born with congenital heart diseases (CHD), from which around 8% suffer from a severe or moderate form that will require surgery (9). Most CHD are caused by genetic defects such as Tetralogy of Fallot (TOF) (also known as the blue baby syndrome) (10), which occurs in approximately 5% of all CHD (11), and mostly requires replacement of the pulmonary heart valve (12,13). Other CHD such as pulmonary atresia can even result in the complete absence of a pulmonary heart valve (14).

1.1.3 What are the current treatment options?

**Surgical intervention**

Surgical procedures of valve repair and replacement used to be done mainly via open-heart surgery (15). These types of operations result in severe tissue damage, and leaves a big scar in front of the chest (16). New evolving technological advances make it now possible to perform increasing numbers of surgical procedures to be executed with so called minimally invasive surgery, by entering the heart using small incisions between the ribs, or via the vascular system using catheter devices. These types of surgical interventions are less invasive and are associated with reduced morbidity (17). Even more so, they improve patient satisfactory and reduce overall health care costs (18).

**Repair surgery**

Depending on the type and severity of the heart valve defect, repair surgery can be an option to fix a malfunctioning heart valve, in which the native valve remains operational. This type of surgery can have advantages in several diseases, such as endocarditis (19),
and is less prone to infections (20). In the case of merged leaflet events, tissue separation can be performed to widen the opening of the valve (21). Decalcification is a technique in which the calcium deposition on the valve is being removed to improve open and closing behavior, however the durability of this technique is still controversial (22). Patching is performed in case of small holes or ruptures in the leaflet tissue (23). And in the case of a dilatation of the annulus, the valve can even be reshaped or supported (24).

**Replace surgery**

A heart valve replacement is required when the disease cannot be treated by means of repair surgery (25). At the moment there are two types of valvular prosthesis that can be chosen from, which are the mechanical heart valve or the biological heart valve, each having their own pros and cons (26). Depending on the age and lifestyle of the patient, the best suitable heart valve prosthesis will be chosen (27).

**Mechanical heart valve**

Mechanical heart valves are entirely made out of non-biological materials and can have different designs, such as ball-and-cage, mono-leaflet, and bileaflet valves, which are embedded in a suture ring to be fixed in the annulus (Fig. 1.2A). Mechanical valves are very durable and can be functional over 25 years (28). However, these valves have suboptimal fluid dynamics and patients must take lifetime blood-thinning medication to prevent blood clot formation (29).

**Biological heart valve**

The alternative to the mechanical heart valve prosthesis is the biological valve that can be made out of animal (xenogeneic), human donor (allogeneic) or patients own (autograft) tissue material (Fig. 1.2B). The xenogeneic valves are sterilized and fixated to prevent tissue deterioration and graft rejection (30). Human donor valves, or even the entire annulus, can also be transplanted (31,32). Limitations of these valves are their availability, but become useful in cases where the annulus needs to be replaced while the human anatomy remains maintained (33). Biological valves have a limited durability, requiring reoperation in 20-30% within 10 years (34,35). However, the need for long term medication can be reduced since these valves are made out of biological material being less prone to thrombosis (36).
1.1.4 **What are we looking for?**

Currently used heart valve prostheses are life saving devices. However, the current available treatment options have some major limitations especially for patients born with congenital heart diseases (1). Besides the lifelong medication treatment for the mechanical prostheses and the limited durability of the biological valves, all available prosthetic valves have limited regenerative and growth capacity (37). These young patients have to undergo multiple staged interventions to compensate for a size mismatch, associated with increasing risks for morbidity and mortality. This emphasizes the urgent need for heart valves prostheses that can fully regenerate and grow with the host. Tissue engineered heart valves are raising high hopes to overcome the current limitations to provide a lifetime solution (2). These valves can be available off-the-shelf (38), and even show evidence to pose regenerative capacity (39). In addition to providing a lifetime solution, these valves are currently under development for their compatibility with minimally invasive surgery (40,41).

1.2 **Heart Valve Tissue Engineering**

1.2.1 **In Vitro Tissue Engineering**

The concept of tissue engineering is to isolate cells from a patient (autologous cells) or donor (allogeneic cells), which will be used for the production of a tissue construct in a
laboratory (42). The first attempt to use tissue engineering for heart valve application, dates back to 1995, where tissue engineered heart valves (TEHVs) were implanted into sheep (43,44). Since then, a lot of progress has been made, taking heart valve tissue engineering to a higher level.

**Scaffold Material**

Proper scaffold material is essential in order to steer and control the shape of the TEHVs, which needs to fulfill certain criteria. These materials have to be biocompatible and bioresorbable, provide sufficient mechanical support, and have optimal architecture properties to support cell infiltration (45). Bioresorbable materials can either be natural-based or synthetic. Advantage of natural based materials is that these are expected to be biocompatible and can be regenerated after implantation (34). Examples of frequently investigated natural based biomaterials in heart valve tissue engineering are collagen and fibrin (46,47). However, these biological materials are hard to obtain from human origin, and are therefore mostly derived from animal origin, increasing the risks of zoonosis (48). Besides, pure natural–based materials are difficult to process and lack sufficient mechanical properties in terms of strength and durability (49). To overcome these limitations, natural-based materials can be combined with, or even be totally replaced by synthetic materials.

The advantage of synthetic materials is that the degradation speed and mechanical performance can be tuned, materials are more easily accessible and have better processability (50). For cardiovascular applications these synthetic materials are mostly aliphatic polyesters based on poly(glycolic acid) (PGA) (43), poly(lactic acid) (PLA) (51), poly(caprolactone) (PCL) (52) and poly(hydroxyalkanoate) (PHA) (53). These polymer materials are susceptible to hydrolysis and enzymatic degradation because of the ester bonds, which is beneficial for in vitro and in vivo applications (54). Since all polyesters have different mechanical properties and degradation speeds, blends can be made and coatings can be applied to meet the desired requirements (50).

Within the past 15 years of heart valve tissue engineering, our group has gathered vast experience in using a combination of porous PGA meshes coated with P4HB (belonging to the group of PHA polymers), providing the right mechanical properties and degradation speed that is desired for this specific application (38-40,55)

**Cell Source**

Different cell types have been investigated for the ability to synthesize extracellular matrix (ECM) for TEHV application. Myofibroblasts are an interesting cell source because of their capacity for fast proliferation and collagen synthesis. These cells can be isolated from the
vasculature of the patient, involving a surgical intervention to obtain a tissue sample (56). To prevent multiple surgical interventions in an autologous approach, alternative cell sources were investigated to be isolated in a less invasive way, and induced towards myofibroblast-like cell types for their use in tissue engineering application, such as prenatal cells from the amniotic fluid (57) and umbilical cord cells (58). Also adult stem cells have been investigated for this application. These can be mesenchymal stem cells originating from the bone marrow (59), or even be adipose stem cells that can be obtained from fat tissue (60).

**Tissue Formation**

Different types of stimuli can be applied during culture to steer cell and tissue formation and orientation in tissue engineered constructs. Tissue producing myofibroblasts can sense environmental cues, such as static and dynamic strains, being present in the heart valves (61). Cells respond to these cues by aligning the collagen fibers in the direction of the largest strain (62). Besides myofibroblasts can sense differences in stiffness, by tuning their internal stiffness, enhance proliferation, and increase traction forces towards more rigid substrates (63-65). Also topographical cues can be used to steer tissue formation. Contact guidance is such an example whereby cells adhere and follow the direction of individual scaffold fibers. In a balance between local fiber stiffness and applied strain, collagen can be guided into a preferred orientation (66). This technique is used in heart valve tissue engineering to steer collagen into a preferred circumferential direction (67). Also constraints can be applied to steer collagen architecture. Myofibroblasts have the capacity to align in the direction of the constraint and compact the tissue in the unconstrained direction, by which collagen alignment can be created (68).

However, there is a delicate interplay between all these individual components, which will eventually define how tissue formation and collagen orientation will evolve in vitro during culture, as well as their preservation and development after implantation.

For in vitro heart valve tissue engineering, all these mechanical cues are combined inside a bioreactor system that is used to stimulate the construct in order to improve tissue formation (69). In these systems, the subjected hydrodynamic load will create flows and pressures over time that will be sensed by the cells, in which tissue formation and collagen alignment will depend on the valvular geometry, scaffold material and scaffold architecture. Different heart valve bioreactors have been developed by different research groups around the world, where each system has its own advantage to promote and control tissue formation by tuning specific individual cues. This makes it that to date, no standardized bioreactor systems are defined that provide the best outcome for heart valve tissue engineering (70).
Chapter 1

**Decellularization**

Implantation of living patient specific constructs was for a long time the standard tissue engineering paradigm. Since the synthesized biological content was not foreign material, it was expected that the body would accept these constructs and prevent immune rejection (71). However, this concept appeared to be time critical for living tissue engineered heart valves and can initiate a response from the host to the implant, resulting in thickened leaflets (40). Other studies in which decellularized matrices or even entire organs were used as bio-inductive materials to be reseeded with autologous tissue producing cells, gained special attention (72). Advantages of these decellularized materials would be that they provide sufficient mechanical integrity and act as inductive templates to cells for constructive remodeling (73). However, their recellularization efficiency is still under debate (74-76). Overall, in order to bring living constructs to the patient, several regulatory and financial hurdles still have to be taken before this approach can be become available on larger scales (77-79).

To overcome these limitations, special interest is currently focusing on the regenerative capacity of decellularized constructs without pre-seeding in the body. Although promising results in terms of host body recellularization was obtained in animal models (80,81), large mortality rates occurred due to foreign body immune rejection when implanting these decellularized xenogeneic valves in children (82). This emphasizes that proper decellularization is extremely important to prevent any immunogenic response, since it has been proven that even small remnants of xenogeneic proteins, can already initiate a large inflammatory response (83,84). Although the usage of healthy human donor heart valves are proper alternatives (85), their limited availability will be the major bottleneck for large-scale applications.

To prevent the lack of xenogeneic disease transmission and to overcome the shortage of donor limitation while maintaining recellularization potential, decellularized tissue engineered heart valve (DTEHV) constructs might represent a promising alternative. This technique has been proven to be functional for heart valve applications showing regenerative potential and offers off-the-shelf available heart valve replacements (39).
1.2.2 IN SITU TISSUE ENGINEERING

The implantation of non-living prostheses to trigger host cell infiltration for future regeneration is a relative new and emerging field, called in situ tissue engineering. Although this approach seems to work for synthetic materials (86,87), the recellularization potential of biological tissue remains arguable (88). Within this scope, either DTEHVs (Fig. 1.3A) or even entirely synthetic constructs (Fig. 1.3B) are being investigated nowadays for heart valve tissue engineering applications (89). Both treatment options have their own pros and cons. A decellularized construct has the advantage that the infiltrating cells can instantly remodel and maintain the implanted matrix, but requires more expensive and labor intense processing. On the other hand, synthetic implants might eventually be cheaper and more scalable since the entire phase for in vitro tissue engineering can be skipped. However, it requires sophisticated engineering to secure the right balance between in vivo degradation and tissue synthesis on the other hand (90).

**Figure 1.3** | In Situ Approaches: In situ heart valve implants can be decellularized matrices (A) or synthetic materials (B), with the aim to regenerate inside the body. Decellularized matrices are expected to be less immunogenic, were the production of suitable synthetic scaffolds is more time efficient. Both pathways are currently being investigated for heart valve applications.
**Immune-Guided Regeneration**

To enable in-situ tissue engineering technology, the regenerative capacity of the immune system needs to be properly activated and controlled to guide regeneration that results in healthy and functional tissue. From basic wound healing it is known that tissue injury or infection is normally addressed in well-orchestrated and defined stages to resolve tissue damage (91). In the first hemostatic phase, proteins from the blood will deposit on the biomaterial to which white blood cells can adhere (92). Also blood platelets get activated and coagulation is initiated. In the second inflammatory phase, immune cells are recruited towards the site of injury via damage associated molecular patterns (DAMPs) or pathogen associated molecular patterns (PAMPs) (93). Initially, these are granulocyte cells from the innate immune system being predominantly neutrophils, but also eosinophils (10%) and basophils (1%) (Fig. 1.4)(94). Later on, phagocytic and antigen presenting cells, like macrophages and dendritic cells will infiltrate, followed by more specialized cells from the adaptive immune system such as T-cells and B-cells (Fig. 1.4) (91). Antigen presenting cells will trigger the activation of the adaptive immune system, and depending on the antigen interaction, will result in cellular activation and create a cytokine environment that is either favorable to tissue regeneration or fibrosis (95).

One of the key players in steering the regeneration process is the macrophage. Upon activation, macrophages can differentiate into certain subsets, driving either a regenerative resolution via a so-called M2 phenotype, or a hypertrophic scarring response via the M1 phenotype (83,96,97). In order to steer the macrophage polarization towards a desired polarization of M2 macrophages, current engineering approaches are incorporating regulating peptides into the scaffold material (98), apply changes in geometrical parameters such as pore size and scaffold fiber diameter (99), or control

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**Figure 1.4** | Immune Cells: Different subsets of blood cells can be defined, each with their own function in the immune system. Most important target cells for immune regeneration are the white blood cells, which can roughly be divided into a granulocyte cell fraction and an agranulocyte fraction.
strain levels (100), which are thought to influence macrophage polarization. Other attempts to obtain a favorable immune response is to pre-seed the implants with freshly isolated bone marrow cells prior to implantation, which principle was shown for heart valve applications in non-human primates (101). This procedure is hypothesized to be immunosuppressive via initial paracrine effects of the bone marrow cells, which resulted in healthy tissue formation in vascular tubes (102,103).

**CIRCULATING PROGENITOR CELLS**

After the first infiltration of immune cells, an endothelial lining and tissue-producing cells are needed to properly maintain the ECM. Therefore, circulating progenitor cells from the peripheral blood are targeted to repopulate these constructs. It has already been shown that endothelial progenitor cells could be obtained from the blood (104). Although the exact origin of these progenitor cells is still under debate, so far it is thought that a small cell fraction in the blood has a high proliferative potential with an endothelial-like phenotype and haematopoietic stem cell markers, that are called endothelial colony-forming cells (ECFC) (105). The interesting finding of these cells is that they can aid in re-endothelialization, which is eventually required for heart valve prosthesis (106). Overall, endothelial cells could than transdifferentiate towards mesenchymal and myofibroblast phenotypes, via a so called endothelial-mesenchymal transition (EndoMT), which cells can produce for instance collagen type I (107). Another interesting circulating cell source with regenerative capacity are the fibrocytes (108). These cells also have haematopoietic stem cell markers and can differentiate into spindle-shaped cells, with the capacity of abundant collagen type I and II formation (109). Also smooth muscle progenitor cells have been described in literature to be circulating in the blood, expressing α-SMA and myosin heavy chain (110). Current research is still ongoing to elucidate the exact repopulation mechanism.

1.3 **STENT DEVICES**

Stents are used in numbers of clinical application to open obstructed tubular constructs, such as vessels, arteries, the esophagus, intestines, the urinary tract, and even the biliary duct (111-114). Also to make heart valve transplantation compatible with minimally invasive surgery, stents play a crucial role in anchoring the valve into the artery instantly after deployment as in transcatheter aortic valve implantations (TAVI) (115).
1.3.1 Metal Stents

Most of the stents are made out of metallic materials. Once brought in place inside the body, the final diameter can be precisely controlled via balloon inflation, giving better clinical outcomes compared to standard angioplasty (116). Overall, these stents can withstand large forces, which is important for calcified regions (117). For stenting interventions in regions that are prone to large mechanical strains, self-expandable stents are more favorable (118), since self-expandable stents can recover in large deformation regions, which is not the case for balloon expandable stents (119,120). For heart valve applications, both stent types are currently used (121).

The metallic stent devices have some major limitations because of their permanent presence that can cause stenosis due to neointima tissue formation (122). Besides they will hamper healthy vessel regeneration and vasomotion (123), and interfere with minimal invasive imaging techniques (124). Especially for pediatric patients, these metal stents have the additional limitation that they do not accommodate for growth, which leads to multiple reoperations (125).

1.3.2 Degradable Stents

Biodegradable stents could theoretically overcome all these limitations. For stenotic vascular applications they have the potential to restore vascular functionality right after implantation, where they disappear once the artery regenerated. But even more appealing for pediatric applications, these stent could thereby accommodate for growth potential (126).

Biodegradable stents are already used for coronary application, were they have shown that by two years of implantation, the vascular motion was restored after the stent was dissolved (127). Biodegradable alternatives for larger diameter applications remain limited. Conventional available degradable polymer materials have difficulties providing sufficient mechanical force causing stent migration (128), where metallic biodegradable materials, such as magnesium, degrade too fast (129). Because of the lack of suitable materials that have the optimal combination of sufficient mechanical integrity, proper degradation speed and the compatibility of handling large strains during minimally invasive surgery, makes it that to date no suitable stents with biodegradable capacity are available for pediatric patients (130).
1.4 Rational and Outline

1.4.1 Current Status

Recently, the feasibility of decellularized tissue engineering heart valves for in situ regeneration, has been investigated in both sheep and non-human primate models (39,55). For the first time it was shown that these prosthetic valves have quick recellularization potential already after 5 hours of implantation. Furthermore, increasing content of ECM was present and the valves remained fully functional for 8 weeks of implantation. However, up to 24 weeks severe insufficiency was developed over time due to leaflet retraction, associated with α-SMA positive cell infiltration from the adjacent vascular wall. In an attempt to find a solution for this retraction problem, the current valvular geometry was investigated via numerical computational simulations (131). From these results it was shown that the current tissue engineered heart valve geometry is prone to tissue compression in radial direction when being exposed to hemodynamic load, which might explain leaflet retraction in the presence of contractile α-SMA positive cells (64,132). Based on these simulations, geometrical improvements have been suggested in an attempt to counteract for these cell mediated retraction forces (131). However, the heart valve geometry can poorly be controlled during in vitro tissue engineering procedures.

1.4.2 Thesis Outline

To address the need for controlling the shape of tissue engineered heart valves as been described above, in chapter 2, a solution is found to impose the valvular geometry during in vitro culture and decellularization, resulting in fully functional DTEHVs with an enlarged coaptation region and profound belly curvature, being less prone to host cell leaflet retraction.

Based on the pre-clinical animal studies, rapid recellularization of DTHEVs with circulating blood cells was observed within hours after implantation (39). Although this fast infiltration response might be beneficial for future regenerative and growth potential, the underlying mechanism is poorly understood. The fast infiltration response was thought to be triggered by the presence of scaffold remnants inside these DTHEVs constructs. In chapter 3 this hypothesis was investigated and confirmed based on in vitro flow experiments, were granulocytes are shown to actively infiltrate into to these non-living constructs and migrate towards the scaffold remnants.
Chapter 1

A growing valve concept in combination with minimally invasive surgery would be applicable for pediatric patients in the future, when the stent is biodegradable. In chapter 4, a proof-of-concept study shows the potential of using polymer materials for biodegradable heart valve stenting applications, investigated via 3D printing technology. Based on computational simulations, functional prototypes were made with competitive mechanical properties to metal alternatives, suitable for minimally invasive application while providing biodegradable capacity.

To see if the geometrical adjustments on the DTEHVs would eventually prevent in vivo leaflet retraction after infiltration of contractile host cells, DTEHVs with improved geometry have been implanted into sheep via minimally invasive transvenous surgery. In chapter 5, preliminary data shows promising results where these valves maintain full functionality up to one year in sheep, with indicative signs of collagen remodeling in circumferential direction, in accordance to computational simulations.

Finally in chapter 6, the main findings of this thesis are being discussed, and their clinical potential is evaluated. Further more, suggestions are made for improvements and how to deal with the next hurdles to bring this promising technology towards clinical application.
CHAPTER 2

IMPROVED GEOMETRY OF DECELLULARIZED TISSUE ENGINEERED HEART VALVES TO PREVENT LEAFLET RETRACTION

The content of this chapter is based on:

2.1 ABSTRACT

Recent studies on decellularized tissue engineered heart valves (DTEHVs) showed rapid host cell repopulation and increased valvular insufficiency developing over time, associated with leaflet shortening. A possible explanation for this result was found via computational simulations, which revealed radial leaflet compression in the original valvular geometry when subjected to physiological pressure conditions. Therefore, an improved geometry was suggested to enable radial leaflet extension to counteract for host cell mediated retraction. In this study, we propose a solution to impose this new geometry by using a constraining bioreactor insert during culture. Human cell based DTEHVs (n=5) were produced as such, resulting in an enlarged coaptation area and profound belly curvature. Extracellular matrix was homogeneously distributed, with circumferential collagen alignment in the coaptation region and global tissue anisotropy. Based on in vitro functionality experiments, these DTEHVs showed competent hydrodynamic functionality under physiological pulmonary conditions and were fatigue resistant, with stable functionality up to 16 weeks in vivo simulation. Based on implemented mechanical data, our computational models revealed a considerable decrease in radial tissue compression with the obtained geometrical adjustments. Therefore, these improved DTEHV are expected to be less prone to host cell mediated leaflet retraction and will remain competent after implantation.
2.2 INTRODUCTION

Annually, approximately 280,000 patients worldwide undergo either a mechanical- or bioprosthetic heart valve transplantation (36). Although these are life saving devices, the lack of growth potential of these prostheses is a major problem for pediatric patients. They have to go through multiple staged interventions to accommodate the increased annulus size, with increasing morbidity and mortality risks (1). Therefore, there is an urgent need for heart valve prostheses with growth capacity that last a lifetime (133,134).

Decellularized tissue-engineered heart valves (DTEHVs) might represent a promising alternative. From our first long-term in vivo experiments, where we implanted DTEHVs in sheep and non-human primates, we learned that the DTEHVs start to repopulate within hours, accompanied by changes in the extracellular matrix after 8 weeks of implantation. Moreover, there was ECM production over time, indicative for tissue regeneration and growth potential (39,55). This in contrast to decellularized xenogeneic heart valves, which only show limited host cell repopulation (37,135). Besides, these DTEHVs could be available off-the-shelf (38). Although these results are promising, there were signs of leaflet shortening and fusion of the leaflets with the wall, ultimately resulting in valvular insufficiency, an effect which is also reported by other groups (136,137). An explanation for this leaflet-fusing and shortening problem might be found in the valve geometry. It was shown from computational simulations by Loerakker et al. (131), that the leaflets in this original valve design were subjected to tissue compression in radial direction when loaded under physiological pulmonary pressures. It might explain why this particular valve geometry, in combination with infiltrated host cell induced remodeling, eventually resulted into reduced leaflet size. Based on these findings, Loerakker et al. also suggested an improved valve geometry that should enable radial leaflet extension during hemodynamic loading to counteract for cellular retraction forces. This required a more profound belly curvature, enhanced coaptation area and predominantly circumferential collagen orientation (131). However, controlling the geometry of tissue engineered heart valves (TEHVs) during culture was limited thus far. Regardless of the initial shape of the scaffold starter matrix, tissue compaction occurred in all possible unconstrained directions in response to the traction forces exerted by the vascular derived cells (myofibroblasts) used to culture the valves (64). This resulted in a flattened leaflet configuration, and absence of coaptation area after culture (138,139).

Therefore, the aim of this study is to find a solution to be able to improve, impose and maintain the DTEHV geometry, in accordance with the suggested geometry from the computational simulations, to reduce leaflet tissue compression in radial direction under
pulmonary loading conditions. A bioreactor insert matching the improved geometry was developed, which will function as an overall geometric constraint during culture. In this way, the leaflets will compact themselves around the bioreactor insert, and when removing the insert after the decellularization procedure, the DTEHV is likely to maintain its shape. This makes it possible to design, impose and maintain the desired DTEHV geometry. Human cell-based DTEHVs were produced, and their functionality and stability were assessed via hydrodynamic and fatigue in vitro tests. The effects of the bioreactor insert on tissue formation and collagen orientation were investigated via histology and confocal microscopy. Furthermore, the mechanical properties were analyzed to investigate the degree of tissue anisotropy and used as input for computational simulations on leaflet tissue loading behavior, in order to analyze the radial strain distribution in the newly designed DTEHV.

2.3 MATERIALS AND METHODS

2.3.1 INSERT MANUFACTURING AND POSITIONING

Based on the mathematical description of Hamid et al. (140), the original valve design was improved by adding coaptation and increasing the curvature in the belly region as being previously described by Loerakker et al. (131). This improved geometry was exported as a .stp file from the simulation software (Abaqus 6.10 Simulia, USA) and imported into computer-aided design software (Autodesk Inventor, USA), to make a one-piece component of 27.8 mm in length and 29.7 mm in diameter, which was compatible with the diastolic pulse duplicator (DPD) bioreactor system (139). The bioreactor inserts were made out of a solid piece of polyether ether ketone (PEEK) by using computer controlled milling technology.

The insert was positioned at the arterial side of the valve to enable tissue compaction around the individual posts. Small holes (0.5 mm in diameter and 1 mm spacing) are covering the insert wall to facilitate nutrient exchange with the adjacent tissue. Three large triangular shaped openings on top were incorporated for medium exchange towards the tissue-engineered valvular wall.

2.3.2 HEART VALVE TISSUE ENGINEERING

Tissue-engineered heart valves (TEHVs) (n=5) were cultured as previously described (139). In short, tri-leaflet heart valves were cut from non-woven polyglycolic-acid meshes (PGA; thickness 1.0 mm; specific gravity 70 mg/cm3; Cellon, Luxembourg), sewn (Prolene 6-0, Ethicon, USA) into a radially self expansible nitinol stent (length=31 mm, ID=30 mm at 37 °C; PFM-AG, Germany), and coated with 1% poly-4-hydroxybutyrate (P4HB; MW: 1 x 10^6;
TEPHA Inc., USA) in tetrahydrofuran (THF; Sigma-Aldrich, USA). The heart valve shaped constructs had an initial coaptation length of 5 mm and a maximal radial belly length of 19 mm. These constructs were sterilized with 70% ethanol (EtOH, VWR international S.A.S. Fontenay-Sous-Bois, France) for 15 minutes, washed 3 times with phosphate buffered saline (PBS) for 10 minutes, incubated in an antibacterial-anti fungus solution (10% penicillin/streptomycin (Pen/Strep)(Lonza, Belgium), 0.5% fungin (InvivoGen, USA)), for 30 minutes, and washed 3 times with PBS for 10 minutes. Hereafter, the valves were incubated overnight in growth medium (Advanced Dulbecco's Modified Eagle Medium (ADMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany), 1% Pen/Strep and 1% Glutamax (Gibco, USA)). Primary isolated human vascular-derived cells were harvested from the human vena saphena magna from a 77-year-old patient, according to the Dutch guidelines for secondary used materials, and seeded (0.3 x 10^6 cells / cm², passage 6) into the valvular shaped scaffolds using fibrin as a cell carrier. The seeded constructs were placed into the DPD bioreactor system together with the newly developed insert, containing growth medium supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma, USA). Pulsatile flow was applied at 1 Hz to the unshielded ventricular side of the valve.

2.3.3 DECELLULARIZATION PROCEDURE

After 4 weeks of culture, the obtained TEHVs (n=5) were decellularized as described by Dijkman et al. (38). Briefly, TEHVs were washed 3 times 10 minutes with PBS and decellularized overnight in detergent solution (0.25% Triton X-100, sodium deoxycholate and 0.02% EDTA), where after the bioreactor insert was removed. Nucleic remnants were enzymatically degraded by using Benzonase (EMD Millipore, USA) incubation steps, diluted in 50 mM TRIS-HCL buffer solution in concentrations of 100 U/ml, 80 U/ml and 20 U/ml for 8, 16 and 8 hours, respectively, on a shaker at 37 °C. Afterwards, the DTEHVs were washed 3 times with PBS and incubated for 24 hours in M-199 medium (Gibco, USA) on a shaker at 4 °C to remove cellular remnants. Valves were washed 3 times with PBS, sterilized with 70% EtOH for 15 minutes, washed 3 times with PBS, and incubated for 30 minutes with an anti-fungi/bacterial solution. After sterilization, the DTEHVs were stored at 4 °C until further use.

2.3.4 IN-VITRO VALVE FUNCTIONALITY

Out of the 5 DTEHVs, 4 were used for in vitro valve functionality assessments, and the remaining valve served as a control, not subjected to fatigue testing.
HYDRODYNAMIC PULSATILE FUNCTIONALITY ASSESSMENT

DTEHV (n=4) were placed inside a silicon annulus of 30 mm inner diameter and positioned into a hydrodynamic pulsatile test system (HDT-500, BDC Laboratories, USA) containing a physiologic saline solution at 37 °C. Valves were subjected to physiological pulmonary conditions (rate of 72 BPM, stroke of 70 ml, maximum diastolic pressure difference of 25 mmHg) for 1 hour. Flow and pressures were measured via a transonic sensor (TS410, Transonic Systems, USA) and pressure sensors (BDC-PT, BDC Laboratories, USA), respectively. Data was collected for 3 seconds at 5 kHz, and functionality was assessed from an average over 10 cardiac cycles by using Statys™ software (BDC Laboratories, USA), to determine the regurgitation fraction, the effective orifice area (EOA), and cardiac output (CO). The regurgitation fraction was defined as being the sum of the leakage volume and the closing volume, expressed as a percentage of the stroke volume. Slow-motion movies were recorded at high-speed burst mode to assess opening and closing behavior of the valves in motion (G15 PowerShot, Canon, USA).

FATIGUE ASSESSMENT

DTEHV (n=4) were placed inside a 30 mm diameter silicon annulus and positioned into a valve durability tester (VDT-3600i, BDC Laboratories, USA) containing physiologic saline solution at 37 °C. Valves were subjected to accelerated opening and closing cycles at a frequency of 10 Hz and a stroke between 1.20 ml and 2.10 ml. Proper opening and closing behavior was assessed by analyzing slow-motion recordings (G15 PowerShot, Canon, USA). During closure, the maximum differential loading was targeted at 28 mmHg and was automatically maintained. After each 3 x 10⁶ cycles, the DTEHV were tested again for their hydrodynamic functionality by using the hydrodynamic pulsatile test system as described above.

2.3.5 Qualitative Tissue Analysis and Global Collagen Orientation

HistoLOGY

Middle sections of the valves (n=5) were fixed overnight in 3.7% formalin (Fluka, USA) at 4 °C. After washing in PBS, the samples were embedded in Tissue-Tek (Sakura, the Netherlands) and cured gradually in liquid nitrogen vapor. Cryosections were cut at 10 μm thickness and stained for Hematoxylin and Eosin (H&E) to assess general tissue formation and the effectiveness of the decellularization procedure, Masson Trichrome (MTC) (Staining kit, Sigma, USA) for the presence and distribution of collagen, and Elastica van Gieson (EvG) (Staining kit, Merck, Germany) for the presence of elastic matrix formation. The samples were embedded in Entellan (Merck, Germany) and analyzed via bright field
microscopy (Axio Observer Z1, Zeiss, Germany) in the mid regions of the heart valve leaflets.

**CONFOCAL MICROSCOPY**

Half leaflet sections of all (n=5) valves were analyzed for the effect of the insert on the global collagen orientation. Samples were stained via a whole mount staining with the collagen specific dye CNA35-OG488 (141), for 1 hour in PBS and visualized with a confocal microscope (TCS SPSX, Leica, Germany). The dye has an excitation and emission profile of 488 nm and 500-550 nm, respectively. Samples immersed in Mowiol (Sigma, USA) were mounted between two preparation glasses. The specimen was observed with a 10X objective and Z-stacks were made throughout 200 μm of the entire arterial side of the sample via stitched adjacent tile scans. Afterwards, a maximum intensity projection was made in Z-direction.

2.3.6 TISSUE MECHANICS AND IN VIVO COLLAGEN REMODELING SIMULATIONS

**BIOMECHANICAL ANALYSES**

Mechanical properties of the control valve were analyzed by using a biaxial tensile tester (BioTester, 5N load cell; CellScale, Waterloo, Canada) in combination with LabJoy software (V8.01, CellScale). Two square samples (36 mm² each) per valve were symmetrically cut from the belly region. Sample thickness was measured at 3 random locations using an electronic caliper (CD-15CPX, Mitutoyo, Japan) and averaged. The samples were stretched equibiaxially in both the radial and circumferential direction up to 20% strain, at a strain rate of 100% per minute. After stretching, the samples recovered to 0% strain at a strain rate of 100% per minute, followed by a rest cycle of 54 seconds. Prior to measuring the final stresses, samples were preconditioned with 5 of these cycles. A high-order polynomial curve was fitted through each individual data set in both the radial and circumferential direction. The stiffness of the tissue was represented by the tangent modulus and was defined as the tangent to the fitted polynomial curve at 20% strain.

**COMPUTATIONAL SIMULATIONS**

Based on the obtained experimental mechanical data of these improved DTEHVs, computational simulations (Abaqus 6.10 Simulia, USA) as described by Loerakker et al. (131), were executed to examine if the implemented changes in valve geometry resulted in reduced radial compression under pulmonary pressure conditions. The original valve design was based on the mathematical description of Thubrikar (142), with the parameters $R_b=R_c=13.5$ mm, $H=19.15$ mm, $H_c=3.15$ mm and $\beta=0^\circ$ without any coaptation. The improved valve geometry was described by Hamid et al. (140), with the parameters $a=b=3.1$ mm, $H=18$ mm, $R=13.5$ mm, with 5 mm coaptation. All values were obtained as
described in the original paper (131) and both designs used the experimentally measured tissue thickness of 0.58 mm.

The total stress in the tissue was given by the stress in the collagen fibers (with volume fraction $\phi_f$) and the isotropic matrix components (with volume fraction $(1 - \phi_f)$) (131):

$$\sigma = \sigma_m + \sigma_f$$

(2.1)

The isotropic matrix stress is equal to:

$$\sigma_m = (1 - \phi_f) \left( \kappa \frac{1n^1 I}{J} + \frac{G}{J} (B - J^3 I) \right)$$

(2.2)

with $B = F \cdot F^T$ the left Cauchy-Green tensor, $F$ the deformation gradient tensor, $J = \text{det}(F)$ the volumetric change ratio. The tissue was modeled as almost incompressible ($\nu=0.498$), and the shear modulus $G$ was set to 10 kPa to prevent numerical instabilities. The compression modulus is defined as:

$$\kappa = \frac{2G(1+\nu)}{3(1-2\nu)}$$

(2.3)

The collagen fibers were modeled with an angular distribution of fibers (resolution of 3°), where the stress if given by:

$$\sigma_f = \sum_{i=1}^{N} \phi_f^i \sigma_f^i \bar{e}_f^i$$

(2.4)

with $N$ the number of fiber directions, $\phi_f^i$ the collagen volume fraction in direction $i$ ($\sum_{i=1}^{N} \phi_f^i = \phi_f$), $\sigma_f^i$ the magnitude of the stress in each fiber direction, and $\bar{e}_f^i$ a unit vector in the deformed fiber direction $i$. The magnitude of the stress is a function of the fiber stretch $\lambda_f$:

$$\sigma_f = \begin{cases} k_1 \lambda_f^2 \left( e^{k_2 (\lambda_f^2 - 1)} - 1 \right) & \text{for } \lambda_f \geq 1 \\ 0 & \text{for } \lambda_f < 1 \end{cases}$$

(2.5)

The collagen volume fractions are described with a Gaussian distribution:

$$\phi_f^i = A \exp \left( -\frac{(\gamma^i - \mu)^2}{2\sigma^2} \right)$$

(2.6)

with $\gamma^i$ the angle of fiber direction $i$ with respect to the circumferential direction, $\mu$ the main fiber angle ($0^\circ$), $\sigma$ the standard deviation, and $A$ a scaling factor to ensure that $\sum_{i=1}^{N} \phi_f^i = \phi_f$. Parameters $k_1$, $k_2$, and $\sigma$ were fitted to the equibiaxial tensile test data of the control valve according to the least squares method. The effect of collagen anisotropy on tissue loading was investigated by comparing the experimentally determined collagen anisotropy to complete collagen isotropy. Eventually, the computational model simulated the radial and circumferential strains in the DTEHV leaflets, by applying a mean pulmonary...
differential pressure of 15 mmHg over the valve, using both the original as well as the improved geometry.

2.3.7 **STATISTICS**

To analyze DTEHV fatigue behavior over time, a linear regression analysis was performed (Prism V6.0d, Graphpad, USA) on the closing volume, leakage volume, cardiac output, and the effective orifice area, with a slope being significantly non-zero for p<0.05. Time points were averaged, and represented by their mean ± standard deviation.

For the biomechanical analysis, samples were averaged per valve, and represented by their mean ± standard deviation. The presence of anisotropy was defined as a significant difference (p<0.05) between the obtained stiffnesses in both radial and circumferential direction, analyzed via a paired student t-test (Prism V6.0d, Graphpad, USA).

2.4 **RESULTS**

2.4.1 **PRODUCTION AND FUNCTIONALITY OF THE BIOREACTOR INSERT**

**PRODUCTION OF THE INSERT**

The improved heart valve geometry as suggested by the computational simulations, was successfully translated into a physical rigid object (Fig. 2.1A), matching the exact same geometry. The surface of the insert was smooth and the holes were equally distributed over the entire surface to enable sufficient nutrient exchange to the scaffold. There was sufficient space between the individual posts to prevent leaflet fusion during culture. The insert fitted nicely into the DPD bioreactor system without obstructing the pulsating flow passing by the ventricular side towards the arterial side (Fig. 2.1B).

**FUNCTIONING OF THE INSERT**

During the 4 weeks of culturing, the leaflets of the TEHVs compacted tightly around the insert, adapting to the imposed geometry. After decellularization, the valves maintained their shape, and there was no leaflet retraction visible upon removal of the insert. The coaptation area was approximately 5 mm in length (Fig. 2.1C), and the belly region of the DTEHVs maintained the imposed curvature (Fig. 2.1D). Tissue was uniformly distributed throughout the leaflets, without any damage or other macroscopically detectable irregularities.
2.4.2 Hemodynamic Functionality and Fatigue Resistance

DTEHVs (n=4) were subjected to physiological pulmonary conditions in a hydrodynamic test setup. A representative graph of one valve is shown in figure 2.2. Overall, all valves opened up completely (Fig. 2.2B-D), and closed symmetrically (Fig. 2.2E-F). Prolapse was not observed in any of the valves.

For the long-term durability assay, valve opening and closing behavior was set to be comparable to the physiological behavior as observed in the hydrodynamic setup. From the DTEHVs (n=4) subjected to fatigue tests, three remained functional up to about 12 million cycles, representative for 16 weeks in vivo follow-up time. One valve failed at 4 million cycles, and was unable to maintain stable pressure conditions during fatigue testing from the start. The valves showed no decrease in functionality over time by having an initial regurgitation fraction of 4.13±1.44 %, consisting out of a closing volume percentage of 3.41±1.42 %, and a leakage volume percentage of only 0.73±0.08 % (Fig 2.3A). The valves maintained a consistent effective orifice area of 2.37±0.04 cm², with a maintained cardiac output of 4.80±0.11 L/min over time (Fig. 2.3B). Loss in functionality was sudden and in all cases the result of leaflet rupture at one of the commissure points.
Figure 2.2 | Hydrodynamic In Vitro Testing: Representative results of a DTEHV subjected to in vitro hydrodynamic physiological pulmonary pulsatile stimulation after 12 million cycles, showed competent valve functioning as indicated in the graph (A). Valves were opening completely (B-D) and closed symmetrically (E,F).

Figure 2.3 | Long Term In Vitro Functionality: Interval measurements of the regurgitation fraction, cardiac output, and effective orifice area, from start (n=4), after 3 million cycles (n=4), 6 million cycles (n=3), 9 million cycles (n=2), and after 12 million cycles (n=2), showing no significant increase in regurgitation over time (p<0.05) (A). Also the cardiac output and effective orifice area maintained stable over time (B). Values are represented as mean values ± standard deviation.
2.4.3 Qualitative Tissue Analysis and Global Collagen Orientation

**Histological Appearance**

Histology was performed on samples of all DTEHVs. Representative photographs in figure 2.4, show that the tissue was uniformly distributed throughout the thickness of the leaflets. Furthermore, no necrotic or damaged tissue was observed. The decellularization procedure successfully removed all cells as demonstrated by the H&E staining, although scaffold remnants were still present in the tissues (Fig. 2.4A). From the MTC staining it appeared that the leaflets are mainly composed of collagen (Fig. 2.4B), with no signs of elastic matrix formation in the VvG staining (Fig. 2.4C), as would otherwise be indicated by black fibers.

![Figure 2.4](image)

**Figure 2.4** | Histology: Representative images of stained sections of DTEHV leaflets with Hematoxylin and Eosin, showing appropriate decellularization, equal tissue formation and the presence of scaffold remnants (A). Masson Trichrome revealed mainly collagen deposition depicted in blue (B), where Elastica van Gieson indicated no elastic matrix formation within the construct (C).

**Global Collagen Orientation**

By analyzing the collagen whole mount stainings, global collagen orientation was visualized throughout ~200 μm in depth, over the entire half of 5 DTEHV leaflets. A representative maximum projection in Z-direction is shown in figure 2.5A. Here, the collagen was clearly aligned in circumferential direction in the coaptation area (Fig. 2.5B) and more randomly distributed towards the bottom region of the belly (Fig. 2.5C).
2.4.4 Tissue Mechanics and Collagen Remodeling Simulations

Biomechanical Properties

The averaged tensile curves from the control valve showed Cauchy stresses ranging between 200-300 kPa in radial direction and 300-400 kPa in circumferential direction at 20% strain (Fig. 2.6A), indicative for tissue anisotropy. From the averaged equibiaxial tensile tests on all valves, it appeared that the tangent moduli were significantly higher in circumferential direction, compared to the radial direction (p=0.035), being 3.59±0.95 MPa and 2.47±0.74 MPa respectively at 20% strain (Fig. 2.6B).
Figure 2.6 | Mechanical Properties: The averaged stress-strain curves obtained from equal biaxial tensile tests of the control group, in both radial and circumferential direction, shows Cauchy stresses ranging between the 200-400 kPa (A). The fitted curves are used as material parameters for the computational simulations (A). The calculated tangent moduli of all DTEHVs showed a significant increase in tissue stiffness in circumferential direction compared to the radial direction (*p=0.035) (B). All values are represented as mean values ± standard deviation.

**Computational Simulations**

Fitting the numerical model on the experimental tensile data of the control group (Fig. 2.6B), resulted in the following parameter values: \( k_1 = 22.0 \text{ kPa}, k_2 = 7.50, \) and \( \sigma = 67.5^\circ \).

At a mean pulmonary differential pressure of 15 mmHg the computational simulations revealed that the original design was subjected to leaflet tissue compression in the radial direction throughout the entire leaflet during loading (Fig. 2.7A). The improved valve design of these DTEHVs showed a considerable decrease in radial tissue compression as compared to the original design (Fig. 2.7B). Strains in the circumferential direction were maintained and comparable in both designs. Besides, the effect of the present collagen anisotropy on tissue loading in the improved design, revealed no changes in radial tissue compression compared to fully isotropic collagen orientation (Fig. 2.7C).
Improved Heart Valve Geometry

**Figure 2.7** | Computational Simulations: Material parameters of the DTEHVs were implemented into an established computational valve model, to assess the strains in radial and circumferential direction in both the original and the improved valve design, simulating 15 mmHg mean pulmonary pressure. In the original valve design the entire leaflet tissue is being compressed in radial direction (A). The improved valve design showed a considerable decrease in radial compression in the belly (B), which is primarily due to geometrical improvements rather than collagen anisotropy (C). Strains in circumferential direction are comparable between both valve designs.
2.5 DISCUSSION

From our first long-term in vivo experiments where we implanted DTEHV in sheep, we observed leaflet fusion with the wall, which resulted in the development of valvular insufficiency over time (39,55). Based on computational simulations it was hypothesized that the original DTEHV geometry had to be adjusted to enable leaflet extension in the radial direction, rather than compression. Therefore, a bioreactor insert was developed to impose the desired valvular geometry during culture, which was maintained after decellularization and removal of the insert. This resulted in DTEHV with an increased coaptation area and a significant radial and circumferential belly curvature.

Adjusting the culture conditions in the bioreactor system by introducing a constraining insert might have affected tissue formation by limiting nutrient and oxygen exchange. However, it appeared that these DTEHV still contain a uniform ECM distribution throughout the entire thickness of the leaflets, consisting mainly of collagen. These histological findings are in agreement with previously cultured human cell-based TEHV having the original valve geometry without the insert (89).

Furthermore, the global collagen orientation was influenced by introducing the insert. During tissue culture, non-woven PGA meshes are known to hydrolyze, thereby losing their mechanical integrity and structural support (45,143,144). This degradation profile in combination with the tension forces exerted by the cells, led to tissue compaction in the unconstrained directions, which resulted in collagen orientation along the constrained direction (145,146).

In this study, tissue compaction against a rigid object was used to impose the DTEHV geometry according to the shape of the bioreactor insert. After 2 weeks of culture, the scaffold loses its mechanical integrity and the tissue starts to compact (64). While compacting, the leaflet tissue is being constrained by the rigid bioreactor insert, except for the tissue at the free edges of the leaflets that could still compact slightly in the radial direction. This resulted in mainly circumferential aligned collagen in the coaptation area, and a more random distribution towards the bottom of the belly.

Other studies in which constraining methods were used to control the geometry of DTEHV are promising, however no long-term functionality up to 12 million cycles have been reported so far (47,146-149). The DTEHV created in the present study showed satisfactory long-term functionality up to 16 weeks in vivo simulation in terms of regurgitation, cardiac output and opening and closing behavior, with only one valve failing after 4 million cycles, being unable to adapt and stabilize to the applied pulmonary pressure conditions during fatigue testing. From previous in vivo implantation studies in
both sheep and non-human primates, host cell repopulation was observed within 5 hours, accompanied by changes in the extracellular matrix after 8 weeks, with evidence of ECM production by these cells (39,55). Therefore, the DTEHVs as developed in this study are expected to be sufficiently fatigue resistant under physiological pulmonary conditions, to provide sufficient time for host cells to repopulate and maintain the ECM.

In addition to the improved valvular geometry in terms of a large coaptation area and an enhanced belly curvature, collagen anisotropy is essential to obtain radial leaflet stretch during dynamic loading, characteristic for native leaflets (150), were anisotropy is expected to further increase after in vivo implantation because of strain-induced collagen reorganization by the repopulating host cells (145). Compared to reported stiffness values (151), human native aortic heart valve leaflets have a tangent modulus in radial and circumferential direction of about 2.0±1.5 MPa and 15.6±6.4 MPa respectively. The reported tangent modulus of the DTEHVs in radial direction is comparable with 2.5±0.7 MPa, but is lower in circumferential direction with 3.6±1.0 MPa.

Despite that local observed differences in collagen anisotropy were not implemented into the model, these computational simulations revealed that the additional effect of the overall collagen anisotropy seems not to influence the tissue loading behavior under pulmonary loading conditions. Therefore the implemented geometrical improvements only, were already sufficient to prevent radial tissue compression almost completely in the entire valve.

The concept of using DTEHVs for human applications still holds great promise in terms of regenerative capacity and growth potential, which would overcome the necessity for multiple re-interventions in young patients. The use of autologous cells is not required and allogeneic cells can be used that simplifies regulations and allows for off-the-shelf availability. Further development on biodegradable stents that are suitable for minimal invasive heart valve implantation should be focus of future studies to complete the growing valve concept.

2.6 CONCLUSION

In conclusion, this study proposes a successful solution to impose a desired three-dimensional curved tissue engineered valvular geometry by using a constraining bioreactor insert during culture, which allows for a maintained shape after decellularization and removal of the insert. This resulted in fully competent off-the-shelf available human cell-based DTEHVs with a large coaptation area and profound belly curvature. Long-term functionality was maintained mainly up to 16 weeks in vivo.
simulation, allowing sufficient time for host cell repopulation. Usage of the bioreactor inserts resulted in homogeneously distributed tissue formation, circumferential collagen orientation in the coaptation region, and overall leaflet tissue anisotropy. Based on the mechanical data, our computational models revealed a considerable decrease in radial tissue compression with the obtained geometrical adjustments. Therefore, these improved DTEHV are expected to be less prone to host cell mediated leaflet retraction and will remain competent after implantation.

2.7 ACKNOWLEDGMENT

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CHAPTER 3

THE EFFECTS OF SCAFFOLD REMNANTS IN DECELLULARIZED TISSUE ENGINEERED CONSTRUCTS ON THE RECRUITMENT OF BLOOD CELLS

The content of this chapter is based on:

3.1 ABSTRACT

Decellularized tissue engineered heart valves (DTEHVs) showed promising results in terms of rapid recellularization within hours after in vivo implantation, which might be favorable for future tissue regeneration. These valvular constructs contain scaffold remnants prior to implantation that could trigger an immune response. To investigate if the presence of these scaffold remnants are responsible for fast recellularization, an in vitro mesofluidic system was used to investigate the effects of different biomaterials, consisting out of decellularized tissue engineered constructs (DTEC) either with or without scaffold material, as well as bare scaffold, on the infiltration of circulation blood cells. Granulocytes and agranulocyte fractions were isolated, stained, brought back in suspension, and subsequently circulated along the biomaterials. After 5 hours of testing, the granulocyte fraction was depleted faster from the circulation than the agranulocyte fraction. Only the granulocytes infiltrated into the DTEC with scaffold and migrated towards the scaffold remnants, while the agranulocyte population was only observed on the outer surface. Active cell infiltration was associated with high levels of MMP-1 secretion in the DTEC with scaffold remnants. Pro-inflammatory cytokines such as IL-1α, IL-6 and TNF-α were significantly upregulated in the DTEC without scaffold remnants. These results indicate that indeed scaffold remnants are responsible for initial cellular infiltration and that their presence can influence the initiated immune response.
3.2 INTRODUCTION

Worldwide on an annual basis, the number of patients that require a heart valve transplantation is expected to increase from approximately 290,000 in 2003, to over 850,000 by 2050 (2). Pediatric patients with congenital heart valve diseases that are treated nowadays still require multiple surgical interventions, since none of the current available prosthetic devices can accommodate for growth (152). This is associated with increasing rates of morbidity and mortality (153).

Decellularized tissue engineered heart valves (DTEHVs) hold great promises to overcome this limitation. From our recent in vivo study, in which DTEHVs were implanted in sheep and non-human primates, rapid recellularization was observed with a first influx of blood derived cells already within hours of implantation, where after a secondary influx of cells from the adjacent native tissue gradually started to infiltrate towards the leaflets within 8 weeks (39,55). Recellularization is required for regeneration and to facilitate growth potential. However, the mechanism that triggers the cellular influx is still unknown. Typically, the infiltrated cells group in the vicinity of the scaffold remnants. Therefore, it is hypothesized that the presence of scaffold remnants might trigger this initial cellular influx. The response of immune cells is known to differ between biomaterials and to play a critical role in guiding the regenerative response (96,154). Depending on the created cytokine environment, cellular polarization of for instance t-helper cells and macrophages can alter and affect regeneration (155).

To allow for the infiltration of such cascades of events towards regeneration, rapid cellular infiltration of implants is required. To investigate if scaffold remnants indeed trigger the initial cellular influx and to investigate the nature of this initiated immune response, a meso fluidic in vitro flow setup was used, in which circulating cells can be brought in contact with biomaterials under physiological conditions (156). In this system, decellularized tissue engineered constructs (DTECs) with and without scaffold material were included, as well as bare scaffold samples. For the circulating cells, human peripheral blood cells were isolated and incorporated in the system. Granulocyte and agranulocyte fractions were isolated, stained and brought into the circulating suspension. Every hour, the composition of the remaining circulating cell population was analyzed via flow cytometry, where after 5 hours the experiment was terminated from which samples from the circulation were taken for ELISA to investigate the released cytokine profiles. The biomaterials were subsequently subjected to scanning electron microscopy (SEM) for surface analyses, confocal microscopy and fluorescence microscopy for cellular infiltration.
3.3 MATERIALS AND METHODS

3.3.1 BIOMATERIALS

BARE SCAFFOLD
Non-woven polyglycolic-acid meshes (PGA; thickness 1.0 mm; specific gravity 70 mg/cm³; Cellon, Luxembourg) were used as the bare scaffold samples (n=4) containing no biological component (Fig. 3.1A).

DTECS WITH SCAFFOLD
DTECs with scaffold (n=4) were grown as a circular shaped sheet with a diameter of 2.5 cm, cut from similar PGA sheets as the bare scaffold samples, sewn (Prolene 4-0, Ethicon, USA) onto stainless steel rings, coated with 1% poly-4-hydroxybutyrate (P4HB; MW: 1 x 10⁶; TEPHA Inc., USA) in tetrahydrofuran (THF; Sigma-Aldrich, USA), placed overnight in a 6 wells plate to evaporate the THF, and cultured as described below prior to use in the meso fluidic setup (Fig. 3.1B).

DTECs WITHOUT SCAFFOLD
DTECs without scaffold (n=4) were grown in circular shaped Velcro rings with a diameter of 2.5 cm, and based on the use of fibrin. These parts were fixed (Silastic MDX4-4210; Dow Corning, USA) onto the bottom of 6 wells plates and cured overnight at 37 °C. These patches were cultured as described below and use in the meso fluidic setup (Fig. 3.1C).

Tissue culture
Sterilizing the biomaterials was performed with 70% ethanol (EtOH, VWR international S.A.S. Fontenay-Sous-Bois, France) for 15 minutes. Thereafter the samples were washed 3 times with phosphate buffered saline (PBS) for 10 minutes, and incubated in an antibacterial-antifungal solution 10% penicillin/streptomycin (Pen/Strep)(Lonza, Belgium), supplemented with 0.5% Fungin (InvivoGen, USA), for 30 minutes and washed 3 times with PBS for 10 minutes. Hereafter the constructs were incubated overnight in growth medium (Advanced Dulbecco’s Modified Eagle Medium (ADMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany), 1% Pen/Strep and 1% Glutamax (Gibco, USA)).

Primary isolated human vascular-derived cells were harvested from the human vena saphena magna of a 77-year-old patient, according to the Dutch guidelines for secondary used materials (0.3 x 10⁶ cells / cm², passage 6), and seeded either into the PGA scaffold, or into the Velcro rings, using fibrin as a cell carrier. The seeded constructs were provided with growth medium supplemented with l-ascorbic acid 2-phosphathe (0.25 mg/ml, Sigma,
USA) and 6-aminocaproic acid (ACA) (1 mg/ml, Sigma, USA) for 2 weeks, where after the ACA was removed from the medium, and patches were cultured for an additional 2 weeks. ACA was supplemented to prevent early fibrin degradation, but was depleted after sufficient tissue was formed.

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**Figure 3.1** | Experimental Overview: Biomaterials of interest used in this study, with bare scaffold (A), decellularized tissue engineered construct with scaffold remnants (B), and decellularized tissue engineered constructs without scaffold remnants (C). Before the experiment, all constructs were acellular. The circulating cell population contained the agranulocyte fraction in green and the granulocyte fraction in red. After 5 hours of the experiment the bare scaffold material contained both cell types, the tissue with scaffold remnants only the granulocyte fraction, and the tissue without scaffold remnants only revealed a surface layer of cells.

**DECELLULARIZATION**

After 4 weeks of culture, all tissue engineered patches (n=8), both with and without scaffold, were decellularized as described previously (38). Briefly, patches were washed 3 times for 10 minutes with PBS and decellularized overnight in detergent solution (0.25% Triton X-100, sodium deoxycholate and 0.02% EDTA). Nucleic remnants were enzymatically removed by using 3 Benzonase (EMD Millipore, USA) incubation steps, diluted in 50mM TRIS-HCL buffer solution in concentrations of 100 U/ml, 80 U/ml and 20 U/ml for 8, 16 and 8 hours respectively on a shaker at 37 °C. Afterwards, patches were washed 3 times with PBS and incubated for 24 hours in M-199 medium (Gibco, USA) on a shaker at 4 °C to remove cellular remnants. Patches were washed 3 times with PBS,
sterilized with 70% EtOH for 15 minutes, washed 3 times with PBS and incubated for 30 minutes in an antibacterial-antifungal solution. After sterilization, patches were stored at 4 °C until further use.

3.3.2 EXPERIMENTAL SETUP

BLOOD COLLECTION AND PREPARATION

Peripheral blood samples (40 ml) were isolated from healthy human male donors (n=4) as approved by the Dutch Medical research Ethics Committees United (NL53574.100.15). The blood samples were divided into separated fractions of plasma, agranulocytes, granulocytes and erythrocytes via density gradient centrifuging at 700g over two stacked layers of histopague with different densities (Histopague 1119 and Histopague 1077, Sigma-Aldrich, USA) and washed with PBS. The isolated agranulocyte fraction was stained with CellTracker Green (Life Technologies, USA) and the granulocyte fraction with CellTracer Orange (Life Technologies, USA) for 30 minutes and washed with PBS. Erythrocytes were discarded, where after the stained granulocyte and agranulocyte fraction were resuspended in the plasma and added up to 40 ml with PBS.

Meso fluidic system

An in-house developed meso fluidic setup was used to investigate the interaction of circulating cells to biomaterials as described by Smits et al. (156). In brief, rectangular strips (10 * 15 mm²) were taken from the individual biomaterials and positioned into three independent fluidic units (Ibidi GmbH, Germany), each containing the prepared blood samples with a total volume of 7.5 ml. Motion of the cell suspension was driven via a pulsatile flow using a pressure pump (Ibidi GmbH). Flow conditions were set at a frequency of 1 Hz, with a peak shear stress and peak pressure of approximately 1.6 Pa and 20 mmHg respectively, to mimic physiological pulmonary valvar hemodynamic conditions. Samples of the circulating cell suspension were taken every hour during flow, using in-line injection ports (Ibidi GmbH), and fixated in formaldehyde (3.7% v/v; Merck Millipore, USA). These samples were later on used for flow cytometry. After 5 hours, the experiment was terminated and the biomaterials were taken out for further analysis. For multiplex ELISA, 300 µl of cell suspension was taken and centrifuged for 1500g to obtain platelet-rich medium. Plasma was transferred to new tubes and centrifuged for 13.000 g to obtain platelet-free plasma and snap-frozen immediately afterwards. Biomaterial samples were fixed in formaldehyde (3.7% v/v; Merck Millipore, USA) for confocal microscopy and histology or fixated in glutaraldehyde (2.5% v/v; Sigma-Aldrich, USA) for scanning electron microscopy.
3.3.3 Analyses

Flow Cytometry

To assess the composition of the circulating cell population, samples (n=4 per time point) were analyzed with a bench-top flow cytometer (Guava easyCyte 6HT, Merck Millipore, USA). Samples were centrifuged at 200 g and resuspended in PBS. Debris was excluded using a threshold on the forward scatter signal and at least 20,000 events were counted per sample. Data analysis was performed using Guava Express Pro software (Guava Express Pro, Merck Millipore, USA) in combination with InCyte software (InCyte v2.7, Merck Millipore, USA). Granulocytes were separated from the agranulocytes by gating on cell size and granularity (forward scatter and side scatter respectively) and on color in a secondary gating step. Data was normalized to the amount of circulating cells at the start of the experiment. The best fit was determined for either a line or one-phase decay.

Confocal Microscopy

Fixed biomaterial samples (n=4 per group) were analyzed with a confocal microscope (TCS SP5X, Leica, Germany) to investigate the distribution of the different cell fractions. Granulocytes were visible in the red and the agranulocyte fraction in green. Scaffold remnants could be visualized via auto-fluorescence in the blue channel. Samples were immersed in PBS and mounted between two preparation glasses. The specimens were observed with a 10X objective and Z-stacks were made throughout a depth of 200 μm. Afterwards, a maximum intensity projection was made in Z-direction.

Fluorescence Microscopy

To investigate the infiltration depth of the cells into the biomaterials, histology was performed. After confocal microscopy analyses, samples were embedded in Tissue-Tek (Sakura, the Netherlands) and cured gradually in liquid nitrogen vapor. Cryosections were cut at 60 μm thickness, embedded in Mowiol (Sigma-Aldrich, USA) and analyzed via fluorescence microscopy (Axiovert 200M, Zeiss, Germany). Scale bars represent 200 μm.

Scanning Electron Microscopy

To investigate cell attachment to the biomaterials, glutaraldehyde fixed samples were washed twice in PBS and dehydrated in a graded ethanol series, starting from 70% (v/v) to 100% (v/v) with 10% increments where after the ethanol was allowed to evaporate. Samples were visualized by scanning electron microscopy (SEM) (Quanta 600F, FEI, the Netherlands) with a 3.0 kV electron beam at vacuum mode.
Chapter 3

**Multiplex ELISA**

Released cytokine profiles in response to the biomaterials were analyzed via multiplex ELISA. These tests have been performed by the Multiplex core facility of the Laboratory for Translational Immunology of the University Medical Center Utrecht in the Netherlands, by using an in-house developed and validated multiplex immunoassay based on Luminex Technology. Samples were incubated with antibody-conjugated MagPlex microspheres (BioRad, Hercules, USA) for one hour at room temperature with continuous shaking, followed by one hour incubation with biotinylated anitbodies, and 10 min incubation with phycoerythrin-conjugated streptavidin diluted in high performance ELISA (HPE) buffer (Sanquin, the Netherlands). Acquisition was done by using a FLEXMAP 3D system controlled with xPONENT software (v 4.1, Luminex, USA). Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software (version 6.1.1, Biorad). Data was normalized to protein levels right after blood collection per donor.

### 3.3.4 Statistics

For flow cytometry, data was represented as mean ± standard error of the mean. Differences in depletion profiles were investigated by using nonlinear regression analysis in terms of a one-phase decay fit with a fixed constrain of 100% at the start. For multiplex analyses, data was represented as mean ± standard deviation and analyzed via one-way ANOVA, followed by a Dunnet’s multiple comparison correction. Statistical analysis were performed using Prism software (v5.0 GraphPad Software, USA) with results considered significant for P values < 0.05.
Figure 3.2 | FACS Analyses: Representative side-forward scatter plot including the applied gates for the granulocyte and agranulocyte fraction (A). A representative green-yellow fluorescence plot with a high and low green gate, a yellow gate and a double stained gate (B). Green fluorescence intensity plot with two distinguishable peaks (C). Yellow fluorescence intensity plot with 1 clear distinguishable peak (D). The counted colour gated fractions represented as percentage, and the associated cell type discrimination fraction (E).

3.4 RESULTS

3.4.1 BLOOD PREPARATION

The purity of the stained cell fractions was analyzed using FACS. Cells were gated in a granulocyte and agranulocyte fraction based on cell size and granularity (Fig. 3.2A). In this way, the granulocyte fraction could clearly be distinguished from the agranulocyte fraction. Secondly, events were gated based on fluorescent intensity (Fig. 3.2B), whereby two green peaks could be observed (Fig. 3.2C), while for the red staining 1 peak appeared
(Fig. 3.2D). Positive stained events were calculated as a percentage of all events. This resulted in stained events for red of 20.65±6.38 %, for green of 26.89±6.99 %, or a double staining of 2.29±1.26 %. From all these single red events, 86.09±5.65 % resides within the granulocyte gate and 6.57±6.49 % in the agranulocyte gate. In case of the single green events, 89.37±7.69 % resides within the agranulocyte gate and 5.91±3.38 % in the granulocyte gate.

3.4.2 CIRCULATING CELLS

Circulating cell population was investigated in time via FACS. It appeared that the granulocyte fraction in the bare scaffold samples were depleted faster from the circulation as compared to the DTECs (p=0.0022) (Fig. 3.3). Here after 60 minutes, the circulating cells were decreased to 46.16±6.47 % of the initial starting value, being significantly lower compared to both DTECs (p=0.0017). Also the reached plateau level of the bare scaffold samples was lower than the DTECs (p=0.0016).

![Circulating Cells](image)

**Figure 3.3** | Circulating Cell Population: Depletion of circulating cells over the course of the experiment. With a fast depletion of the granulocyte fraction (A) compared to the agranulocyte fraction (B) and a faster depletion and a lower plateau level of granulocytes in the bare scaffold samples compared to the tissue engineered constructs (A).
3.4.3 **Cellular Distribution**

Cellular distribution of the cells was analyzed with confocal microscopy. In all the biomaterials, cells were observed homogeneously distributed over the sample. In the bare scaffold biomaterial, cells were located in the vicinity of the scaffold fibers (Fig. 3.4A). These were both red and green stained cells. No cells were located in between the scaffold fibers. In the DTEC with scaffold, cells also seemed to be located near the scaffold fibers (Fig 3.4B). Especially the red cells appeared near the fibers, were the green cells were also observed in between the scaffold fibers.

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

**Figure 3.4 | Cellular Distribution:** Maximum projection of confocal Z-stacks throughout the different biomaterials. Bare scaffold shows homogeneous cell distribution with both granulocytes and agranulocytes adherent to the scaffold fibers (A). Decellularized tissue engineered constructs with scaffold remnants, shows the granulocyte fraction in the vicinity of the embedded scaffold fibers (B). The decellularized tissue engineered constructs without scaffold remnants also show a homogeneous cellular distribution (C). Red cells represent granulocytes, green cells the agranulocytes and blue the scaffold.
3.4.4 CELLULAR INFILTRATION

To see to what extent cells actually infiltrated into the biomaterials, cross sections of the individual biomaterials were made. In the bare scaffold samples, cells were observed throughout the entire thickness of the biomaterial (Fig. 3.5A). In the DTECs with scaffold, red cells were observed in the core of the biomaterial, where as the green cells were located at the surface (Fig. 3.5B). Therefore, it seems that within 5 hours, the red cells are actually capable of infiltrating into the core of the biomaterial. In case of the DTECs without scaffold, both red and green cells were located at the surface of the biomaterial. No cellular infiltration was observed in these constructs.

![Image A](image1.png)

![Image B](image2.png)

Figure 3.5 | Cellular Infiltration: Representative cryosections. Bare scaffold samples show cellular distribution throughout the entire thickness (A). Only granulocyte infiltration in the decellularized tissue engineered constructs with scaffold, and a layer of agranulocytes on the surface was observed (B). Both granulocytes and agranulocytes on the surface of decellularized tissue engineered constructs without scaffold (C). Scale bars indicate 200 μm. Red indicated the granulocytes, and green the agranulocytes.
3.4.5 Surface Profile

A surface analysis was made via scanning electron microscopy (SEM). On the bare scaffold samples, cells were located homogeneously distributed along the individual scaffold fibers (Fig. 3.6A). On the DTECs with scaffold, cells were adhering to the surface of the tissue. Scaffold fibers were nicely embedded into the tissue and not exposed to the surrounding (Fig. 3.6B). In the DTECs without scaffold, cells were observed adhering to the surface (Fig. 3.6C).

![Figures A, B, C](image)

Figure 3.6 | Surface Analyses: Scanning electron microscopy images on bare scaffold samples show cells adhering to the scaffold fibers (A). Decellularized tissue engineered constructs with scaffold remnants shows cells on the outside and complete embedding of the scaffold material into the tissue (B). Decellularized tissue engineered constructs without scaffold reveals a smooth surface with cells adhering (C). Scale bars indicate 100 μm.

3.4.6 Cytokine Release

After 5 hours, the experiment was terminated and the circulating medium was analyzed for the enzyme release profile (Fig. 3.7). What was observed is that inflammatory regulating cytokines TNFα (p=0.0353), IL-6 (p=0.0116) and IL-1α (p=0.0166) in the DTECs without scaffold were all upregulated compared to the initial levels. The level of monocyte recruiting cytokine MCP-1 was upregulated in both DTECs without scaffold (p=0.0142) and
DTECs with scaffold (p=0.0024) compared to the control group. When looking at the protein MMP-1, which is associated with active cell infiltration by means of collagen degradation, this protein is significantly upregulated only in the DTECs with scaffold material (p<0.0001), indicative for active cellular infiltration into this biomaterial.

**Figure 3.7 | Cytokine Release:** Cytokine release profiles after 5 hours of testing. Significant increase of the pro-inflammatory cytokines TNFa, IL-6, and IL-1α, in the tissue constructs without scaffold remnants. MCP-1 is upregulated in both tissue engineered constructs. MMP-1 is only and significantly upregulated in the tissue construct that does contain scaffold remnants, indicative for active cell infiltration.
3.5 Discussion

From previous in vivo experiments, rapid cellular infiltration was observed in explanted DTEHVs from sheep (39), which can be promising for future regeneration capacity and is limited in currently used bio prosthesis (37,135). Based on histological findings, these infiltrated cells were mainly observed in the vicinity of scaffold remnants. This raised the question whether scaffold material, residing inside these DTEHVs, was triggering active cell infiltration. In this study three different biomaterials were produced and analyzed by using a meso fluidic bioreactor system that circulate cells under physiological conditions. Decellularized tissue engineered constructs were successfully produced, either with or without scaffold material, and their effects on the circulating cells were compared to the bare scaffold material.

Blood was collected where after cells were isolated and stained either red for the granulocyte fraction and green for the agranulocyte fraction. FACS analyses revealed however three clear distinguishable cell populations with one clear peak of the red signal originating from the granulocyte cell fraction, while the green population revealed two clear distinguishable peaks. Even in the control samples containing only the agranulocyte fraction, two clear distinguishable peaks could be observed (results not shown). The dye used in this experiment contains a group that is using an intracellular glutathione S-transferase-mediated reaction to activate fluorescence. This reaction is known to play a role in the metabolic activity of eukaryotic and prokaryotic cells and that levels of glutathione differ based on mitochondrial activity (157). Glutathione levels in the different subsets of blood cells also differ, with higher contents in the monocyte fraction compared to the lymphocyte fraction (158). This might explain why in this agranulocyte fraction, the expected monocyte population as being indicated in green, gives a brighter signal compared to the expected lymphocyte fraction indicated in blue (Fig. 3.2A and 3.2B).

Based on the flow cytometry analyses, after 5 hours of circulation, the agranulocyte population is still above 50% of the initial concentration (Fig. 3.3). When comparing this to the granulocyte fraction, it appears that the curve is still in its linear phase when being above its half time value. Therefore, it can be assumed that the agranulocyte fraction might eventually also decrease in similar exponential way, but that up to 5 hours this time period is to short to observe this behavior.

A clear difference in infiltration speed and total amount of cell infiltration was observed in the granulocyte fraction of the bare scaffold sample compared to both DTECs. This can be explained by the porous three-dimensional open structure of the biomaterial. Since all the individual scaffold fibers are in direct contact with the cells, the total surface area to
which cells can adhere is much larger compared to the other biomaterials. Interestingly, no differences in granulocyte depletion were observed between the two types of DTECs. Despite that it appeared that the granulocytes actively infiltrate into the tissue-engineered samples with scaffold as was shown from the fluorescent cross-section analyses, the total amount of infiltrating cells from this fraction was not significantly larger.

What can be seen from the infiltrated cells in the tissue engineered sample with scaffold (Fig. 3.4B), is that especially the granulocytes were observed mainly near the scaffold fibers as been depicted in blue. This result suggests that the infiltrated cells actively migrate toward the scaffold remnants, which are embedded into the tissue. Granulocyte infiltration into the core of the construct can be seen from figure 3.5B, where as from the SEM pictures depicted in figure 3.6B, no scaffold fibers appear on the outside of the tissue engineered construct. This might explain the up regulation of MMP-1 that was probably secreted by the granulocyte fraction in order to be able to actively infiltrate the tissue construct.

Here, the observed rapid recellularization of granulocytes and the increase of pro-inflammatory cytokines, might not be in favorable for tissue regeneration. However, this 5 hour experiment is expected to show mainly the innate immune response, where the long-term outcome will be defined by the adaptive immune response.

In addition, the granulocytes infiltrate into the constructs with scaffold, but not in those without scaffold. Therefore, it seems that cells are able to sense the presence of the encapsulated remnants already in the circulation. Although it is still not fully understood how these immune cells sense the presence of the foreign material, it appears that toll-like receptors play an important role and are able to detect polymer biodegradable products (159). These tissue engineered constructs have been cultured for 4 weeks, in which PGA scaffold starts hydrolyzing after 2 weeks, where after 4 weeks most of the material has been degraded (64). As a result of the hydrolyses, local pH will drop making the environment acidic (160). Although it is known that an acidic environment can be beneficial for integrin activation, thereby affecting cellular adhesion and migration (161), there is also contradicting literature available describing the impaired effects of a low pH on granulocyte migration and infiltration (162).

Despite the effects on cellular migration, changes in extracellular pH can heavily affect immune activity and result in a diminished release of pro-inflammatory cytokines when these secreting cells are present in acidic environments (163). In this study, a decreasing trend of pro-inflammatory cytokine release of IL-1α, IL-6, and TNFα with the increasing amount of scaffold content could be observed. Therefore it can be hypothesized that
these fast degrading polymers create a local acidic environment that can suppress a pro-inflammatory response. It has to be mentioned that the enzyme secretion over time is not further investigated and is therefore a limitation of this study. Despite, this study shows the importance of including granulocytes in studies that investigate the regenerative response of immune cells to biomaterials. Where current research is mainly focusing on the response of peripheral blood mononuclear cells (PBMCs), the lack of granulocytes in these cell populations and their influence on the interpretation of the outcomes needs to be further evaluated.

### 3.6 Conclusion

This study was aimed at finding the explanation why our implanted decellularized tissue engineered heart valves showed fast initial cellular infiltration within hours of implantation. An in vitro setup was used to bring blood cells in contact with tissue engineered biomaterials either with or without scaffold remnants compared to bare scaffold material. These results show that granulocyte fraction are activated faster and mainly within 5 hours. These cells have shown to infiltrate into the core of the construct only in the tissue-engineered sample that does contain scaffold material. Once inside the construct, granulocytes migrate toward the present scaffold remnants (Fig. 3.1). Even more so, MMP-1 is significantly upregulated in the tissue engineered constructs including scaffold remnants. These results confirm the hypothesis that scaffold remnants are indeed responsible for early cell recruitment from the blood. However, pro-inflammatory cytokines as IL-1α, IL-6 and TNF-α are only significantly upregulated in the constructs without scaffold remnants after 5 hours of exposure. All these results together, show that scaffold remnants are responsible for initial cellular infiltration and that their presence can influence the initiated immune response.

### 3.7 Acknowledgment

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CHAPTER 4

COMPUTATIONAL DESIGNED 3D PRINTED SELF-EXPANDABLE POLYMER STENTS WITH BIODEGRADABLE CAPACITY FOR MINIMALLY-INVASIVE HEART VALVE TRANSPLANTATION

A proof-of-concept study

The content of this chapter is based on:


* Shared first author
4.1 Abstract

Due to the rapid development of minimally invasive implantation techniques and the promising in vivo remodeling potential of decellularized tissue engineered heart valve prostheses, there is an increasing need to provide stent alternatives that allow for growth in order to make these techniques available for pediatric patients. By means of computational tools and 3D printing technology, this proof of concept study demonstrates the design and manufacture of a polymer stent with a mechanical performance comparable to a conventional nitinol stent used for the same purpose. A commercially available 3D printing material was selected and computational and experimental crush and crimping tests were conducted to validate the results predicted by the computational model. Finally, the degradability of the material was assessed and confirmed via accelerated hydrolysis in terms of loss in mechanical properties and a decrease in molecular weight.
4.2 INTRODUCTION

Every year, approximately 280,000 patients undergo heart valve surgery worldwide (164) and the number of patients requiring heart valve replacement is expected to triple by 2050 (2). Bioprosthetic and mechanical valves are currently available life-saving substitutes (26). Nonetheless, the lack of repair and growth capabilities of these alternatives demands staged interventions in pediatric patients as they outgrow the prostheses. Recent developments in decellularized tissue engineered valves (DTEHVs) are promising in terms of remodeling potential. These valves were implanted minimally invasively in sheep and demonstrated rapid host cell repopulation and extracellular matrix production, indicative for growth potential (165). Stent devices for minimally invasive heart valve implantation are crucial to enable delivery and provide instant support to the prosthesis after deployment. However, the stent is only required until the heart valve is fully integrated into the host tissue. In addition, for DTEHVs to be considered as an alternative for pediatric patients, it is of importance that the stent can accommodate for growth. The currently used metal stents, which reside permanently inside the body, lack growth capacity and may incur long term complications such as hyperplasia (166,167). Biodegradable polymers are interesting alternatives, broadly investigated for their applicability in stent devices (168). These materials are not only of interest due to their short-term presence, but also because they might enable other benefits, such as low late thrombus formation and less interference with minimally invasive techniques such as magnetic resonance imaging (MRI) (169).

Polymer stents for minimally invasive heart valve applications have to meet a set of criteria such as sufficient radial force after deployment to maintain its position and limited plastic deformation to secure initial valve functioning until host body integration. In this proof of concept study, we aim to produce a computational-based, self-expandable polymer stent prototype with biodegradation potential. Our goal is to achieve the same mechanical performance of a polymer stent as with a nitinol stent currently used for preclinical implantation studies of DTEHVs in sheep (165). In order to design a stent with such mechanical requirements, a previously developed finite element (FE) computational model of a nitinol stent was used (170). To translate the computational simulations into physical prototypes, fused deposition modeling (FDM) rapid prototyping technology was combined with a commercially available flexible thermoplastic copolyester elastomer (TPC). Copolyesters are an interesting category of biodegradable polymers since the ester bonds are susceptible to hydrolysis. The TPC was mechanically characterized by tensile tests and used as input for the computational model. Based on this computationally
engineered design, 3D printed stents were obtained and subjected to crush and crimping tests for mechanical validation. Ultimately, an accelerated in-vitro degradation test was performed on the polymer stents to assess the biodegradability of the TPC via hydrolysis. Degradation over time was determined via mechanical tensile tests, differential scanning calorimetry (DSC), gel permeation chromatography (GPC) and scanning electron microscopy (SEM).

4.3 MATERIALS AND METHODS

4.3.1 COMPUTATIONAL SIMULATIONS

POLYMER MECHANICAL CHARACTERIZATION

Wires of FlexiFil (FormFutura, the Netherlands), a flexible TPC filament used for FDM applications, and 3D printed dog bone samples of this material were tested (n=3 each) to take into account the effects of the production procedure. Both groups were evaluated by uniaxial tensile tests in a universal testing machine (Zwicki-Line, Zwick/Roell, Germany) equipped with a 2.5 kN load cell. Samples were loaded at ambient temperature until breaking to assess the mechanical properties. Stress versus strain curves were determined and compared. Finally, the mechanical properties of the 3D printed samples were used as input for the computational models.

STENT DESIGN

The stent geometry used for the computational model is based on a self-expanding laser-cut nitinol stent used for animal trials of tissue-engineered heart valves (pfm medical, Germany). The stent is composed by a repeating design with three rings of 40 struts, connected by bridges. Three-dimensional computational models simulating stent crushing, crimping and self-expansion were developed using FE software (ABAQUS 6.14, Dassault Systèmes Simulia Corp. Providenc, USA). These models were validated for nitinol in a previous study with a stent geometry used for minimally invasive heart valve implantation in sheep (170). To obtain a new polymer stent design with comparable mechanical performance to the nitinol stent, the number, thickness and width of the struts were adjusted. The design was optimized simultaneously by means of simulated crush and crimping tests with the nitinol stent model and different designs of the polymer stent.

COMPUTATIONAL CRUSH TEST

Two plane rigid surfaces were created to simulate parallel plates (initially separated by 31 mm) and the stent was compressed until a clearance of 5 mm by displacement of the
upper plate. Afterwards, the plate returned to its initial position (170). The vertical reaction force and the displacement of a reference point placed on the upper plate were registered. Force versus displacement curves of nitinol and polymer stents were compared. The stent design was modified until the crush response of the polymer stent was equal or higher than the nitinol stent.

**Computational crimping test**

A cylindrical rigid surface was used to simulate a crimping tool and calculate the radial forces (170). The stent was crimped from its nominal diameter of 31 mm until 12 mm, in accordance with transapical implantation requirements, and allowed to self-expand. The radial reaction forces on the crimping tool were determined and plotted versus the stent outer diameter (OD). The design was modified to make the stent crimpable minimizing self-contact of the struts. Force versus diameter curves of nitinol and polymer stents were compared. Furthermore, to illustrate the result of design parameter modifications, the effects of reducing the polymer strut thickness was assessed.

**4.3.2 Prototyping and validation**

**Prototyping**

Based on the computational model, an optimal stent geometry in terms of radial force, crimpability and recovery was converted to a stereolithography file format via MakerWare software (V 2.4.1.35, MakerBot, USA). The stent design was translated into physical prototypes by using fused deposition modeling (FDM) 3D printing technology (Replicator™ 2X, MakerBot USA). Printer settings were specified for a nozzle temperature of 210 °C and a plateau temperature of 130 °C. Layer height was set at 0.2 mm with a polymer extruding speed of 90 mm/s and a nozzle travel speed of 150 mm/s. To account for the differences between the computationally derived stent geometry and the 3D printed prototype, a digital image of a stent portion was acquired (100 X, Keyence Digital Microscope VHX-500F) from which a strut profile with more accurate dimensions was sketched and incorporated in the computational models repeating the crush and crimping simulations.

**Experimental crush tests**

Polymer stent prototypes (n=5) were crushed in a universal testing machine (Zwick/Roell, Germany) equipped with a 2.5 kN load cell in an ambient environment. The stents were compressed from their nominal diameter until a 5 mm clearance between plates at a speed of 0.2 mm/s. Without a holding time, crush plates returned (at similar speed) back to their initial position. Force versus plate displacement was measured. Experimental and computational crush results were compared overlapping the displacement versus force curves.
Experimental Crimping Tests

Polymer stents (n=3) were crimped in a radial force machine (RX 650, Machine Solutions Inc., Flagstaff, Arizona) at a speed of 0.2 mm/s. A crimping head composed by 12 movable wedges disposed about a rotational axis crimped the stents from their nominal diameter until 12 mm. The displacement of the head was later reversed allowing self-expansion of the stent. Experimental and computational crimping results were compared by overlapping the diameter versus force curves.

4.3.3 In Vitro Degradation Characterization

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) analyses were employed to characterize the melting and crystallization behavior of the polymer in order to set the appropriate temperature for enhanced degradation (823e/700, Mettler-Toledo). A sample was heated to 300 °C at 20°C/min, maintaining this temperature for 5 minutes, and cooled down to 25 °C at the same speed. In a subsequent cycle, heating and cooling was performed without holding time. The recorded heat flow was normalized by sample mass. Curves from the first cooling and second heating were computed.

Enhanced Hydrolysis

Polymer stents (n=15) were subjected to enhanced hydrolysis in a temperature controlled water bath, containing demineralized water maintained at 90 °C. Every 7 days, 3 stents were retrieved, freeze dried overnight and stored in an air tight sealed container at room temperature until mechanical testing.

Mechanical Analysis

Diamond shaped samples were cut from the stents and tested uniaxially, by stretching in the circumferential and longitudinal direction (n=2 per direction and time point) until break to assess the loss of mechanical properties by hydrolysis over time.

Gel Permeation Chromatography

The effect of hydrolysis on the molecular weight of the polymer was assessed via gel permeation chromatography (GPC). The GPC measurements (n=18) were performed on a Mixed-D column (300×7.5 i.d. 5 μm particles, Agilent Technologies) using chloroform at 1mL/min as the mobile phase and a photo diode array (PDA, SPD-M20A, Shimadzu) as the detector. The chromatograms were recorded for the UV absorption of 254 nm. The system was calibrated with polystyrene standards.
**Scanning Electron Microscopy**

To investigate the microscopic effects of hydrolysis on the polymer stents, samples (n=18) were analyzed via scanning electron microscopy (SEM) (UHR-SEM, FEI, USA). Freeze dried samples were analyzed at low vacuum mode with a secondary electron detector at 10 kV.

![Polymer Mechanical Properties](image)

**Figure 4.1** | Polymer Stent Design: Stress-strain curves of the polymer wire and dogbone samples. Results are represented as mean values ± standard deviation (A). Based on computational iterations, the final stent design was translated into a physical prototype by using 3D printing technology (B). The computational geometry was adjusted taking into account the dimensions of the resulting prototype (C).
4.4 Results

4.4.1 Polymer Stent Design

Mechanical Characterization of the Polymer

The material properties of the TPC used in this study were determined via uniaxial tensile tests. To assess the influence of 3D printing on the mechanical properties of the material, stress-strain curves obtained from the polymer wires and 3D printed dog bone samples were compared (Fig 4.1A). The printed samples deviate from the linear behavior for lower stress values than the wires. However, despite these differences, the decay in tensile properties is not drastic. The averaged stress-strain curve from the printed dog bones was used as input for the simulations.

Polymer Stent Prototyping

Based on the geometry of the nitinol stent used in preclinical studies (165), the design was iteratively adjusted to improve the performance of the polymer prototype based on computational crush and crimping results. The final design (Fig. 4.1B) resulted in a stent geometry with 24 struts, an outer diameter of 30.5 mm and a length of 30 mm. The prototyped stent (Fig 4.1B), however, slightly differs from the computationally derived geometry due to factors inherent to the 3D printing manufacturing process. To account for these differences, a strut sketch was derived from a digital image of the stent prototype (Fig 4.1C) and incorporated in the simulations.

Figure 4.2 | Crush Test Results: Computational crush test showing the equivalent plastic deformation (PEEQ) and the deformed geometry after crushing and self-expansion overlapped with the original geometry (grey) (A) Experimental and computational force-displacement curves of polymer stent with mean values ± standard deviation (B).
4.4.2 Computational Analysis and Experimental Validation

Crush Tests

The simulation shows the presence of plastic deformation at the sides of the stent creating an oval geometry after load removal (Fig 4.2A). Experimental force versus displacement curves resulting from the crush tests of the polymer 3D printed prototypes were obtained and compared with the computational crush simulation (Fig 4.2B). Experimental results are in good agreement with the computational models.

Figure 4.3 | Crimp Test Results: Computational crimp test showing the original (grey) and deformed geometry after crimping and self-expansion represented as the equivalent plastic deformation (PEEQ) (A). Experimental and computational diameter-displacement curves of polymer stent with mean values ± standard deviation (B).

Crimp Tests

The simulation shows a plastic deformation repetitive pattern that is more severe at the zones where two struts meet, causing a reduction in stent diameter after load removal (Fig 4.3A). Experimental diameter versus displacement curves resulting from the crimp tests of the polymer 3D printed prototypes were obtained and compared with the computational crimping (Fig 4.3B). Also here good agreement between computational predictions and experimental results can be observed. However, the diameter after self-expansion could not be properly captured, were the computational values go back to ±25 mm, the experimental recovery is ±28 mm. Computationally, loading and unloading slopes are coincident. Experimentally, the unloading slope is less steep than the corresponding loading curve. Comparing experimental and computational results, only the loading curves overlap causing a diameter mismatch during unloading.
**Comparison with Nitinol Stent**

Computational crush polymer stent results were compared with equivalent simulations performed on the nitinol stent that served as a reference (170). The resulting crush response of the polymer stent is higher than the nitinol stent (Fig 4.4A). Due to the presence of plastic deformation in the polymer, the unloading curve does not overlap with the loading curve for low values of plate displacement as it happens with the nitinol stent due to the superelastic nature of this material.

The computational predictions of the crimping behavior of the polymer and nitinol stents were as well compared showing that both alternatives can provide the same radial force for a diameter of 22 mm (Fig 4.4B). For smaller diameters, the polymer stent provides even a higher radial force than the nitinol stent. The presence of plasticity is not
evidenced in the nitinol stents and the initial diameter is recovered upon unloading whereas the polymer stent experiences a diameter reduction. This situation suggests that, having both stents with a similar nominal diameter, the polymer stent would be suitable for implantation in smaller arteries due to its lower diameter after self-expansion. Comparing the unloading curves of both stents, it can be evidenced that the slope of the polymer stent is steeper than the one of the nitinol stent in the vicinity of the intersection point between both lines. In order to soften the force variation with respect to diameter changes, the thickness of the polymer stent was consequently reduced. This situation causes a reduction of the radial force of the stent while keeping the diameter after self-expansion constant (Fig 4.4C).

4.4.3 IN VITRO DEGRADATION CHARACTERIZATION

DIFFERENTIAL SCANNING CALORIMETRY

Polymer phase transition was analyzed by DSC (Fig 4.5A). It can be observed that this copolyester has a crystallization temperature of 133 °C and a melting temperature of 184 °C. Therefore, an enhanced in vitro degradation study conducted in 90 °C water is expected only to enhance hydrolysis speed, and not affect phase transition.

GEL PERMEATION CHROMATOGRAPHY

Figure 4.5B shows the evolution of the molecular mass of the 3D printed scaffolds over the course of the degradation experiment. As shown, the molecular mass (Mₙ) dramatically decreases, from 25063±3671 g/mol to 6141±356 g/mol after 35 days of enhanced hydrolysis. This suggests that this flexible TPC does hydrolyze over time, which is beneficial for in vivo degradation application.

Figure 4.5 | Enhanced Degradation: DSC analysis for identification of crystallization and melting phase regions (A). GPC analysis shows the molecular mass (Mₙ) over time, during enhanced degradation at 90 °C with mean values ± standard deviation (B).
Figure 4.6 | Mechanical Properties During Hydrolysis: Maximum force at break in the circumferential and longitudinal direction as a function of time (A). Maximum strain at break in the circumferential and longitudinal direction as a function of time (B). Results shown as mean values ± standard deviation.

Figure 4.7 | Scanning Electron Microscopy: Surface analyses during enhanced degradation of polymer stents. Before degradation, the surface was smooth (A), over time the surface roughness increased (B-C). After 14 days, holes appeared in the material (D-F). Scale bar represents 500 μm.
MECHANICAL ANALYSIS

Diamond shaped samples obtained from the polymer stent prototype were loaded uniaxial in the circumferential and longitudinal directions showing a decay in the maximal applicable stress over time. After 10 days of enhanced in vitro degradation, mechanical properties dropped until half of the initial force (Fig. 4.6A). The maximal strains dropped by 50% around 7 days of enhanced degradation (Fig. 4.6B). Despite the fused polymer deposition printing in longitudinal direction, the obtained maximal breaking stresses and strains in the circumferential and longitudinal direction are comparable.

SCANNING ELECTRON MICROSCOPY

The surface of the 3D printed polymer stents was smooth before starting enhanced degradation (Fig. 4.7A). Over time, surface roughness increased (Fig. 4.7B-F). Furthermore, the samples became porous after 14 days of enhanced degradation (Fig. 4.7C-F).

4.5 DISCUSSION

In this proof of concept study, the combination of computational modeling and 3D printing technology was investigated to design large diameter biodegradable polymer stents that can be minimally invasively implanted allowing growth of the patient.

The bottleneck for large diameter biodegradable polymer stents production is currently their lack of sufficient mechanical properties (171). Conventional biodegradable polymers such as poly(lactic acid) PLA require sophisticated material processing to enable sufficient radial force (127) and are only applicable to small diameters, where other large diameter stents deal with stent migration problems due to low mechanical forces (172). In this study we showed that rapid prototyped polymer stents could reach similar levels of radial force compared to nitinol alternatives. However, since polymers in general have a lower Young’s modulus than metals, geometrical compensation in terms of strut thickness and width is required, which could lead to stenosis (173). For pediatric applications where a growing stent is required, further adjustments on the stent size and thickness of our prototype need to be performed to adapt to the size of the patient and the force requirements. The effect of decreasing the strut thickness is depicted in Fig 4.4C. This technique offers a good alternative to reduce the radial force while maintaining the amount of oversizing constant and providing a more gradual force decay during stent deployment. An additional benefit of a lower radial force is that after crimping, less force would be required to push the stent out of the delivery device during implantation, by which valvular positioning can be better controlled.
The nitinol stent that we have used as reference for the design of our polymer stent has been previously used successfully for DTEHV implantation in adult ovine pulmonary arteries of 24-26 mm (165). In this diameter range, the corresponding computational radial force is between 15-17 N (Fig 4.4B). The oversizing of the nitinol stent with respect to the adult ovine arteries is therefore in between 18.5-28.3 %. The polymer stent has shown an experimental loss of 6% in diameter after crimping and self-expansion (Fig 4.3B). Aiming to have the same degree of oversizing as the nitinol stent, would mean that the polymer stent is suitable for implantation diameters in arteries of 22.4-24.43 mm. For these diameter ranges, the experimental force of the polymer stents is between 13.5-18.9 N, being comparable to the nitinol alternative.

A limitation of the computational simulations is that the polymer material model is merely elasto-plastic and cannot capture time nor temperature dependent behavior. For the application of DTEHVs, the total crimping time during implantation could range between 10 to 20 minutes. Therefore, experimentally, the effect of stress relaxation could result in a smaller final diameter after deployment than the one anticipated by the model. Furthermore, despite the good level of agreement between the computational and experimental crimp models, the diameter after self-expansion could not be simulated with a higher accuracy. This situation can be explained by the nature of the material model, which does not account for differences in the elastic behavior between tension and compression. These effects have to be carefully investigated for future applications.

FDM printing technology is an evolving and promising approach for polymer processing. It can be used for a broad range of thermoplastic materials where other rapid prototyping methods, such as laser sintering or stereolithography, offer fewer options due to their specific processing technique (174). The downside of this method is that the layered nature of the processing technology is less accurate and might present local regions with different mechanical performance. However, 3D printing was considered to be an acceptable prototyping technique for the initial phase of biodegradable stent design, providing a good compromise between material properties and the feasibility to produce prototypes with reliable outcomes.

In this proof of concept study, a commercially available TPC was used to manufacture 3D printed stents. Apart from proper mechanical characteristics and degradation capacity, special attention has to be paid to the biocompatibility and degradation products of the copolyester to be non-toxic. Aromatic polyesters, such as poly(butylene terephthalate) (PBT) and poly(ethylene terephthalate) (PET), which are largely used in the commercial industries (175) and investigated for their biocompatibility (176,177), have excellent mechanical properties, but are more resistant to hydrolytic degradation (178). Where
interesting biocompatible aliphatic polyesters as poly(lactid acid) (PLA) and poly(caprolactone) (PCL) (45) have good biodegradative properties in terms of hydrolysis, but lack proper mechanical behavior (179). Blending both aromatic and aliphatic building blocks seems feasible and might be tunable to meet appropriate mechanical and hydrolysis properties for large diameter stent applications (180). The speed at which hydrolysis of polyesters occurs in vivo depends on multiple factors, such as monomer composition, relative humidity, temperature and bioactivity of present enzymes (181). Besides hydrolytic degradation, oxidative and enzymatic degradation also contribute to in vivo polymer degradation (182). However, to what extent these pathways are affecting the total degradation speed has to be further investigated. But so far, this proof-of-principle study showed that stents made out of TPC are susceptible to hydrolysis, which is crucial in order to facilitate future growth.

4.6 Conclusion

In order for DTEHVs to be minimal invasively implanted, while maintaining the capacity to grow with the patient, the technical feasibility for a suitable biodegradable stents was investigated. In this proof-of-concept study it was shown that computational-based 3D printed self-expandable stents with biodegradable potential could successfully be designed and produced. These stents show a reasonable degree of plastic deformation during minimally-invasive procedures, while maintaining radial forces comparable to nitinol stents. The computational simulations have demonstrated the capacity to build models with realistic outcomes, based on uniaxial material characterization. In addition, the FDM 3D printing technique is a promising manufacturing technique to translate computational models into reliable prototypes that can be used to assess the mechanical performance of stent designs, where the chosen copolyester material has shown to have biodegradation potential via hydrolysis.

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CHAPTER 5

IN VIVO REMODELING OF DECCELLULARIZED TISSUE ENGINEERED HEART VALVES DURING ONE YEAR FOLLOW UP

Preliminary results in a sheep model

The content of this chapter is based on:


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5.1 Abstract

Decellularized tissue engineered heart valves (DTEHVs) are a promising alternative to current heart valve prostheses in view of the rapid repopulation with host derived cells, creating a living heart valve. Previous in vivo studies, however, resulted in the long-term (months) development of valvular insufficiency because of cell mediated leaflet retraction. Computational simulations of the leaflet deformation in response to transvalvular pressures, suggest that a change in valve geometry may counteract this leaflet retraction by enhancing radial stretch in the leaflets. In this study we have investigated if the proposed geometrical adjustments indeed result in long-term in vivo functionality.

We present the first results of a larger study. For the first time, we have obtained successful one-year functionality (n=3) in sheep using decellularized tissue engineered heart valves. Prior to implantation, (n=3) valves were analyzed in vitro to calculate an averaged regurgitation fraction of 9.35±400 %, from which only 1.64±0.67 % was caused by the leakage volume. Valves (n=3) were implanted transvenously, and maintained stable in vivo performance up to 52 weeks. The explants showed smooth and pliable leaflets, without macroscopic signs for thrombus formation or calcification. Leaflets were significantly (p=0.0004) shortened over time, without affecting valve performance. This observation was explained by numerical models to be the result of tissue regeneration, leading to circumferential collagen remodeling and leaflet shortening, without affecting valve functionality. Circumferential collagen organization was confirmed by confocal microscopy together with a significant increase (p=0.035) in tissue stiffness being 5.44±1.18 MPa in circumferential direction, compared to 3.05±0.41 MPa in radial direction. Low amounts of α-SMA positive cells were observed in the explants, which was explained by computational simulations to be beneficial in terms of collagen distribution and valve functionality. These results are part of a larger, ongoing study, but so far the available data is providing a strong indication that the implemented improvements to the current valve geometry are decisive for long term in vivo performance, where valves have shown to remodel towards native like characteristics, holding significant potential for future clinical applications.
5.2 INTRODUCTION

Annually, 60,000 patients in Europe receive a heart valve prosthesis. In order to treat these patients with a lifetime solution, decellularized tissue engineered heart valves (DTEHVs) may represent an interesting alternative for future applications (55). The main advantage of the DTEHVs is that they have shown to possess the remarkable capacity to rapidly repopulate with host cells after implantation (39). This unique feature, which is lacking in current heart valve prosthesis (37,135), is raising the question if these valves possess the capacity to actually remodel over time. So far, we have shown that the content of infiltrating cells from the host as well as the amount of synthesized extra cellular matrix (ECM) is increasing gradually over time (39). These infiltrating cells were α-SMA positive, which may cause retraction of the heart valves leaflets. Based on computational simulations, geometrical improvements for these DTEHVs have been suggested to promote radial tissue stretch, in an attempt to counteract cell mediated leaflet retraction (131). For this purpose, bioreactor constraints were developed and implemented into the valve culture procedure, which resulted in proper in vitro functioning DTEHVs that maintained the imposed shape (183). However, the question still remains if these geometrical adjustments will indeed prevent cell mediated leaflet retraction in vivo. Therefore, the aim of this study is to investigate the effects of the implemented geometrical adjustments of these DTEHVs on tissue remodeling and functionality, during long-term in vivo follow-up. The preliminary data of this study is using DTEHVs (n=7), from which three have been implanted transvenously in sheep as a pulmonary valve replacement. These have been assessed monthly via cardiovascular MRI (cMRI) for regurgitation measurements. The valves have been explanted after reaching a one-year follow-up. The remaining valves (n=4) were not implanted and served as a control group. Functionality of the valves prior to implantation was assessed using an in vitro test setup, being subjected to physiological pulmonary conditions. All valves were inspected first on their macroscopic appearance, where after they were divided into parts for further analyses. Via conventional histology on the explants, the level of recellularization for α-SMA positive cells was analyzed. Biomechanical analysis and whole mount staining for collagen using confocal microscopy, were performed to investigate collagen remodeling. In addition, leaflet shortening was quantified for both the control valves as well as the explanted valves. To elucidate the robustness of the valve design, the output from the experimental data was used as input for the computational models to predict and understand valvular tissue remodeling behavior under pulmonary loading conditions, taking into account different levels of tissue thickness, and cell contractility.
5.3 MATERIALS AND METHODS

5.3.1 HEART VALVE TISSUE ENGINEERING

**GENERAL CULTURE PROCEDURE**

Tissue-engineered heart valves (TEHVs) (n=7) were cultured as previously described (139). In summary, tri-leaflet heart valves were cut from non-woven polyglycolic-acid meshes (PGA; thickness 1.0 mm; specific gravity 70 mg/cm³; Cellon, Luxembourg), sewn (Prolene 6-0, Ethicon, USA) into a radially self-expansible nitinol stent (length=31 mm, ID=30 mm at 37 °C; PFM-AG, Germany), and coated with 1.75% poly-4-hydroxybutyrate (P4HB; MW: 1 x 10⁶; TEPHA Inc., USA) in tetrahydrofuran (THF; Sigma-Aldrich, USA). These constructs were sterilized with 70% ethanol (EtOH, VWR international, France) followed by an anti-fungi/bacterial treatment containing 10% penicillin/streptomycin (Pen/Strep)(Lonza, Belgium), supplemented with 0.5% Fungin (InvivoGen, USA). Primary isolated ovine vascular-derived cells were seeded (0.3 x 10⁶ cells / cm², passage 6) onto the valvular shaped scaffolds using fibrin as a cell carrier. The seeded constructs were placed into the bioreactor system containing growth medium, supplemented with L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma, USA), and were subjected to 1 Hz pulsatile flow, including geometry constraining inserts, to maintain the imposed leaflet geometry, as being described elsewhere (183).

**CULTURE PROCEDURE ADJUSTMENTS FOR OVINE APPLICATIONS**

Previous work describes the culture of human DTEHVs with improved leaflet geometry (183). Due to the increased traction forces and tissue production of ovine vascular-derived cells compared to human cell based applications (184), adjustments were made to the current bioreactor setup and culture procedure. To prevent tissue retraction in radial direction, heart valve leaflets were grown in merged configuration and supported by an additional insert from below to define and maintain the coaptation length during the culture procedure (Fig. 5.1B). To prevent hampering of medium exchange due to the introduction of this additional tissue constraint, the top insert was adjusted by enlarging the opening area near the coaptation area (Fig. 5.1A). To maintain continued medium supply in both top and bottom region of the valve, unidirectional pressure driven fluid flow was applied by using one way valves (Qosina, USA), in which the circulation was spitted at the pressure tube and directed to both top and bottom region (Fig. 5.1C).

In the valves for implantation (n=3), the culture medium contained 0.5% lamb serum (Gibco, USA) and the top insert was included in the bioreactor system from the start of the culture procedure. To improve the amount of tissue during the course of this study,
the serum content was increased to 1%, and the top insert was placed in the bioreactor system after 13 days of culture in the control group (n=4). This, to enhance medium exchange to the cells in the early stage of tissue production, by which compaction is not yet occurring.

**Decellularization Procedure**

After 4 weeks of culture, the TEHVs (n=7) were decellularized as described by Dijkman et al. (38). Briefly, TEHVs were decellularized overnight in detergent solution (0.25% Triton X-100, sodium deoxycholate and 0.02% EDTA), where after the bioreactor inserts were removed. Nucleic remnants were enzymatically degraded by using Benzonase (EMD Millipore, USA) incubation steps, diluted in 50 mM TRIS-HCL buffer solution in concentrations of 100 U/ml, 80 U/ml and 20 U/ml for 8, 16 and 8 hours, respectively, on a shaker at 37 °C. Afterwards, the valves were washed with PBS to remove cellular remnants, and sterilized with 70% EtOH and an anti-fungi/bacterial solution as described above. After sterilization, these decellularized tissue engineered heart valves (DTEHVs), were stored at 4 °C until further use.

**5.3.2 Functionality Assessment**

**In Vitro Functionality**

All DTEHVs (n=3) were tested on there functionality prior to implantation. DTEHVs were placed inside a silicon annulus of 30 mm inner diameter and positioned into a hydrodynamic pulsatile test system (HDT-500, BDC Laboratories, USA) containing a physiologic saline solution at 37 °C. Valves were subjected to physiological pulmonary conditions (rate of 72 BPM, stroke volume of 70 ml, maximum diastolic pressure difference of 25 mmHg) for 1 hour. Flow and pressures were measured via a transonic sensor (TS410, Transonic Systems, USA) and pressure sensors (BDC-PT, BDC Laboratories, USA), respectively. Data was collected for 3 seconds at 5 kHz, and functionality was assessed from an average over 10 cardiac cycles by using Statys™ software (BDC Laboratories, USA), to determine the regurgitation fraction being defined as the sum of the leakage volume and the closing volume, expressed as a percentage of the stroke volume. Slow-motion movies were recorded at high-speed burst mode to assess opening and closing behavior of the valves in motion (G15 PowerShot, Canon, USA).

**In Vivo Functionality**

To investigate *in-vivo* functionality and regenerative capacity, DTEHVs (n=3) have been implanted via a percutaneous trans-venous approach in adult grey horned sheep as a pulmonary valve replacement. The implantation was conducted at the German Heart Institute in Berlin. Briefly, angiography and pressure measurements were performed in
the right ventricle and pulmonary artery. The DTEHVs were crimped and loaded into a custom-made delivery system with an inner capsule diameter between 8-10 mm, and inserted via the jugular vein of the sheep under visualization of fluoroscopy. After implantation, the position and functionality of the DTEHVs were assessed using angiography and intracardiac echocardiography (ICE). Anticoagulation therapy was maintained for 5 days after implantation, and valve functioning was assessed every 4 weeks via cMRI, including ICE at the final time point. Animals were sacrificed and valves were explanted using a study-endpoint of >30% regurgitation, or when a 1 year follow-up was reached. After explantation, valves were evaluated macroscopically and divided into individual parts for further analysis. Approval to conduct this study was given by the animal ethical committee (Regional Office for Health and Social Affairs Berlin), in compliance to the guidelines of the European and German Societies of Laboratory Animal Science (FELASA, GV-SOLAS).

5.3.3 Tissue Analysis

Histology

To investigate the infiltration of α-SMA positive cells after one-year of implantation, sections of (n=3) explants were fixated in 3.7% formalin (Fluka, USA) and imbedded in paraffin for histology. Coupes of 2-4 µm thickness were cut and stained for α-smooth muscle actin (α-SMA, Dako, IR611), during a 60 min incubation at room temperature. Primary antibodies were detected using iVIEW DAB detection kit. The samples were embedded in Shandon-Mount (Thermo Scientific, 1903331) and analyzed via bright field microscopy (Mirax Midi Slide Scanner, Zeiss, Germany).

Leaflet Dimensions

To investigate if the leaflets shortened during implantation, measurements on the explant were performed. First, the distance of the hinge towards the end of the stent was measured, to see if over time the leaflets were merging with the wall (Fig. 5.4A-B). The other measurement concerned the total length of the leaflet, from the hinge to the free edge, to quantify the leaflet shortening over time (Fig. 5.4A-B). Measurements were taken by using an electronic caliper (CD-15CPX, Mitutoyo, Japan), from 3 leaflets per (n=4) control valve, as well as from the (n=3) explanted valves.

Confocal Microscopy

Half leaflet sections of all (n=4) control valves and (n=3) explants were analyzed to investigate the global collagen orientation. Samples were stained via a whole mount staining with the collagen specific dye CNA35-OG488 (141), for 1 hour in PBS and visualized with a confocal microscope (TCS SP5X, Leica, Germany). The dye has an
excitation and emission profile of 488 nm and 500-550 nm, respectively. Samples immersed in Mowiol (Sigma, USA) were mounted between two preparation glasses. The specimen was observed with a 10X objective and Z-stacks were made throughout ±200 μm of the entire arterial side of the sample via stitched adjacent tile scans. Afterwards, a maximum intensity projection was made in Z-direction.

**IMAGE ANALYSES**

The images obtained from confocal microscopy were analyzed for collagen orientation by using the directionality analysis plugin from Fiji (ImagJ, 2.0.0-rc-14/1.49g, USA). With the software embedded local gradient orientation method, the calculated directionality was provided as the main angle and standard deviation from the data fit of a Gaussian distribution.

**BIOMECHANICAL ANALYSES**

Mechanical properties of all (n=4) control valves and (n=3) explants were analyzed by using a biaxial tensile tester (BioTester, 5N load cell; CellScale, Waterloo, Canada) in combination with LabJoy software (V8.01, CellScale). Two samples (36 mm² each) per valve were obtained from the belly region from which the thickness was measured by using an electronic caliper (CD-15CPX, Mitutoyo, Japan). The samples were stretched equibiaxial in both the radial and circumferential direction up to 20% strain, at a strain rate of 100% per minute. After stretching, the samples recovered to 0% strain at a strain rate of 100% per minute, followed by a rest cycle of 54 seconds. Prior to measuring the final stresses, samples were preconditioned with 5 of these cycles. A high-order polynomial curve was fitted through each individual data set in both the radial and circumferential direction. The stiffness of the tissue was represented by the tangent modulus and was defined as the tangent to the fitted polynomial curve at 20% strain.

5.3.4 **COMPUTATIONAL SIMULATIONS**

**VALVE GEOMETRY AND MATERIAL BEHAVIOUR**

Based on the experimental data of the control valves, tissue remodeling of the DTEHVs was analyzed using computational simulations (Abaqus 6.14, Dassault-Systèmes Simulia Corp, USA) as described by Loerakker et al. (185,186) and compared to the obtained experimental data of the explants. The initial valve geometry in the model was equal to the geometry imposed on the DTEHVs during the in vitro culture procedure (183), where the leaflet thickness in the model was equal to the average leaflet thickness of the control valves being 0.52 mm. The tissue was modeled as a mixture of contractile actin stress fibers, collagen fibers, and isotropic matrix constituents, where the stress fibers and collagen fibers were distributed over different directions. The details of the constitutive
equations that describe the material behavior of each tissue component are detailed in Loerakker et al. (185,186). Briefly, the stress exerted by the actin stress fibers was dependent on the maximum intrinsic cell contractility and the current strain and strain rate experienced by each stress fiber. The stress exerted by the collagen fibers was described using an exponential stress-stretch law to capture the nonlinear behavior, where the amount of stress was determined by both the external hemodynamic load imposed on the valve, as well the contraction of the collagen fibers due to cell traction. Finally, the isotropic matrix components were modeled as an elastic Neo-Hookean material.

**Tissue Remodeling**

Remodeling of the tissue was simulated by including mechanisms for tissue compaction and collagen remodeling (185,186). Tissue compaction was included via cell-mediated contraction of the collagen fibers, which leads to compaction when the external load is too low to counteract cell traction forces. Collagen remodeling was simulated via isotropic synthesis of collagen in combination with strain-dependent degradation. For the latter, it was assumed that strain protects collagen fibers from degradation.

**Material Parameters**

The shear modulus $G$ of the isotropic matrix components was set at 10 kPa to prevent numerical instabilities. Next, the material parameters of the collagen fibers in the model were fitted to the biaxial tensile test data of the control valves as been described elsewhere (183). For the stress fibers, the original parameter values described in Obbink-Huizer et al. (187) were used, as stress fibers were absent in the tissues subjected to the biaxial tensile tests. These parameter values mostly represent a myofibroblast phenotype.

**Remodeling Simulations**

Multiple simulations were performed to analyze tissue remodeling in the DTEHVs, where in every simulation a dynamic variation in transvalvular pressure was applied (maximum diastolic pressure of 15 mmHg, 71 BPM). Tissue remodeling was simulated using a range between 20% to 100% of the maximum myofibroblast contractility, to investigate the effect on the remodeling process and valve functionality. In addition, simulations with different initial leaflet thicknesses were performed to determine the sensitivity of the remodeling process to this parameter.

**5.3.5 Statistics**

To assess the *in vivo* development of the measured regurgitation fraction over time, values were averaged where after a linear regression analysis was performed. Elongation
of the distance of the hinge region as well as shortening of the leaflet between the control valves and the explanted valves was investigated via an unpaired student t-test. For the biomechanical analysis, the presence of anisotropy between the obtained stiffnesses in both radial and circumferential direction was analyzed via a paired student t-test. All the data were averaged and represented as the mean ± standard deviation (SD), and considered significantly different for p-values < 0.05. Statistical analyses were performed using Prism software (V6.0d, Graphpad, USA).

Figure 5.1 | Valve Culture: Adjusted top bioreactor insert to define valve geometry (A) and the bottom insert to define the coaptation length (B). Adjustments to the bioreactor system applied pulsatile flow to both top and bottom regions of the valve (C). After culture, valves for implantation showed a maintained geometry after decellularization in both the valves for implantation (D-E), as well as the control valves (F-G).
5.4 RESULTS

5.4.1 DTEHV PRODUCTION

Based on macroscopic appearance, sufficient tissue was formed throughout all the valves, without showing weak spots of tissue formation. After removing the inserts, the imposed geometry in terms of belly curvature and coaptation length was fully maintained (Fig. 5.1D-G). The valves for implantation had thin and flexible leaflets, in especially the belly region (Fig. 5.1D-E). The control valves from the optimized culture protocol, showed thicker tissue formation throughout the leaflets (Fig 5.1F-G). All valves maintained equal coaptation lengths after culture.

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**Figure 5.2 | Valve Functioning:** Representative curves of in vitro valve testing, applying physiological conditions prior to implantation (A). The averaged regurgitation fraction of the valves upon implantation was $9.35 \pm 4.00 \%$, from which the closing volume was $7.71 \pm 4.62 \%$, and the leakage volume $1.64 \pm 0.67 \%$. These regurgitation fractions remained stable during in vivo implantation, confirming long-term valve competence up to one year (C).
5.4.2 IN VITRO FUNCTIONALITY

Prior to implantation, valve functionality was assessed in vitro, being subjected to physiological pulmonary testing conditions. All valves were fully opening and closing symmetrically, showing no signs of stenosis and having flexible leaflets (Fig. 5.2A). After 1 hour of testing, the implants had an averaged regurgitation fraction of 9.35±4.00 % (Fig. 5.2B). From this, 7.71±4.62 % was a result of the closing volume of the valve, causing the majority of regurgitation. The leakage volume was accountable for the remaining 1.64±0.67 %.

5.4.3 IN VIVO FUNCTIONALITY

After implantation, valve functionality was assessed periodically using cMRI, from which the in vivo regurgitation was calculated. Over time, the averaged regurgitation fraction of the valves was 7.36±1.53 % directly after implantation and 10.62±3.64 % after one year of follow up (Fig. 5.2C). No significant increase or decrease in regurgitation fraction was observed over time based on an linear regression analyses, having an estimated initial regurgitation fraction of 8.27±1.27 %, and a slope being non-significantly different from zero.

![Figure 5.3](image)  
**Figure 5.3** | Valve Explant: One year explants showed flexible and smooth, native-like leaflets (A), where no signs of thrombus formation or calcification could be observed on a macroscopic scale (B).

5.4.4 MACROSCOPIC VALVE APPEARANCE

After successful functioning for one year in sheep, valves were explanted and observed macroscopically, revealing smooth and thin leaflets (Fig. 5.3A). Furthermore, the valves maintained their nice flexible leaflets, showing no macroscopically detectable signs of calcification or thrombus formation (Fig. 5.3B).

5.4.5 LEAFLET DIMENSIONS

After obtaining individual parts of the explants, leaflets were measured to investigate signs of leaflet shortening and fusion of the leaflets with the wall (Fig. 5.4A-B). This
showed a significant decrease (p=0.0004) from an initial length of 18.40±0.40 mm in the control valves to 12.38±0.80 mm in the explants (Fig. 5.4C). Comparing the location of the hinge region relatively to the stent, revealed no significant difference, being 6.35±0.37 mm in the control valve and 6.89±0.11 mm in the explant (Fig. 5.4D), indicating that there are no signs of fusion of the leaflet with the wall.

**Figure 5.4** | Leaflet Retraction: The control valves (A) were compared to the explants (B) by measuring leaflet length (C) and positioning of the hinge region relatively to the stent (D). The explants revealed significant (p=0.0004) shortening of the total length of the leaflets (C), but no signs of leaflet merger with the wall (D).
5.4.6 Cell contractility

The presence of α-SMA positive cells within the leaflets was investigated using histology. A merged tile scan was made from sections of the explants (Fig. 5.5A), in combination with local magnifications from the tip (Fig. 5.5B), middle (Fig. 5.5C-D) and hinge region (Fig. 5.5E) of the leaflets. Cells were observed from the hinge to the tip region throughout the leaflets. However, only very limited amounts of α-SMA were present, as can be observed from the brown color intensity.

Figure 5.5 | Contractile Cells: The presence of α-SMA positive cells throughout the explants was analysed using histology (A). Here, only very limited amount of α-SMA is observed throughout the leaflets, as can be observed from the low brown colour intensity. (B-E)
5.4.7 Collagen Orientation

Leaflets of the explants and the control group were analyzed for their collagen orientation by using confocal microscopy. The control valves showed a homogeneous collagen distribution throughout the entire construct (Fig. 5.6A1). In the belly region, no preferred collagen orientation could be identified (Fig. 5.6A2). However, in the explants (Fig. 5.6B1) a clear top layer of collagen in circumferential direction was observed that spread along the belly region (Fig. 5.6B2). Using image analysis software, collagen anisotropy was confirmed in all explants, showing a strong overall alignment in circumferential orientation (Fig. 5.6C).

![A: Control Valve](image1.png) ![B: One Year Explant](image2.png) ![C: Collagen Distribution](image3.png)

**Figure 5.6** | Collagen Orientation: Whole mount collagen staining was performed on the control valves (A) and the explants (B). Random collagen organization was observed in the belly region of the control valves (A2), while circumferential orientation was seen in the explants (B2). Here, the observed collagen organization in the explants is nicely in line with computational prediction (C).

5.4.8 Mechanical Properties

Biaxial tensile tests were performed to analyze the tissue properties of both the control valves as well as the explanted valves. In the control valves at 20% strain, stress levels of 353±85 kPa and 364±29 kPa in both circumferential and radial direction were obtained respectively (Fig. 5.7A). From these curves, tensile moduli of both 7.31±1.54 and 7.73±0.45 MPa were obtained, which were not significantly different (Fig. 5.7B). In case of the explants, stress levels at 20% strain were 217±41 kPa in circumferential and 105±14 kPa in radial direction, having a tensile modulus of 5.44±1.18 MPa and 3.05±0.41 MPa respectively. For the explants, the tensile modulus in circumferential direction was significantly higher than in radial direction (p=0.0354) (Fig. 5.7B).
5.4.9 COMPUTATIONAL SIMULATIONS

Computational simulations were performed to understand the underlying mechanism of tissue regeneration and collagen remodeling. In addition, to investigate the robustness of these DTEHVs, the degree of cell traction and the initial leaflet thickness were varied to assess their influence on the remodeling process and valve functionality. The material parameters of the collagen network were fitted to the tensile test data, confirming that the collagen network exhibits a highly nonlinear mechanical behavior ($k_1 = 8.45$ kPa and $k_2 = 9.91$), and is almost isotropic ($\sigma = 108.63^5$) before implantation. With high levels of cell contractility up to 100%, the valves were predicted to be insufficient (Fig. 5.8A-B) with a valve opening during diastolic loading of even 12% of the total orifice area (Fig. 5.8D).

Only upon decreasing the cell contractility towards 20% of the original value, the simulations predicted the valve to close properly (opening area of <1% in combination with a solid coaptation area) (Fig. 5.8D-E). The collagen architecture was expected to be almost random at high degrees of cell traction (Fig. 5.8C), where if the cell contractility was decreased towards 20%, circumferential collagen orientation is more favored (Fig. 5.6C). A 25% decrease in leaflet thickness would cause an increase in valve closure, which was demonstrated by the decreased valve opening during loading, and the increase in coaptation area (Fig. 5.8D-E). Furthermore, an increased collagen alignment in the circumferential direction was predicted in these simulations compared to the original thickness (Fig. 5.8C). Conversely, a 25% increase in leaflet thickness resulted in decreased valve closure and a less pronounced collagen orientation in the leaflets (Fig. 5.8A-E).
Figure 5.8 | Computational Simulations: The influence of leaflet thickness in combination with increasing cell contractility was investigated computationally, to analyse their effect on collagen distribution and valve competence. Strain distribution throughout the leaflets was shown from a top (A) and side view (B), together with the expected collagen distribution (C). In addition, valve opening (D) and coaptation area (E) were calculated.

Geometrical improvements have been made to DTEHVs, for enhancing leaflet stretch in radial direction to be able to counteract for cell mediated leaflet retraction, as described previously (183). This study was conducted to investigate if these geometrical adjustments indeed led to an enhanced in vivo performance, and to elaborate on the robustness of this design in terms of tissue remodeling potential and their effect on valve functionality. So far, this is the first time that DTEHVs remain fully competent in vivo up to one year.
Prior to implantation, the regurgitation of the valves was analyzed *in vitro* and calculated to be 9.35±4.00 %, showing a proper closing with minor leakage of only 1.64±0.67 % (Fig 5.2B). When looking at the *in vivo* valve performance over time, linear regression analyses calculated a stable regurgitation fraction of 8.27±1.27 % over time, being not significantly different from the obtained *in vitro* results. This is giving a clear indication that during the period of 52 weeks, these valves were still closing completely under physiological loading conditions, and did not loose competence due to leaflet retraction. This result is supported by the macroscopic appearance of the explants, showing long and thin, native-like leaflets without any macroscopic detectable sign of thrombus formation or calcification. Also no merging of the leaflets with the wall was seen in the explants, as observed with the previous valve geometry (39,55).

Although all the valves remained stable over time, leaflet shortening did occur without affecting valve functionality. The computational model was able to explain (at least part of) this result, as leaflet shortening in the radial direction was predicted to occur due to tissue remodeling. Tissue compaction in the model primarily occurs in the radial direction due to the presence of the free edge, which offers the least resistance. The actual degree of compaction then depends on the balance between contractile cells and the applied hemodynamic load. When cell traction overruled radial tension in response to transvalvular pressure, leaflet shortening causing valvular insufficiency was indeed predicted by the numerical simulations. These also predict that the combination of thin leaflets with low expression of α-SMA is the most favorable combination to result in circumferential collagen reorientation, while maintaining complete valve functionality. To position the experimental results in the context of these numerical outcomes, the implanted valves were expected to be thinner than the control valves due to suboptimal culture conditions, being in the range of 75% of the initial leaflet thickness. In addition, histological findings revealed very limited amounts of α-SMA to be present in the leaflets, supporting low cell contractility in the numerical model. In this combination, the computational outcomes are supported by the observed circumferential collagen organization from the confocal images, as well as the biaxial tensile results, and where maintained functionality was confirmed from the *in vivo* performance.

During the course of the study, valve culture protocols were optimized in order to enhance tissue formation, making the leaflets less prone to rupture during crimping procedures for implantation. Hence, the numerical simulations expect these thicker valves to have less circumferential collagen orientation compared to the current explants, but to remain competent if the amount of α-SMA is limited. This makes the robustness of this valve design dependent on the presence of contractile host cells. Therefore, to translate this technology to future clinical applications, specific focus has to be paid to the donor
variability on α-SMA expression, which is known from literature to alter between patients (188).

However, these encouraging preliminary results are providing a strong indication that the implemented improvements to the current valve geometry are decisive for long term in vivo performance, where these valves have shown to remodel towards native like characteristics, holding significant potential for future clinical applications.

5.6 Conclusion

For the first time, DTEHVs have remained fully functional in vivo for up to one year. Explanted valve leaflets were smooth and pliable, without macroscopic observable signs of thrombus formation or calcification. In addition, the explants showed significant anisotropic circumferential collagen organization. Numerical analysis demonstrated that tissue remodeling is highly sensitive on the degree of cell contractility, where in these explants low amounts of α-SMA expression was observed throughout the leaflets. Despite the maintained long-term performance, leaflets shortened over time due to in vivo tissue remodeling, without affecting functionality. Numerical simulations predicted and explained the observed remodeling process based on leaflet thickness and cell contractility, both in relation to valve functionality and collagen distribution. Future analyses on the remaining valves that are currently included in this ongoing study need to confirm these first outcomes. So far, the current preliminary results are promising, and confirm that the implemented geometrical adjustments indeed lead to an improved long-term in vivo performance.

5.7 Acknowledgment

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6.1 MAIN FINDINGS

6.1.1 STARTING POINT

For almost 15 years, intensive research at the Eindhoven University of Technology has focused on heart valve tissue engineering, with the ambitious goal to provide a lifetime solution for especially pediatric patients born with congenital heart diseases. Over the years, big hurdles have been taken that has led to further progression of the technology. Decellularized tissue engineered heart valves (DTEHVs) could be produced that were functional for 8 weeks of implantation and showed quick recellularization after hours of implantation (39). Although this fast host cell repopulation might be promising for regenerative potential, the exact infiltration mechanism remained unknown. A major limitation thus far has been that these DTEHVs lack long-term in vivo functionality due to leaflet shortening (55). Based on computational simulations, this failure mechanism was explained by the heart valve geometry that required further optimization in order to maintain long-term functionality (131). In addition, DTEHVs have shown to be implantable with minimally invasive techniques, but suitable biodegradable stents to make this technology compatible with pediatric applications that can accommodate for growth are lacking thus far (130).

6.1.2 ACHIEVEMENTS

In order to improve DTEHV geometry, adjustments to the culture procedure were made. In chapter 2, a solution to improve, impose and maintain the valvular geometry is described (183). The solution to overcome this problem was to include an insert that defined the shape as derived from computational simulations to counteract for cell mediated tissue retraction by increasing the leaflet strain in radial direction. Improvements included a more profound belly curvature and an enlarged coaptation region. In this way leaflets could compact around the insert due to cell-mediated contraction, which by the end of the culture procedure maintained the imposed shape. It was shown that in this way functional human cell based DTEHVs could be made that were functional for up to 16 weeks in vivo simulation in a test set up, and that these design improvements resulted in a considerable decrease in radial tissue compression under pulmonary loading conditions. Therefore, the improved DTEHV geometry is expected to be less prone to in vivo leaflet retraction.

To elucidate the underlying mechanism of the rapid host recellularization within hours after implantation, the influence of scaffold remnants in decellularized tissue engineered constructs (DTECs) on cell recruitment was investigated in chapter 3. Here it was shown
that predominantly granulocytes from the blood infiltrated into the DTECs including scaffold remnants after already 5 hours in a mesofluidic in vitro test setup. After infiltration, cells migrated to the core of the constructs in the vicinity of scaffold remnants. Furthermore, significant up regulation of MMP-1 was indicative for active cell infiltration.

To translate the growing valve concept to pediatric patients, further research in chapter 4 focused on a proof-of-concept to develop stents for minimally invasive heart valve transplantation applications with biodegradable potential. In this study we showed that co-polyester polymer based stents could be designed and produced for trans-apical implantation procedures. Such stents could be crimped to the required diameters with sufficient recovery after deployment. Furthermore, we showed that by using co-polyesters, these stents are prone to hydrolysis by decreasing their molecular weight over time, as well as their mechanical performance.

In order to show that the geometrical adjustments made to these DTEHV indeed result in long-term functionality in vivo, valves were implanted via minimally invasive trans-venous implantation in the pulmonary position in sheep, as described in chapter 5. Up to one year of follow up, the first preliminary results show that these valves were fully competent without any signs of increased regurgitation. Furthermore these valves show tissue remodeling behavior by means of collagen reorientation and increased tissue anisotropy in circumferential direction. Besides, computational simulations could explain this tissue remodeling behavior, which confirms that the valvular design leads to circumferential collagen reorientation while remaining competent, as long as infiltrated host cell traction is limited.

6.1.3 END POINT

This thesis has provided solutions, mechanical explanations, and proof-of-concepts that is of added value in the field of heart valve tissue engineering. 1: Heart valve geometry can now be improved and controlled. 2: Scaffold remnants were found to be the trigger for fast recellularization capacity in the DTEHVs. 3: A proof-of-concept was given that biodegradable stents for heart valve transplantation application is feasible. 4: It was shown that geometrical improvements to DTEHVs enabled successful one-year follow-ups in sheep with maintained competence and tissue remodeling behavior as the first in the field.

6.2 CONSIDERATIONS

6.2.1 GEOMETRICAL IMPROVEMENTS
With the introduction of bioreactor inserts to define the heart valve shape, major improvements in valvular geometry have been made. However, there are still opportunities for further improvement. In the current design of the DTEHV, sharp transition regions from the belly towards the coaptation area exist, which resulted in weak tissue formation at these specific locations that are prone to tissue rupture during the crimping procedure. In native configurations, the coaptation region makes a smooth transition towards the belly and continues this smooth transition to the wall region, where the pulmonary valve, as well as the aortic valve, has incorporated sinuses, which geometry can be described by three spheres that meet in the center (189). The incorporation of the sinus in the valve has a considerable influence on valve closing behavior, were the blood flow is able to smoothly close the valve in the presence of the sinus (190). In addition, the valve is currently defined as a perfect cylindrical shape. However, due to anatomical constraints, the valve mostly is more oval shaped once being deployed, which could lead to valvular insufficiency after deployment (191).

Therefore, further improvements to the valvular geometry must be included that take into account a smooth transition from the coaptation via the belly towards the sinus and look into the possibilities to account for the oval shaped host artery.

6.2.2 Collagen Anisotropy

The bioreactor inserts appear to have an affect on the collagen orientation and overall remodeling. In chapter 2, where valves have been cultured in open configuration, collagen anisotropy was observed in circumferential direction in the coaptation area. Probably this was possible since the tissue was unconstrained at the free edge and could therefore slightly compact in radial direction, resulting in collagen anisotropy. However, in the case of the ovine cell based DTEHVs in chapter 5, circumferential collagen orientation in the coaptation region could not be observed. In these valves, leaflets were cultured in a closed configuration and could not compact in the radial direction. This additional constraint most likely prevented radial tissue compaction during culture and resulted in isotropic collagen distribution.

From our first preliminary results as been described in chapter 5, leaflets shortened over time as a result of in vivo collagen remodeling. In order to maintain the initial geometry of the valve as much as possible to prevent valvular insufficiency, initial collagen anisotropy of the implant in circumferential direction is preferred. A possible way to steer collagen orientation during valve culture is using contact guidance (192). Unfortunately, current available PGA/P4HB scaffold meshes that are used today to make the DTEHVs are not available in a preferred scaffold fiber alignment. Future research on suitable scaffold materials including predefined circumferential orientation might be needed.
6.2.3 Cell Source

The cells used in this thesis to produce the DTEHVs originate from primary isolated vena saphenous cells obtained from ovine or human origin. However, the question remains if these cells are the most ideal for tissue production. Since decellularized implants have proven their functionality in vivo, an autologous approach of using donor specific cells might no longer be required (39,55). Tissue production of cells depend on age, origin and passage number, more consistent outcomes in terms of tissue production might be reached by using cells from a mixed donor population or by using an immortalized cell line (188).

Over time, different cell types from various sources have been used for heart valve tissue engineering (56,57), (58,59), (60). However, if patient specificity is no longer a requirement, new possibilities become available to use cells from valvular origin, the so called vascular interstitial cells (VICs), for tissue engineering purposes which might be beneficial in terms of a more native like tissue composition (193).

*Figure 6.1 | α-SMA Expression Between Studies: Progression of α-SMA positive cell infiltration over time during implantation, where clear differences can be observed between both implantation studies with comparable DTEHVs (A-B). Here, even after 24 weeks scaffold remnants were still present as been indicated by the arrows (C).*
6.2.4 Tissue Remodeling

In our first long term follow up study we observed an initial influx of cells after hours of implantation, most likely by blood born cells. However, over time more α-SMA positive cells were present in tissue-engineered construct (Fig. 6.1A). Although the exact origin of these cells have not been confirmed yet, they could have migrated from the native pulmonary wall, originate from endothelial cells via EndoMT (107), transdifferentiate from infiltrated macrophages (100), or even be circulating progenitor cells from the bloodstream (104).

Despite the exact origin of these α-SMA positive cells, their influence on valvular remodeling and long term functioning have been shown in chapter 5 to be of major concern. Between donors, the level of expression of α-SMA and number of infiltrating cells can largely differ between patients (194,195). Based on unpublished data from associated implantation studies within LifeValve, where heart valves have been implanted in sheep via both the transapical (Fig. 6.1A) and the transvenous approach (Fig. 6.1B), the amount of α-SMA positive cells is varying largely between explants. This observation makes it clear that the remodeling response can largely deviate between hosts and that the occurrence of these variations needs special attention for future human applications.

6.2.5 Scaffold Remnants

In chapter 3 the influence of scaffold remnants on cell recruitment is shown. It was suggested that the presence of these remnants had a suppressive effect on pro-inflammatory cytokine expression. The presence of foreign material and its influence on tissue regeneration is currently under investigation by multiple research groups (196,197). Although the presence of scaffold remnants seems to be beneficial for early cell recruitment, the long-term effects remain to be investigated. Via histology on the explants, local irregularities inside the tissue constructs still indicated the presence of scaffold remnants after 24 weeks of implantation (Fig. 6.1C). Since potential remnants from PGA are known to degrade in vivo within a month (198), most likely these are P4HB remnants. This would be in agreement with literature describing the full degradation of P4HB in vivo between 12 and 18 month (199). Although the safety of P4HB has been proven and usage is clinically approved, the long-term presence in DTEHVs and the influence on tissue regeneration needs to be further investigated.

6.2.6 Stent Improvements

The stents currently used for heart valve tissue engineering are the support basis for the construct, where the metal parts are used as anchor points that contribute to the initial shape of the valve. However, the currently used meshed design might require further
improvements to optimize these stented valves. First of all, the stent struts needs to be equally dividable by three, to maintain symmetry and have struts in equal dividable parts, providing sufficient anchoring points to secure the valve and maintain the required shape. Concerning future improvements in the valvular geometry in terms of incorporation of sinuses in the wall, these adaptations need into be introduced to the stent as well.

Besides geometrical adjustments, further research needs to be conducted to look for suitable biocompatible material alternatives that have the optimal combination of both mechanical forces and degradation speed to match the arterial size and growth potential of the patient, while maintaining shape after the minimally invasive implantation procedure. To further develop these biodegradable devices, it needs to be investigated until what period of time the mechanical integrity needs to be maintained to secure proper integration of the valve with the native artery, where after degradation is allowed. The downside for using biodegradable stents for DTEHVs application might be the limited shelf life, since these valves needs to be stored in an aqueous environment that will initiate degradation already during storage.

6.3 Future Directions

6.3.1 Simplify Bioreactor System

The initial function of the DPD bioreactor system has been to mimic in vivo mechanical conditions and “train” the tissue to become strong enough to maintain in vivo functionality. With the introduction of the insert, the use of the DPD bioreactor system becomes questionable, since the insert hampers cyclic loading of the valve. Although it has been shown from previous a study that proper tissue formation can be obtained by flow, and tissue orientation by mechanical stimuli (69), this might provide new opportunities for future applications in which less complicated bioreactor systems may be used for heart valve tissue engineering. This would be beneficial for translating this technology to clinical application in terms of up scaling, controllability, safety and cost perspective. Perfusion flow could for instance be obtained by positioning the valves on a shaking plate, by which medium is continuously being mixed. In this way, valves have been made from PCL based scaffold materials (Fig. 6.2A). Further research to less complex bioreactor systems could be an advantage for future clinical translation.

6.3.2 Control Scaffold Degradation

The balance between proper tissue formation and scaffold degradation is important. During the culture of the ovine valves in chapter 5, the quality of tissue formation was varying in the presence of the inserts. Maintenance of scaffold architecture over the time
that tissue is being produced by the cells is important for the quality and quantity of tissue formation. However, decreased tissue formation can be the result of faster scaffold degradation rates and/or a lowered tissue production capacity by the cells (200). From previous research it is known that vascular derived cells used for in vitro tissue engineering, are able to create sufficient contractile force after 2 weeks of culture (64) to compact the PGA scaffold, which is known to lose mechanical stability after 2 weeks of culture conditions (201). This makes there a delicate balance between tissue formation and scaffold degradation. Having more control over the scaffold degradation process during culture might be beneficial for the final outcome of the tissue integrity after culture, to overcome the imbalance between scaffold degradation and tissue production.

Previous studies have been investigating the use of slow degrading scaffold materials, such as PCL, but unfortunately these polymers have weak fatigue properties and lose mechanical support after several millions of cycles (202). However, this material might be an interesting scaffolding material if during culture the presence of this scaffold can be removed in a controlled manner after sufficient tissue has been formed. From recent literature it is know that PCL can be degraded enzymatically by including the enzyme lipase in the culture medium (192,203,204). In this way, scaffold support material could be degraded in a controlled manner.
In addition to enzymatically scaffold removal, PCL is also prone to dissolve in xylene (205). To investigate the effects of scaffold removal after culture, PCL based DTEHVs were first dehydrated with increasing ethanol series and afterwards treated with xylene for 10 minutes (Fig. 6.2B), followed by a rehydration series (Fig. 6.2C). After the treatment, tissue engineered heart valve leaflets were much more flexible and even remained functional inside *in vitro* test setups under physiological pulmonary conditions (Fig. 6.2 D). Further research on the effects of xylene treatment on the engineered tissue needs to be investigated. Such a potential post culture treatment for scaffold removal prior to implantation could be beneficial for long-term tissue regeneration outcomes.

![Figure 6.3](image_url)  
*Figure 6.3* | Control Collagen Alignment: Enhancing circumferential collagen orientation based on aligned electrospun PCL matrix (A). After culture and scaffold removal, tissue anisotropy was confirmed by biomechanical analyses (B) where a collagen staining (C), shows perfect circumferential alignment even in the belly region (D)*
6.3.3 IMPROVE COLLAGEN ANISOTROPY

As previously mentioned, commercial available non-woven PGA sheets are not available with predefined scaffold fiber alignment, although the effects of predefined circumferential scaffold fiber alignment has been shown to be beneficial for circumferential collagen orientation (206). If this holds for all types of scaffold materials, or also depends on the stiffness of the fibers to be able to overrule the effect of tissue compaction has to be investigated. However, caution needs to be paid by the amount of orientation in relation to the pore size. The more the fibers align in one direction, the less void space will remain. This can hamper cellular infiltration that might have negative effects on tissue production in the core of the scaffold (207).

In this scope, PCL was used to electrospin scaffold fibers in predefined circumferential orientation (Fig. 6.3A). Dual nozzle spinning was used to spin aligned and less aligned fibers simultaneously in an attempt to have an overall circumferential fiber orientation with sufficient void space. This approach appeared to be functional and has resulted in predominant circumferential collagen orientation throughout the entire leaflet, which was verified by using biomechanical analyses as well as whole mount collagen staining (Fig. 6.3B-D). This could have a great future advantage in controlling collagen orientation.

6.3.4 INVESTIGATE GROWTH POTENTIAL

Tissue engineered vascular constructs have already been proved to posses growth potential (208), but thus far growth of DTEHVs still remains to be seen. For future research on growth, smaller sized tissue engineered heart valve constructs needs to be developed for implantation in juvenile animal models. Either smaller sized DTEHVs could be implanted via open heart surgery, while for minimally invasive implantation procedures, functional biodegradable stent alternatives remain to be developed.

For the production of smaller diameter valves and future commercial implementation, a pilot study was conducted where PCL based DTEHVs were produced by using small size 3D printed inserts in combination with adjusted 3D printed stents (Fig. 6.4A). Valves were seeded with ovine cells and cultured in a jar on a shaking plate (Fig. 6.4B). Removal of the PCL material after the culture procedure by mean of xylene (Fig. 6.4C), resulted in fully functional valves that could withstand in vitro testing conditioning, taking into account the increased heart rates and lower stroke volumes related to juvenile applications (Fig. 6.4D-E). Further development on suitable small diameter DTEHVs is important to perform new studies for investigating growth potential.
6.3.5 TARGET THE T-CELLS

Current focus of *in situ* tissue engineering is investigating the possibilities to control the initiated immune response towards favorable healthy tissue regeneration (90). Here, the main goal is to steer the initiated immune cascade towards a favored regeneration response (83,103). Depending on the trigger the immune system receives, this response will be either favorable for regeneration, or result in an unfavorable response such as chronic inflammation or fibrotic tissue (209). What is known from literature is that macrophages play an important role in steering this immune response (210). Multiple subsets of macrophages have already been identified with different characteristics based on their intercellular content, membrane proteins and response to different cytokine environments in response to the presence of IFN-γ and IL-4 (211). Current topic of research interest is to incorporate proteins or peptide into the scaffold material to steer the immune system (212,213). The main reasoning is that by doing so the infiltrating macrophage will be exposed to an engineered cytokine environment that is beneficial for regeneration and could be of use to steer polarization towards a regenerative macrophage, also known as macrophage type 2 (M2) phenotype (214).
Although this can be an interesting approach to affect local cell behavior, it remains important to take into account the influence of the whole systemic immune cascade in response to the biomaterial as well. The pitfall underlying the local approach is that the main secretors of both IFN-γ and IL-4 in the immune response are the T-helper (Th) cells, being Th1 and Th2 respectively (215), which can potentially overrule this local microenvironment. Both Th cell populations have their own specific function in the immune system. Where small pathogens like small microbes can be phagocytized by macrophages, their M1 polarization and activation is regulated via Th-1 cells in response to the secretion of IFN-γ (216). When bigger pathogens invade the body like helminthes, these are too big for macrophage to phagocytose. Therefore, another defense mechanism of the immune system is to activate mast cells and eosinophils, which secrete their toxic granules to terminate the pathogen in response to IL-4 that is secreted by Th2 cells, but which response will also damage the surrounding tissue (217). This might explain why in this last defensive mechanism the macrophages are polarized towards a M2 phenotype to enable tissue repair. The entire cascade of macrophage activation via Th cells is such a tight controlled and effective mechanism of the human body, that it will become a challenge to counteract their response via the incorporation of peptides into these relatively small biomaterials (218).

A suggestion for an alternative powerful target cell to aim for with immunoengineering, rather than directly try to steer the macrophages, would be the antigen presenting cells (APCs) that activate these different Th cells subsets (219,220). If it would be possible to steer the immune system on a higher level up in the control unit of the adaptive immune system, it might be possible to steer the desired Th response that could lead to a favorable macrophage polarization.

*Figure 6.5* | Target Th-Cells: In response to a biomaterial, APCs can polarize Th cells towards a Th1 or Th2 response, which can activate respectively a M1 and M2 response. Activation goes via direct cell contact and cytokine secretion. A suggestion to target the APC as an effective immunoengineering approaches is provided in the text.
APCs process antigens and present them on their membrane to interact with naive T cells. To activate the T-helper cell population, the biomaterial should be processed by the APC in a way that it interacts via class II major histocompatibility complex (MHCII) with the naïve T cells, which will cause their differentiation toward Th cells (221). The polarization towards Th1 is depending on the presence of IFN-γ and IL-12, where polarization towards Th-2 is depending on the presence of IL-4 (222). But how can Th cells be polarized towards Th-2 if they are the main cell sources that produce IL-4? Although it is still not entirely known how this works, is seems that the polarization depends on the secretion of IL-12 by the APCs (223). If IL-12 is secreted upon activation of the APCs, Th cells polarize towards Th1 and start secreting IFN-γ, where as if IL-12 is absent, Th cells start producing IL-4 themselves and thereby initiate their own polarization towards Th2 (224). A schematic overview of these pathways is provided in Fig. 6.5. Therefore, new intelligent approaches to skew the APC towards a favorable Th2 cell initiation might be an interesting route to further explore.

6.3.6 Go to Disease Models

From a clinical point of view, patients that require a heart valve transplantation mostly suffer from underlying associated health problems (5,6). The effect of these systemic diseases on tissue remodeling needs to be further investigated. As explained above, the influence of the adaptive immune system can largely influence the outcome of tissue remodeling and should be taken into consideration in diseased patients (225). The risk in these patients is that the immune system is already activated. If the Th response is already skewed towards a Th1 response because of the underlying disease, it might become difficult to switch it towards a Th2 response (226). And even in the case it would succeed, what are the consequences on the underlying disease? However, certain diseases might also be beneficial for tissue regeneration. If the heart valves will be implanted in patients that already have an onset Th2 response, tissue regeneration might be more favorable. Even more so, if a pathogen could be identified that is causing an enormous Th2 response, it could be argued to treat patients receiving a heart valve transplant, with antigens from a specific pathogen to skew the immune response towards a favorable Th2 response (227). Research in disease animal models might be the next step to investigate remodeling capacity under influence of certain diseases. Typically, these model systems are initiated in rodent models, such as specific genetic knock-out models for diabetes and even obesity. To be able to use these models, tissue engineered heart valves need to be further downscaled to sizes that match the vessel diameter of rat models, and even further for mouse models. This will push the available technology to its limitations and will require precise and controlled scaffold manufacturing, small-scale insert production and the development of small-scale biodegradable stents in the near future. To investigate the
technological boundaries, first pilot studies have been initiated where we have been able to spin small PCL tubes for rat applications. From these tubes, small tri-leaflet heart valves were obtained (Fig. 6.6C). Even small inserts that could maintain structural integrity as was shown in chapter 2 to control the valvular geometry, were produced (Fig. 6.6B). Since flow appeared to be of crucial importance for tissue formation, the IBIDI system as used in chapter 3 has been transformed to a bioreactor system, were the valvular construct including the inserts could be incorporated. In this way, the first small scale DTEHVs could be produced (Fig. 6.6D). Concerning stent applications, the material that has been used in chapter 4 for the proof-of-concept study to produce biodegradable stents, has been used to make small size stents for rat implantation applications via CNC milling technology (Fig. 6.6A). Taking this all together, from an engineering point of view, there are a lot of opportunities to translate DTEHV technology to rodent models to investigate the effects of certain diseases on tissue remodeling.

**Figure 6.6** | Small DTEHVs for Rat: As a proof-of-concept, tissue engineered heart valves in a polymer based stent were made for rat applications to test the effect of diseases on tissue regeneration. Small stents were made out of co-polyester using CNC milling (A), where compatible inserts to guide tissue formation (B) and small diameter heart valves based on PCL (C) were made as well. Valves were cultured using the IBIDI flow system and rat vena saphenous cells (D).
6.4 CONCLUSION

Promising results are obtained in the currently ongoing one-year follow up study where we implanted geometrically improved DTEHVs in sheep. Before reaching clinical implementation, important questions on the underlying regeneration mechanisms and the effects on patient variations remains to be answered, as well as the robustness of this current valvular design. However, important steps have been taken that has brought this promising technology closer to application.

The geometry of DTEHVs can now be controlled and has proven to be functional up to one year in vivo implantation. Insight is provided on their fast recellularization potential as well as their regeneration capacity, and a proof-of-concept has been given for stents with biodegradable potential. Furthermore, suggestions have been given on how this technology can even be further optimized.

All together, this thesis describes the important improvements and next steps, towards a tissue engineered heart valve for life.
APPENDIX

PATENT APPLICATION

The content of this appendix is based on:

Appendix

**SUMMARY OF THE INVENTION**

The present invention provides devices, methods of using these devices and systems for controlling tissue engineered heart valve leaflet geometry by using predefined inserts during tissue culture. The inserts are referred to herein as (leaflet) shapers and (leaflet) spacers, which can be used individually or in combination with each other mostly depending on the type of cells cultured with the tissue growth materials and level of geometry shaping/control.

The first insert is a leaflet shaper and has been described herein with several different variations of embodiments. Since we observed that the cells build up tension in all constrained directions, we make use of this effect by inserting a rigid, concave construct that has the shape of the leaflet. The tension that develops in the leaflets will cause the leaflets to compact against the shaper, which acts as a constraint and is capable of controlling the curvature and coaptation of the leaflets.

In some embodiments, the leaflet shaper is covered with small holes to achieve proper nutrient exchange between the medium and the tissue that compacts around the insert. The shaper does not cover the wall of the heart valve such that nutrients and oxygen can be supplied to the wall. Because the tissue compacts against the concave aspect of the shaper, there is no need for a second valve shaped insert shaper on the other side of the valve leaflets.

The second insert is a leaflet spacer and has been described herein as one embodiment that can be used in combination with the various shapers. When the leaflets are cultured in a closed configuration, the spacer will prevent retraction of the leaflets in the radial direction to constrain the height, and therefore control the size of the coaptation area. It will also enable maintenance of a predefined coaptation area. Hence, this leaflet spacer will constrain the height of the leaflets. A second advantage of the leaflet spacer is to prevent the leaflets from merging over the coaptation area during culture. Since the spacer will be positioned in between the individual leaflets, there is no chance for leaflet concrescence.

An advantage of using the embodiments presented in this invention is that it can result in circumferential collagen orientation in the cultured heart valves, which is beneficial for heart valve functionality.

Another advantage of using the embodiments presented in this invention is that it enables us to culture heart valves without the need of using a complex bioreactor system. In fact, the use of a simple jar would be sufficient. One of the functions of the bioreactor system
was to impose the right geometry to the valves by dynamically loading them. But this inserts can achieve the same objective, which is to constrain the imposed geometry.

**CLAIMS**

1. A heart valve cell culturing device, wherein the heart valve comprises at least two leaflets, comprising:
   
   (a) a support base; and
   
   (b) at least two inner arms each capable of supporting a tissue growth material to form one of the leaflets,
      
      • wherein each of the inner arms has a first portion and a second portion,
      
      • wherein the first portion is disposed normal to the support base and disposed proximal to a center of the support base,
      
      • wherein the second portion is nonlinear and disposed distal to the support base and bends away from the center of the support base, wherein the at least two inner arms are distributed in a pattern at the support base, and
      
      • wherein the at least two inner arms are spaced from each other defining enough space to fit at least the respective tissue growth materials.

2. The heart valve cell culturing device as set forth in claim 1,
   
   • wherein each of the inner arms further comprises a canopy growth surface expanded from the second portion of the respective inner arms,
   
   • wherein each of the canopy growth surfaces define a concave surface when moving away from the center of the support base in outer direction.
   
   • wherein each of the canopy growth surfaces is supported by the first portions of the inner arms and a pair of outer arms defined for each of the inner arms, wherein each of the outer arms have a first portion disposed normal to the support base and disposed distal to the center of the support base, and wherein each of the canopy growth surfaces are capable of supporting the respective growth materials.

3. The heart valve cell culturing device as set forth in claim 2, wherein the canopy growth surfaces comprise holes to allow exchange of nutrients.
4. The heart valve cell culturing device as set forth in claim 2, wherein each of the canopy growth surfaces further span to the base of the support surface along the radial separation of the respective outer arms and inner arm, wherein each span capable of supporting the respective growth material.

5. The heart valve cell culturing device as set forth in claim 2, wherein each of the canopy growth surfaces further span to the base of the support surface and form meshes surfaces between the respective outer arms and inner arm each capable of supporting the respective growth material.

6. The heart valve cell culturing device as set forth in claim 5, further comprising a spacer to fit in the space left to fit at least the tissue growth material to separate the tissue growth materials supported by the meshed surfaces.

7. The heart valve cell culturing device as set forth in claim 5, wherein the meshes surfaces comprise holes to allow exchange of nutrients.

8. The heart valve cell culturing device as set forth in claim 2, wherein the combinations of each of the first portions of the inner arms with their respective pair of outer arms define wedge-shape growth surfaces each capable of supporting the respective growth material.

9. The heart valve cell culturing device as set forth in claim 8, wherein the wedge-shape growth surfaces comprise holes to allow exchange of nutrients.

10. The heart valve cell culturing device as set forth in claim 8, further comprising a spacer to fit in the space left to fit at least the tissue growth material to separate the tissue growth materials supported by the wedge-shape growth surfaces.

11. The heart valve cell culturing device as set forth in claim 1, further comprising a spacer to fit in the space left to fit at least the tissue growth material to separate the tissue growth materials supported by each of the linear portions of the inner anus.

12. The heart valve cell culturing device as set forth in claim 1, wherein the at least two inner arms have three inner arms and the pattern is a triangular pattern.
Figure Appendix | Patented Bioreactor Inserts: Illustrations that support the claims from the patent.
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SAMENVATTING

RICHTING EEN GEKWEEKTE HARTKLEP VOOR HET LEVEN

Ieder jaar zijn er wereldwijd 280.000 mensen die een hartklep transplantatie ondergaan. De huidige protheses kunnen levens redden, maar niet mee groeien met het lichaam. Dit vormt een groot probleem voor kinderen die geboren worden met een hartklep afwijking en hierdoor meerdere operaties moeten ondergaan om deze aan te passen tijdens hun groei. Dit leidt tot een verhoogd risico op complicaties, ziektes en zelfs de dood.

Gedecellulariseerde, gekweekte hartkleppen (GGH) hebben een grote potentie om deze limitatie te overwinnen. Uit vorige lange termijn studies in vivo, bleek dat deze hartkleppen snel nieuwe cellen uit het lichaam bevatten, maar dat deze kleppen in de loop van de tijd ook ernstige insufficiëntie ontwikkelden. Het onderliggende probleem was een geleidelijke verkorting van de vliezen dat waarschijnlijk een effect was van het terug trekken van het weefsel. Gebaseerd op computer simulaties werd het dit bezwijkingssmechanisme verklaard door de vorm van de hartklep. Onder fysiologische belasting was het weefsel onderhevig aan compressie in plaats van uittrekking.

Er werd een verbeterde klep geometrie bepaald welke het weefsel zou moeten oprekkken en cellulair gegenereerde weefsel contractie tegen zou gaan, door het coaptatie deel te verlengen en de bolling van het vliez te vergroten. Om deze aanpassingen door te voeren tijdens de productie van de hartkleppen, is er een gepatenteerd onderdeel ontworpen dat de vorm kan controleren tijdens het kweken, waarmee GGH gebaseerd op menselijke cellen zijn gemaakt. Deze hartkleppen bleven functioneel in een in vitro opstelling waarbij succesvol 16 weken van implantatie gesimuleerd kon worden, en een aanzienlijke afname in radiale weefsel compressie werd aangetoond.

Momenteel worden deze GGH minimaal invasief geïmplanteerd via een bloedvat, waarbij gebruik gemaakt wordt van een metalen stent. Om in de toekomst de groei van een hartklep in een stent te realiseren, zijn de mogelijkheden voor een zelf expandeerbare, biodegradeerbare stent onderzocht, met behulp van 3D printen en een flexibel thermoplastisch copolyester. Computer modelen zijn gebruikt om de stents te ontwerpen zodat deze voldoende radiale kracht kunnen genereren welke vergelijkbaar is met een metalen stent, en minimale plastische vervorming vertoont na het krimpen.
Samenvatting

Het is aangetoond dat deze GGH de unieke capaciteit hebben om snel nieuwe cellen vanuit het lichaam op te kunnen nemen. Deze hartkleppen bevatten dan nog drager materiaal na implantaat, wat cellen uit de bloedbaan aan zou kunnen trekken. Gebaseerd op in vitro experimenten waarbij het weefsel en drager materiaal blootgesteld werd aan fysiologische vloeistof stromingen, is aangetoond dat bloedcellen vanuit de circulatie actief het weefsel infiltreren en migreren richting de resten van het drager materiaal. Hierbij werd de uitscheiding van ontstekings cytokines onderdrukt in de aanwezigheid van resten drager materiaal.

Om de effecten van de geoptimaliseerde hartklep vorm op de mechanische functionaliteit en weefsel remodelering te onderzoeken, zijn deze GGH minimaal invasief geïmplanteerd in schapen waar ze gedurende een jaar volledig functioneel bleven. Deze resultaten laten het belang zien van de juiste vorm van de hartklep en het effect op cel gecontroleerde weefsel retractie. Daarnaast laten deze eerste resultaten zien dat het weefsel tijdens implantaat remodelleert, waarvan de uitkomsten aan de hand van computer simulaties vooropgesteld kunnen worden.

Samenvattend, dit proefschrift beschrijft een oplossing om de hartklep vorm te verbeteren en te controleren. Verder blijkt dat resten van het drager materiaal ervoor zorgt dat cellen actief vanuit de bloedbaan het weefsel infiltreren. Daarnaast is aangetoond dat zelf expandeerbare, biodegradeerbare polymere stents voor minimaal invasieve hartklep vervanging gemaakt kunnen worden. Als laatste onderschrijft dit proefschrift het belang van de juiste vorm voor deze GGH, welke nu een jaar lang volledig functioneel zijn gebleven in schapen en weefsel remodelering laten zien.

Deze verbeterde GGH hebben een grote potentie om uiteindelijk een geschikte gekweekte hartklep voor het leven te zijn.
DANKWOORD

Dit proefschrift zou er nooit zijn geweest zonder de hulp en steun van een hele hoop lieve mensen om mij heen. Daarom verdienen zij hierin ook een speciale plek.

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Anita, bedankt dat je mijn copromotor wilde zijn. Als een van de grondleggers op het gebied van hartkleppen kweek kon ik me geen betere begeleiding wensen. Naast je grondige analyses en nauwkeurige correcties op mijn werk hebben we samen ook vooral veel lol gehad. Zo kan ik me nog menige keren herinneren dat we met tranen over de waxen met elkaar, maar vooral ook om elkaar hebben gelachen.

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Dankwoord

eelkander klagen als de wetenschap weer eens voor ging op de voorbereidingen voor het kerstdiner.

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Sandra, jij hebt het LifeValve project gered. Dankzij jouw deskundigheid en mooie computer modellen heb je het fundamentele probleem aan het licht gebracht. Hierdoor hebben we de kleppen kunnen verbeteren en het probleem op weten te lossen. Bedankt voor je hulp en het beantwoorden van al mijn vragen rondom deze complexe numerieke materie!

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128
De consequentie van een promotie onderzoek is vaak dat je tot diep in de nacht achter je laptop zit, door de bewakers ’s nachts uit laboratoria gesleept wordt, en je het wel en wee van je cellen verkiest boven dat van je familie. Het nadeel van zo’n eigenzinnige levenswijze is dat je de sociale omgeving soms wat te kort doet. Desondanks hebben familie en vrienden mij altijd gesteund wanneer ik weer eens een afspraak was vergeten, te laat was, of af moest zeggen. Daarom wil ik jullie allemaal heel erg bedanken!

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Bart
Dankwoord
CURRICULUM VITAE

Bart Sanders was born on May 10, 1987 in Eindhoven, the Netherlands. After graduating in 2005 from the Eckart College in Eindhoven, he studied Biomedical Engineering at the Eindhoven University of Technology. During his study he performed a research internship at the University of Pennsylvania in Philadelphia in the United States, to investigate the influence of Wnt-3a on c-kit⁺ cell proliferation. For his graduation project in the group Soft Tissue Biomechanics and Engineering at the Eindhoven University of Technology, he focused on tissue engineering vascularized muscle constructs using electrospun scaffolds. After he graduated in 2011, he started his PhD in the same group working on the prestigious European LifeValve project, resulting in the research as presented in this thesis. In 2015 he was awarded with the STW Take-off Phase I grant to continue further development and valorization of biodegradable stents for cardiovascular applications.
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