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Experimental studies on hemodialysis access innovations

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W.J. Geelhoed

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Wouter Jan Geelhoed

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Experimental studies on hemodialysis access innovations

Ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
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Chapter 1

General introduction

From normal kidney function to hemodialysis

The kidneys are responsible for the filtering of blood and allow toxins, metabolic waste, and excess ions to be removed from the body while retaining essential substances within the blood. End-stage kidney disease (ESKD) is an irreversible decline in an individual's kidney function which would lead to death in the absence of dialysis or a kidney transplantation[1,2]. ESKD patients are therefore dependent on receiving a transplant, or must start with dialysis. Worldwide, over 2 million people are dependent on dialysis for their survival [3]. The two modalities of dialysis are hemodialysis (HD) and peritoneal dialysis (PD). HD is the process of removing water, and waste products such as urea and creatinine, from the blood stream. Blood is first removed from the body through a vascular access site, after which it is passed through a dialyzer that removes the water and waste products and returned to the body through the same vascular access site. PD functions by running dialysate through the abdominal cavity, allowing the diffusion of water and waste through peritoneal membrane. HD is the most frequently used modality of dialysis. According to the Dutch Kidney Foundation, 6500 patients were reliant on dialysis in The Netherlands in 2019, of which 83.8% were on HD [4]. Similarly, in the United States in 2016, 87.3% of individuals requiring dialysis began with HD[1].

ESKD and dialysis also have a substantial impact on the QOL of patients[5] and the societal burden of ESKD is enormous. Of the total Medicare budget, over $30 billion was spent on ESKD in 2016 [1]. Of this budget, nearly $3 billion was spent in 2016 on the maintenance of vascular access sites, such as addressing restenosis, bleeding complications, and thrombotic events. This figure is estimated to be around $4 billion if private care costs are included [6]. In the Netherlands a dialysis treatment costs around €80 -€120 thousand euros per patient per year [4]. Considering the increasing trend of ESKD prevalence worldwide, its societal burden is likely to increase. Contributing factors to the high costs of the procedures is the frequency by which HD occurs (~4 times per week), the large volumes of dialysate required, and the substantial costs of maintaining a vascular access site.

Vascular access for hemodialysis

Despite early attempts at developing a dialysis procedure dating as far back as 1913, the true revolution in the field of nephrology came in 1943 when the Dutch scientist Willem Kolff began dialyzing patients using a rotating drum HD machine [7-9]. His first 15 patients did not survive the procedure. In 1945 however, he dialyzed a 67-year old woman presenting with signs of ESKD for two-and-half days, leading to her full recovery a week later [8]. Although Kolff had shown that a dialysis procedure was possible, a reliable access to the patient's circulation for multiple purposes did not yet exist, making vascular access the Achilles heel of the dialysis procedure. One early attempt at creating a long-term vascular access site was the Scribner shunt, which used the principle of connecting an artery and vein via Teflon tubing leading outside the body allowing its first patient to survive 11 years following the insertion of the first shunt. The real revolution in vascular access however came in 1966 with the publication of the paper "Chronic hemodialysis using venipuncture and a surgically created arteriovenous fistula" by Brescia, Cimino and Appell, where they describe a side-

to-side anastomosis between the radial artery and the cephalic vein [10]. This was the first description of the current gold standard of vascular access; the arteriovenous fistula (AVF). Except for the introduction of expanded polytetrafluoroethylene (ePTFE) synthetic arteriovenous grafts (AVG) and CVC's in the 1970's, the field of vascular access has not changed much with the general concept of the AVF as described Brescia, Cimino and Appell, still being the preferred method of vascular access today. Data from 2018 shows that in the United States only 18.9% of patients had a CVC, only 16.6% had an AVG, while 64.5% used an AVF as hemodialysis access [1].

The limitations of the CVC, AVG, and AVF

CVC's are generally not intended for long-term use and thought to result in inferior patient outcomes. The use of CVCs may however lead to bias in study outcomes as the CVC may be considered a surrogate marker of sicker patients, as few deaths in patients with CVCs are directly linked to CVC related complications[11].

Both AVFs and AVGs are mainly limited by venous stenosis, characterized by intimal hyperplasia in a process that is not completely understood to this day [12]. A notable advantage of AVG's is that they can be used almost immediately, while AVFs must undergo a maturation phase. Generally, a newly placed AVF may start with a shunt flow around 250ml/min, while a shunt flow of at least 600ml/min is required for an effective dialysis procedure to occur. During maturation, the venous segment of the AVF remodels in an outward direction over the course of several weeks or months in order to increase in size and accommodate the increased blood flow required for dialysis. Therefore, the process of outward remodeling of the AVF is crucial, as inward remodeling results in failure of the AVF (Figure 1).

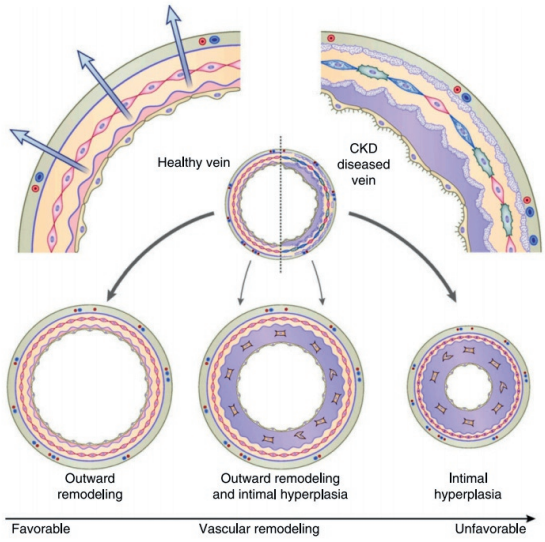

Figure 1. Showing the outward and inward remodeling responses of an AVF [13].

The process of AVF maturation itself is complex and multi factorial involving turbulent blood flows, inflammation, and numerous other processes (Figure 2), and although AVFs are considered superior to AVGs, their patency outcomes are similar when primary failure is included [11].

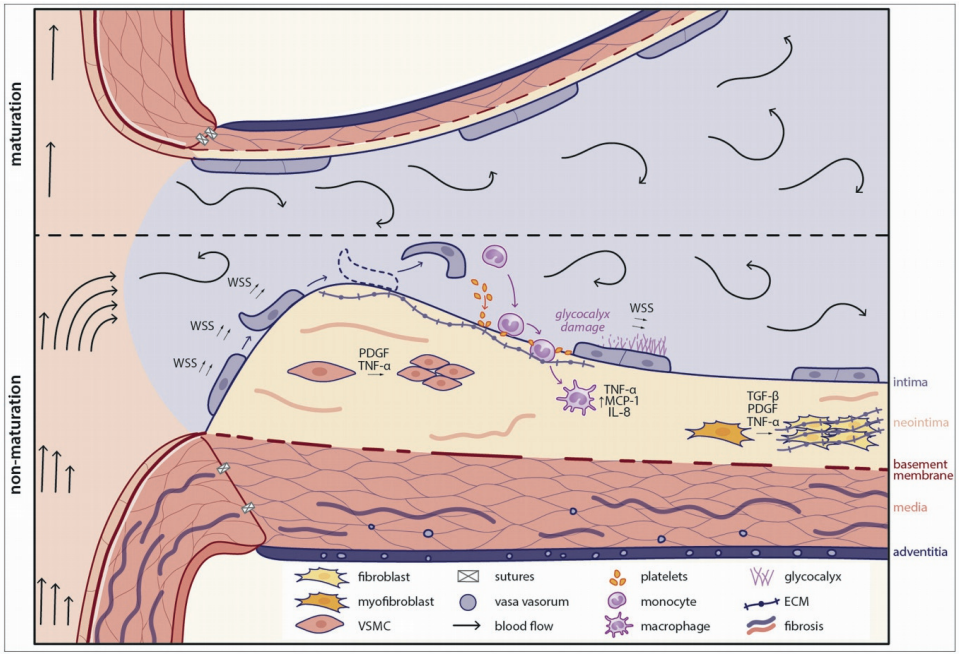

Figure 4. Mechanisms of arteriovenous fistula (AVF) maturation failure. Shown are the proposed cellular mechanisms associated with fistula non-maturation. Because of the formation of the arteriovenous fistula, the already very high blood flow shifts from a laminar to turbulent system, exercising high wall shear stress (WSS) on the venous cells. Endothelial dysfunction, damage, and de-endothelialization of the intimal layer due to WSS occurs, thus, exposing the subendothelial extracellular matrix (ECM) directly to flow. The exposed subendothelial layer results in thrombus formation as well as the adhesion and activation of platelets and monocytes. Monocytes infiltrate the vessel and differentiate to macrophages, releasing proinflammatory cytokines, such as TNF-, monocyte chemoattractant protein (MCP)-1, and IL-8. Meanwhile, vascular smooth muscle cell (VSMC) migration and proliferation are promoted by platelet-derived growth factor (PDGF) and TNF-. Driven by cytokines such as transforming growth factor (TGF)-, TNF-, and PDGF, fibroblasts differentiate to myofibroblasts, which proliferate and excrete ECM components [12].

Although much progress has been made in the field of vascular access for hemodialysis, vascular access still remains the Achilles heel of the hemodialysis procedure. AVGs are hindered by venous stenosis, while for AVFs both the failure to mature and late AVF occlusion due to stenosis in the venous outflow tract are limiting factors.

TEBV as an alternative

One of the proposed alternatives to the use of conventional vascular access modalities is the utilization of tissue engineered blood vessels (TEBVs). The field of vascular tissue engineering (VTE) is an upcoming field in which the aim is to engineer, replace or regenerate tissue suitable for vascular grafting. There are numerous approaches to vascular tissue engineering [14] and one such approach, the mandrel-based method, will be discussed in detail in this thesis in **chapters 2** and **4**.

The cannulation of the vascular access

In order to transport blood to and from the arteriovenous access, this conduit must be cannulated using dialysis needles. These are relatively large (15-16 gauge) needles, two of which are required each dialysis session. As an average patient may have up to 4 sessions per week, the vascular access site is cannulated over 400 times per year. According to literature, at least 5% of these cannulations result in major fistula infiltration and the formation of a hematoma [15]. In order to decrease the incidence of this complication of cannulation, a novel fluid driven automatically retracting needle has been developed, which will be discussed in **chapter 5** of this thesis.

Scope of this thesis

This thesis includes the pre-clinical evaluation of mandrel-based TEBVs in a goat model of arteriovenous grafting. In these experimental studies, the mechanical properties, histology as well as thrombogenicity are assessed, and both stenosis formation and patency are compared with conventional prosthetic AVGs. In addition, a novel non-invasive imaging modality is designed and evaluated with the aim to visualize in situ TEBV maturation upon subcutaneous implantation of the mandrel.

In addition, a novel fluid driven automatically retracting needle system for dialysis vascular access is conceived and assessed in a bench top setting.

In **chapter 2**, we review the literature available on autologous in vivo TEBVs and discuss the current state of development of the various approaches.

In **chapter 3**, we compare 3 methods for the assessment of mechanical properties of TEBVs, and question if these are truly applicable for the assessment of biological tissue.

In **chapter 4**, we assess the functionality of TEBVs *in situ* that are grown by utilizing the foreign body response to subcutaneously implanted polymeric rods, which were specifically designed for this purpose. These TEBV were then implanted as arteriovenous graft in goats and compared to conventional ePTFE grafts.

In **chapter 5**, we describe the implementation of a novel imaging modality for the non-invasive monitoring of TEBV maturation and wound healing.

In **chapter 6**, proof-of-concept experiments are carried out for a novel fluid driven automatically retracting needle system.

Finally, in **chapter 7**, we discuss the results of this thesis, and consider its implications on future research.

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Utilizing the Foreign Body Response to Grow Tissue Engineered Blood Vessels in Vivo

Wouter J. Geelhoed [1,2] · **Lorenzo Moroni** [3] · **Joris I. Rotmans** [1,2]

Abstract It is well known that the number of patients requiring a vascular grafts for use as vessel replacement in cardiovascular diseases, or as vascular access site for hemodialysis is ever increasing. The development of tissue engineered blood vessels (TEBV's) is a promising method to meet this increasing demand vascular grafts, without having to rely on poorly performing synthetic options such as polytetrafluoroethylene (PTFE) or Dacron. The generation of in vivo TEBV's involves utilizing the host reaction to an implanted biomaterial for the generation of completely autologous tissues. Essentially this approach to the development of TEBV's makes use of the foreign body response to biomaterials for the construction of the entire vascular replacement tissue within the patient's own body. In this review we will discuss the method of developing in vivo TEBV's, and debate the approaches of several research groups that have implemented this method.

Vasculature · Vascular access · Animal models · Translational · Graft patency · Foreign body response

Introduction

Globally there is an immense and ever increasing need for vascular grafts for use as vessel replacement in cardiovascular diseases (CVD), or as vascular access site for hemodialysis. It is widely known that the incidence of CVD is increasing, a trend expected to continue in the foreseeable future [1, 2]. This coincides with an increase in the number of end-stage renal disease (ESRD) patients requiring a vascular access (VA) site [3]. Ideally, autologous veins or arteries are used as grafts, as these are associated with superior patency [4, 5]. However, due to a relative lack of donors, previous harvesting, or the poor state of the patients own vessels, native arteries and veins are not available for grafting in a substantial portion of patients. In such cases prosthetic grafts offer a suitable alternative and are frequently utilized. However, the primary patency for these synthetic grafts is dismal, both as arterial bypass, and arteriovenous graft for hemodialysis [6]. This failure of synthetic vascular grafts is primarily due to intimal hyperplasia, thrombosis and infection [7, 8].

Tissue engineered blood vessels (TEBV's) may be a promising alternative for patients requiring a vessel replacement or VA site. Numerous approaches to the development of tissue engineered grafts have been described, and extensively reviewed [9–11]. The majority of these approaches tend to involve complex in vitro preparation steps, decellularized constructs, or the incorporation of synthetic materials onto the TEBV. Ideally, a vascular replacement is made completely out of cellularized autologous tissue, thereby not causing any immune reaction, and retaining the ability to remodel in vivo.

In the present review, we will discuss approaches that [illegible] lize the host reaction to an impl[illegible]

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Chapter 2

Utilizing the foreign body response to grow tissue engineered blood vessels *in vivo*

W.J. Geelhoed, L. Moroni, J.I. Rotmans

J Cardiovasc Transl Res. 2017 Apr;10(2):167-179

Abstract

It is well known that the number of patients requiring a vascular graft for use as vessel replacement in cardiovascular diseases, or as vascular access site for hemodialysis, is ever increasing. The development of tissue engineered blood vessels (TEBVs) is a promising method to meet this increasing demand vascular grafts, without having to rely on poorly performing synthetic options such as polytetrafluoroethylene (PTFE) or Dacron. The generation of *in vivo* TEBVs involves utilizing the host reaction to an implanted biomaterial for the generation of completely autologous tissues. Essentially this approach to the development of TEBVs makes use of the foreign body response to biomaterials for the construction of the entire vascular replacement tissue within the patient's own body. In this review we will discuss the method of developing *in vivo* TEBVs and debate the approaches of several research groups that have implemented this method.

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Tissue engineered blood vessels (TEBVs) may be a promising alternative for patients requiring a vessel replacement or VA site. Numerous approaches to the development of tissue engineered grafts have been described, and extensively reviewed [9-11]. The majority of these approaches tend to involve complex *in vitro* preparation steps, decellularized constructs, or the incorporation of synthetic materials onto the TEBV. Ideally, a vascular replacement is made completely out of cellularized autologous tissue, thereby not causing any immune reaction, and retaining the ability to remodel *in vivo*.

In the present review, we will discuss approaches that utilize the host reaction to an implanted biomaterial for the generation of completely autologous TEBVs *in vivo*. In other words, this approach to regenerative medicine and the development of TEBVs aims to make use of the body's foreign body response to biomaterials and exploit the host environment as a bioreactor for the generation of new tissues, essentially allowing for the construction of the entire vascular graft within the patient's body. Interestingly, most biomedical research concerning the foreign body response (FBR) is conducted with the aim of minimizing, or abolishing the cascade resulting in this host reaction, as propagation of the FBR is commonly associated with a decrease in implant functionality [12,13]. Yet, by utilizing the FBR to generate tissue constructs in a controlled setting, various groups have developed methods to construct TEBVs [14-17]. This involves an implantable biomaterial, which elicits a FBR to allow the growth of tissue around it (Figure 1.). TEBVs made in this way would be non-toxic, elicit no immune response, and be free of pre-existing disease as the tissue is completely autologous, none of the initial foreign material remains in the body to propagate an immune response [18].

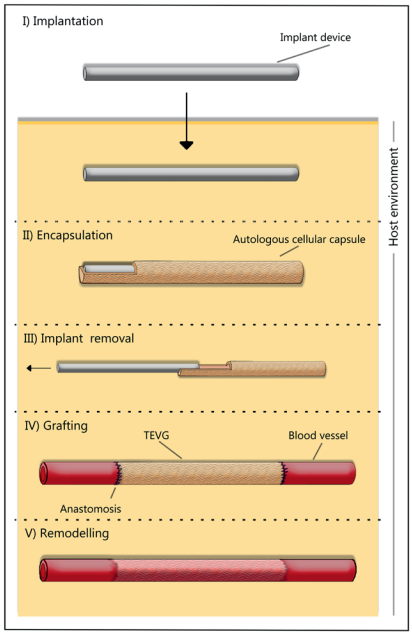

Figure 1. An overview of the in vivo based concept. I) a biomaterial is implanted in the host. II) The host environment acts as a bioreactor, leading to the encapsulation of the biomaterial with a cellularized fibrous tissue capsule. III) The implant device is removed, leaving only the tissue capsule. IV) The tissue capsule is grafted to the vasculature, creating a TEBV. V) Over time remodeling occurs, where the TEBV transdifferentiates to attain characteristics of a native blood vessel.

There are several requirements to which a TEBVs must adhere in order to be considered as a promising vessel substitute. These factors must be taken into account when through the developing a TEBV. An overview of the requirements of TEBVs is provided in Table 1.

Table 1. An overview of the requirements of a TEBV.

I)	the mechanical properties of the vessel must be sufficient to withstand the pressure caused by flow for extended periods of time without resulting in an aneurysm or bursting;
II)	the vessel should be sufficiently compliant to avoid a compliance mismatch, a known factor for graft failure [19];
III)	the surgical suitability must be considered; the graft must have sufficient suture retention strength, and be easy to handle by the surgeon;
IV)	the vessel must be compatible with the host, i.e. not elicit an inflammatory reaction, be non-toxic, and non-carcinogenic;
V)	the vessel should be capable of remodeling to meet the demands of the vasculature;
VI)	the surface of the graft should not be prone to thrombus formation.
VII)	be produced in a large scale, cost effective manner, and available in various dimensions;
VIII)	show acceptable variation between batches.

The concept of using the host as an *in vivo* bioreactor is not new. Already in 1961, Schillings et al. attempted to make autologous grafts by implanting stainless steel mesh cylinders subcutaneously for 4 months in 12 dogs. Five of these grafts failed due to thrombosis,

technical errors and bleeding. However, the remaining 7 grafts showed an impressive patency of 3 years. Yet, it was Sparks who truly pioneered the application of autologous capsulated tissue as vascular grafts grown *in vivo* in the 1960's, with the grafts being briefly applied in clinical applications [20,21]. Dacron fabric grafts, covered by layers of fibrous tissue that formed as a result of the subcutaneous implantation of silicone rods, were clinically implemented as arterial bypass grafts in the late 1960's. However, the application of this TEBV approach was reportedly low, as follow-up studies showed that there were various complications concerning the technique [22-25]. A main issue was the long incubation time that was required for the formation of the tissue around the implant, although this did not always yield a suitable graft. Thrombosis and stenotic occlusion of the graft was the main reason for late graft failure, while 20% of the grafts experienced aneurysm formation[23]. Little information is available on the mechanical properties of the Spark's graft, which could give an indication of why aneurysm formation occurred. However, it is likely that a main downside of the Spark's graft is the lack of sufficient mechanical properties (concluded by the high rate of aneurysm formation). Incorporation of the polymer into the tissue and the low cell density of the graft may be one of the explanations behind the aneurysm formation of the graft.

The pioneering work by Sparks illustrated that the host foreign body response could be tailored for *in situ* tissue engineering purposes. Although aneurysm formation limited its further clinical application, subsequent research groups aimed to fine tune and reinvigorate this approach for the generation of TEBVs, by making use of the increasing knowledge of biomaterials and the dynamics of the FBR.

This review will discuss vascular tissue engineering (VTE) approaches that utilize the patient's body as a bioreactor for the development of completely autologous TEBVs. The context of these studies and their approach will be discussed. Furthermore, the complex process of clinical translation of and the use of appropriate animal models will be debated.

The foreign body response

In order to understand how the host environment can contribute to the formation of new tissues in response to an implanted biomaterial, it is important to understand the cells and pathways involved in the FBR. All biomaterials elicit a cellular and tissue response when implanted *in vivo*, known as the FBR [13,26]. If a foreign body is small and superficial enough it will be extruded from the body. If the foreign body is too large to extrude, it will be encapsulated to ensure there is a safe barrier between it and the host. Initially, the implantation of a biomaterial causes mechanical damage to the vascularized connective tissue at the implant site. In this very early process of the FBR, blood material interactions caused by implantation result in the immediate adsorption of proteins onto the surface of the implant creating what is known as the provisional matrix [27].

The provisional matrix is composed of numerous bio-reactive agents, including fibronectin, complement components, albumin, and vitronectin. This makes it crucial in determining the activity, proliferation, migration, and differentiation of inflammatory and wound healing cells

[26]. Notably, fibrinogen can absorb directly on the biomaterial surface, creating a dense fibrin network, which sequentially promotes leukocyte adhesion [28,29]. This presence of fibrinogen is vital, as it has been shown that mice with depleted fibrinogen were unable to initiate an inflammatory response to implanted biomaterials[30]. Complement factors can spontaneously adsorb to the biomaterial, which can lead to the activation of the alternative complement pathway [31-33]. Due to the mechanical nature of the formation of the provisional matrix, its composition can vary greatly depending on the implant location, and surface properties of the implant [27]. Furthermore, variations in protein adsorption occur due to what is known as the Vroman effect, which describes a competitive protein exchange on biomaterial surfaces, i.e. the competitive displacement of adsorbed proteins by other proteins with stronger binding affinities [34].

Following the formation of the provisional matrix, the initiation of the acute inflammatory response is the next phase of the FBR, characterized by the infiltration of neutrophils. In this phase the wound site is cleaned, vessels dilate and blood flow to the injury site increases. A variety of cytokines and growth factors are released, monocytes infiltrate the implantation site and begin to differentiate to macrophages [26]. It has been shown that histamine inhibition significantly decreased phagocyte recruitment, elucidating the importance of mast cells and histamine in the acute FBR [35].

If the inflammatory stimulus persists, in case the biomaterial is not removed; the inflammatory response enters a chronic phase. Normally, the acute phase lasts from several hours to several days; chronic inflammation generally lasts no longer than two weeks as long as the inflammatory stimulus does not persist any longer [26]. Yet, the extent of damage that occurs at the implant site is vital in determining the length and severity of the acute, and the chronic inflammatory phases. This phase is characterized by monocyte infiltration, macrophage activation and angiogenesis of the site of tissue injury [26]. Angiogenesis is essential to support the wound healing process with a supply of nutrients. Following an early angiogenic pulse caused by fibrin [36], histamine [37], and VEGF (released by platelets), the angiogenic process is later maintained by hypoxic macrophages and fibroblasts in the new tissue [38].

Monocyte and macrophage recruitment to the wound site is driven by numerous chemoattractants such as IL-1β, IL-4, TNF-a, and CCL2, which facilitate proliferation, and the extravasation of leucocytes [39-41]. Macrophages have been shown to initially secrete IL-β, and IL-6, and eventually express more IL-10 as time progressed, indicating a phenotypic shift from a pro-, to an anti-inflammatory state [42]. If the foreign body is too large to be phagocytize activated macrophages fuse together to form multinucleated foreign body giant cells (FBGC's), a characteristic feature of chronic inflammation. Vitronectin, commonly present in the provisional matrix has been shown to support macrophage adhesion, and foreign body giant cell formation [43]. If the FBGC's are unable to remove the foreign body, the process of encapsulation is initiated by surrounding the implant with a dense collagen matrix [44]. Macrophages and FBGC's stimulate fibroblasts to proliferate and overproduce components of the extracellular matrix (ECM), including collagen, by releasing TGF-β, IL-4, IL10, IL13, TNF-a, and IL-1 [45,46]. It should be noted that TGF-β is regarded as the most potent inducer of the ECM formation[38]. Granulation tissue is characterized by the immigration

of fibroblasts, angiogenesis in the newly developed tissue, and the presence of a layer of macrophages or FBGC's lining the implant. If the stimulus is not resolved, a thick fibrous capsule ultimately forms that is very rich in collagen, (myo-) fibroblasts, and one or two layers macropaghes and FBCG's [26,38]. It is proposed that if the inflammatory stimulus remains for multiple months, the tissue becomes less cellularized, ECM rich scar tissue as was seen in the Sparks' graft. An overview of the FBR for biomaterials aimed at the generation of *in vivo* autologous TEBVs is provided in Figure 2

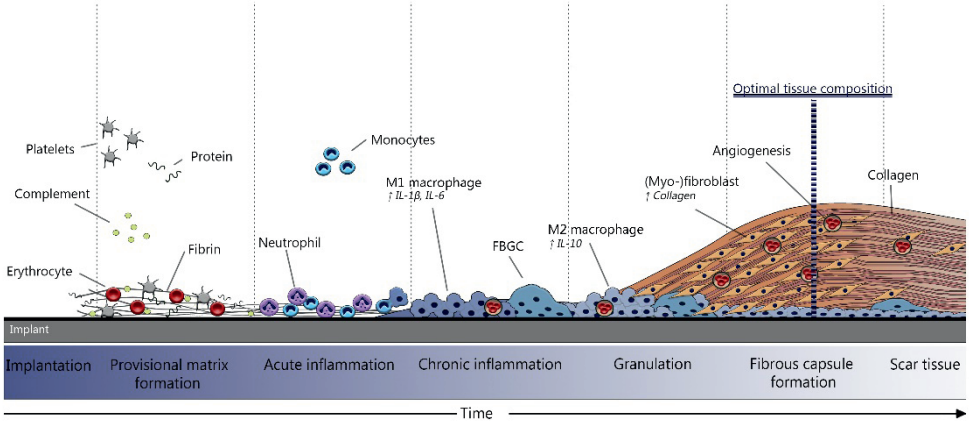

Figure 2. An overview of the foreign body response. Following implantation, a provisional matrix immediately forms around the implant. Following provisional matrix formation acute inflammation is mainly characterized by the presence of neutrophil, and some monocyte infiltration and differentiation. Chronic inflammation is characterized by the infiltration of monocytes that differentiate to macrophages, and neovascularization. Fibroblasts then proliferate and begin to produce ECM components including collagen. A fibrous capsule forms composed out of a (myo-)fibroblasts, ECM components (mainly collagen), and a one- to two-layer of macrophages. Over time scar tissue forms mainly composed of ECM and collagen, with less fibroblasts. The optimal tissue composition for use as TEBV is a thick cellularized fibrous capsule, which is collagen rich with relatively few inflammatory cells.

Tailoring the FBR

The host FBR to an implant is a complex reaction, where host features, implant characteristics, and implantation duration all contribute to the response generated. However, in its complexity it is clear that many factors can influence the host response to a biomaterial. Regardless, the host environment and surrounding tissue are an interesting cellular source for *in vivo* tissue regeneration strategies [47,48]. Since the application of the Spark's graft, much research has been done on the FBR and how it could be modulated to provide a cellular source for the construction of new tissue structures *in vivo*.

Firstly, biomaterial characteristics are critical in defining the FBR response that is generated. The chemical composition, hydrophilicity, topography, and coating of the biomaterial can critically influence the initial cell-material interactions that occur [49-51]. Therefore, by altering the composition of the biomaterial the resulting foreign body response can be modulated. For example, if the composition and adherence of the provisional matrix is altered, it can

impact the following cellular reaction [52]. Moreover, macrophage adherence is known to be essential in driving the FBR [26]. If a material can alter macrophage adherence, this will also greatly influence the resulting FBR. For example, solvent etching and gas plasma treatment is known to affect cell attachment to biomaterial surfaces [53,54]. **Secondly**, the length of implantation period is vital in determining the tissue structure that is developed. Very early in the foreign body response, the tissue will be largely composed of neutrophils, have a highly inflammatory profile, and low collagen content (and therefore poor mechanical properties). Yet, a too long incubation step (several months) will result in largely acellular tissue unable to adequately remodel in the vasculature potentially leading to aneurysm formation, as was seen in the Sparks graft. **Thirdly**, implantation location is vital in determining the FBR to a biomaterial. Fibroblasts differ at varying anatomical sites, and with disease processes [55]. Moreover, fibroblasts from separate tissues differ in their production of matrix metalloproteinases (MMP's), the production of collagen, and proliferation, meaning that the degree of encapsulation is likely to vary between tissues [56].

Overview of approaches for in situ vascular tissue engineering

Since the application of the Sparks graft in the 1960's, the group of Campbell was the first to reconsider the utilization of the body as a bioreactor for the generation of new tissue, by using the peritoneal cavity as implant location. The motivation behind this approach is that besides showing a sufficient FBR to the implanted material, mesothelial cells can be recruited to the tissue capsule. Mesothelial cells and endothelial cells have been shown to have various similarities, including a non-thrombotic tendency [57].

In both rat and rabbit models, a piece of silastic (an inert silicone elastomer) tubing was implanted in the peritoneal cavity. Two weeks after implantation the implants were harvested and the silastic tubing removed from surrounding tissue capsule. The resulting tissue capsule was covered by layers of myofibroblast, and a single layer of mesothelial cells. Next, the tissue capsules were reverted, so that the outer mesothelial layer of the capsule lined the lumen during grafting. The tissue capsules were implanted as arterial interposition, left in place for a maximum of 4 months. Ultimately, the TEBVs showed an overall patency of 67% in rats, and 70% in rabbits. Prior to grafting, the granulation tissue was shown to be rich in β-actin, and desmin indicating the contractile capacity of the cells. However, low levels of smooth muscle myosin heavy chain (a marker for smooth muscle cells (SMC's)) were observed in the capsules. One month after implantation, arterial levels of myosin heavy chain were reported, indicating a phenotypic shift of the myofibroblasts to a SMC-like cell. Furthermore, 1 month after implantation the presence of an internal elastic lamella was observed. The TEBV was shown to respond positively when treated with contractile and relaxing agents. However, only 50% of the implants produced suitable TEBVs, occasionally producing no usable tissues in an animal, a notable limitation of this method [58].

Subsequently, the approach was attempted in 15 mongrel dogs, where various types of implant materials were assessed for their suitability as TEBV. Some of the implants were foreseen of an external mesh. 3-3.5mm in diameter TEBVs were implanted in either the peritoneal or pleural cavity for 3 weeks, allowing the formation of tissue capsules around

the implants, and then grafted as arterial interposition in the femoral artery. In this model the TEBVs were not everted prior to grafting. The mesh implants produced usable TEBVs in all cases yet had a poorer patency of 60% between 3 and 6.5 months. Only half of the implants without a mesh produced usable TEBVs yet had a patency of 83% between 3 and 6.5 months. The uncoated TEBVs showed an impressive remodeling of its cellular composition. The myofibroblasts appeared to undergo a phenotypic switch to SMC like cells, and endothelization of the TEBV was observed as well. Furthermore, the TEBV had an adequate burst pressure of 2500mmHg, allowing safe implantation into the vasculature [59].

More recently, the method was improved by allowing the TEBVs to be generated under pulsatile conditions, significantly improving the mechanical properties of the vessels. The application of sheer stress has been shown to promote ECM protein production [60].

Clearly, the group of Campbell had shown the potential of a TEBV formed by the FBR within a host organism. A major advantage of the technique being everting the TEBVs to expose mesothelial cells before grafting, whereas the invasive peritoneal implantation of the silastic tubing, comprises a relative disadvantage of this technique when compared to subcutaneous approaches that are discussed below.

Following the example of the group of Peirce et al., who in 1953 attempted to make vessel constructs out of aortic collagen, Tsukagoshi et al. attempted to create autologous TEBVs using subcutaneously implanted silicone surrounded by a layer of fascia [61]. A major advantage of a subcutaneous implant location is the rapid regenerative capacity of the skin [62], which could lead to rapid development of tissue surrounding an implant. Besides only a silicone implant, a biological component from the host was added to the biomaterial in order to reinforce the TEBV. A 10x40mm segment of fascia from the dorsum and medial thigh of 15 rabbits was removed, wrapped around silicone tubes, and implanted in subcutaneous pockets. Four weeks later, the tubes were removed from the body and the silicone tube was removed from the fibrous capsule composed of fascia and a fibrocollagen mesh. The exposed collagen promoted platelet adhesion and thrombus formation [63]. A lumen composed entirely of collagen could therefore be highly thrombogenic. It was then interposed into the femoral artery of the rabbits as an end-to-end graft. Patency rates of 80 percent were reported at 5 and 8 weeks, and re-endothelialization of the TEBV was shown to occur. No aneurysm formation was reported, indicating that the method provided adequate mechanical strength. However, intimal hyperplasia was reported at both ends of the TEBV of 70% of the lumen, yet not in the center of the TEBV. This would indicate an increased proliferation of cells near both anastomoses likely as a result of the turbulent flow [16].

Another interesting approach has been developed by the group of Nakayama, who aims to create TEBV with a silicone implant. They have termed the fibrous tissue capsule that grows around their silicone implant ‘biotubes’. In their first report of the biotube they reported well-formed grafts a bursting pressure of at least 200 mmHg [64]. Subcutaneous implantation is advantageous due to the large population of dermal fibroblasts, which could promote the formation of new tissue.

In their first grafting study in rabbits, an 82% patency rate of the biotubes was reported at 12 weeks. Again, as the tissue capsules are largely composed of myofibroblasts and collagen. To reduce the risk of thrombosis, the potent anticoagulant Argatroban was administered to the fibrous capsule prior to grafting. Argatroban is commonly used in patients with heparin induced thrombocytopenia (HIT) requiring an antithrombotic therapy [65]. The tissue capsule around the biotube was composed of fibroblasts, whereas after grafting circumferential collagen, myofibroblasts and possible SMC were reported [66]. In a subsequent study, the TEBV remained patent for an impressive 2 years in one animal, and showed signs of both elastin formation and endothelialization [67]. The extremely limited sample size does fundamentally limit the conclusions that can be drawn. The concept appeared to be somewhat less successful in a rat model, where a patency of 67% at 12 weeks was reported. Again, elastin formation and endothelialization of the TEBV was reported. Several creative improvements to stimulate tissue growth were implemented on the implants, such as the addition of nicotine, optical stimulation using LED's, and the addition of eosin-Y, which were all shown to stimulate fibrous capsule formation [68-70].

In a recent study the acute phase patency of a new biotube type, designed around a silicone cage as appose to a plain rod, was compared to the original silicone rod mediated biotube design in 6 beagle dogs. Following a 4-week implantation in the dorsal subcutaneous pocket, the silicone implant was removed, and the resulting tissue capsule was grafted into the femoral artery. The new biotube design was shown to have a burst pressure of 1825mmHg, compared to 944mmHg for the original biotube [15]. At 7 days the acute patency was observed by means of angiography. The new biotube design had a 100% patency, compared to a patency of 33% for the original biotube [15]. This research again underlines the potential of the *in vivo* bioreactor approach.

Our group has also attempted to generate autologous grafts *in situ* by focusing on the surface characteristics implanted biomaterials. It is known that the surface characteristics of an implanted biomaterial are key in driving the FBR and fibrous capsule formation [18,51]. Ultimately, we aim to develop a TEBV to be used as vascular access site for hemodialysis. Our reasoning is that, due to the growing number of patients requiring hemodialysis treatment and limited vascular access options, TEBVs could fundamentally improve this huge clinical problem for hemodialysis patients.

In a study in 15 rats, various surface modifications of a few materials were assessed in their effectiveness in propagating an encapsulating response. These included gas plasma treatments, collagen I and TGF-B coating, and chloroform etching. While the thickest tissue capsules were produced using TGF-B and collagen coating, this tissue was less uniform and had a low cell density. It was shown that the copolymer poly(ethylene oxide terephthalate/ polybutylene terephthalate) (PEOT/PBT) which was chloroform etched provided the most ideal tissue; providing a thick collagen layer with a high cell density of circumferentially aligned myofibroblasts and initial signs of elastin formation. It was shown that chloroform etching increased the surface roughness and oxygen content of the polymer, resulting in an enhanced cell adhesion [51].

Sequentially, the method was assessed in a porcine model. Chloroform etched rods were implanted subcutaneously in the abdomen of 4 pigs. Four weeks later the polymer implants were removed, and two tissue capsules were implanted bilaterally as carotid interposition grafts. A 1-week patency of 100%, and 4-week patency of 88% was reported. Prior to grafting, the tissue capsules were shown to be largely composed of collagen, glycosaminoglycans, fibroblasts, and some myofibroblasts. Directly lining the lumen leucocytes were observed, with hardly any FBGC's. After grafting an increase in luminal diameter was shown, where the luminal side of the tissue capsule was covered by a monolayer of endothelial cells. The initial leucocytes lining the lumen was no longer present, most likely due to hemodynamic factors. The remaining cells in the tissue were mainly SMC like cells. The potential of modifying the surface characteristics of a biomaterial to modulate the FBR after implantation was shown to be a viable method of producing a cell rich TEBV. Clearly, the FBR can be steered to generate tissues with for varying purposes, including TEBV generation.

An overview of the animal studies carried out by the groups discussed above, in which TEBVs were grafted into the vasculature is provided in table 2.

Remodeling of TEBV after implantation in the circulation

Ideally, tissue engineered grafts mimic the composition of native vessels. These are composed of a thin layer of healthy endothelial cells, surrounded by layers of connective tissue, SMC's, and elastic laminae depending on the location, diameter and function of the vessel. Grafts derived from fibrous capsule formation tend to be rich in collagen, myofibroblasts, and have one- to two-layers of macrophages directly surrounding the implant. Remodeling in circulation is an essential step in the maturation of these *in vivo* grown grafts to attain attributes that more accurately mimics native vessels.

A hallmark of a functional vessel is the presence of a healthy endothelial monolayer, as this reduces the thrombogenicity and promotes the homeostasis of a vessel [71,72]. A major struggle for all tissue engineering approaches is the incorporation of a functional endothelium that can handle the arterial flow rates following a grafting procedure and remain in place long term [73]. Numerous attempts have been made to seed endothelial cells on both TEBVs and vascular grafts prior to grafting, however this has proven to be deceptively complex [74]. Exposed collagen on the lumen of *in vivo* grafts provide a potentially thrombogenic surface shortly after insertion in the circulation. Campbell et al. already showed an elegant approach to this problem by lining the luminal side of the TEBV with autologous mesothelial cells [58]. Despite the difficulty of seeding an endothelium upon TEBVs prior to grafting, all studies using the *in vivo* grafts have shown endothelialization of the lumen [14,16,59,75]. Although, the function of endothelial progenitor cells is decreased in patients with chronic kidney disease[76]. Spontaneous endothelialization of decellularized TEBVs has recently been demonstrated in ESKD patients [77]. It remains to be determined whether this endothelialization of the grafts results from circulating EPCs that adhere to the surface of the TEBV or result from the migration of endothelial cells from neighboring vessels to the graft.

Tissues grown as a result of the FBR are rich in fibroblasts and myofibroblasts, but not SMC's. SMC's are important in maintaining vascular homeostasis notably with regard to the vasoreactivity of vessels. Various studies have shown that after grafting, populations of (myo-)fibroblasts have either transdifferentiated to, of been replaced by SMC like cells [14,58,59,66]. A histological overview of the differentiation of the tissue capsules towards blood vessel-like structures that we observed in our pig studies is provided in Figure 3. Moreover, Campbell et al. had reported an increased vasoactivity of the grafts after being implanted for several months [58,59].

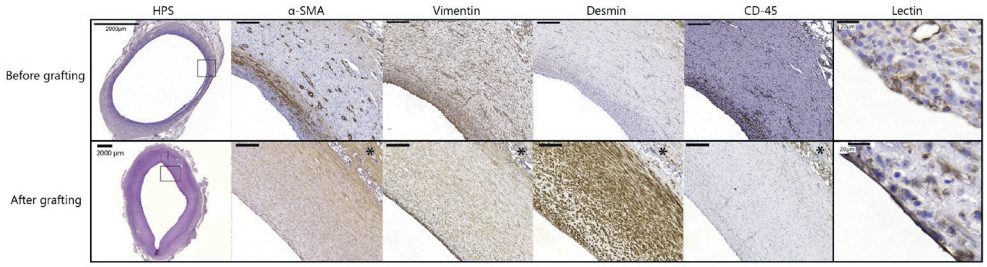

Figure 3. Adapted from Rothuizen et al. Showing an autologous tissue engineered blood vessel before, and after grafting. Before grafting α-SMA, vimentin positive (myo-)fibroblasts are present, with frequent CD-45 positive leucocytes and no endothelium (lectin negativity). After grafting the cells are α-SMA, desmin positive SMC like cells with no CD-45 positive cells and an endothelial monolayer 14.

The structural integrity of a vessel is largely determined by the ECM and is able to fundamentally modulate various aspects of cell biology in addition to its structural role [78,79]. Here, collagen is the determining factor of the strength of the vessel [80]. Prior to grafting, TEBVs that have grown as a result of a FBR will have a different ECM as well as cellular composition than native arteries. A breakdown of the ECM could therefore lead to diminished mechanical properties of the graft, and even aneurysm formation as was frequently seen in the Spark's graft[20]. In circulation, collagen, which accounts for the mechanical strength of the ECM, can be broken down by matrix metalloproteases (MMP's), in particular MMP-2 and MMP-9 [78]. This underlines the importance of a cellularized graft, which could allow the production of ECM components in vivo after it has been grafted into the circulation.

Another important component of the vessel wall is elastin, which is essential for vascular compliance [81]. Native elastin is immensely durable with a half-life of approximately 70 years [82], and relatively resistant to chemical and biological degradation[83,84]. Thus far, the incorporation of elastin into TEBVs which can remain functional for a long time, appeared to be extremely challenging [85,86]. As elastin synthesis only rarely occurs in adult life, the incorporation of exogenous elastin fibers into the tissue might favor methods that depend on *in vivo* synthesis of elastin [85,86]. Ideally, cells within the *in vivo* TEBV could remodel and begin to produce elastin after grafting, although the process by which this occurs in this the setting of FBR mediated TEBVs is not entirely understood. Nayakama et al. for example, had reported the presence of elastin after grafting of the biotubes [75]. However, it remains to be demonstrated if functional elastic fibers are formed which have a positive impact on vessel

compliance. Recently, we showed that miRNA29 inhibition could be an attractive method for elastogensis, with a superior elastogenic potential when compared to IGF-1, TGF-β1, and minoxidil [87].

Advantages and disadvantages of in situ vascular tissue engineering

There are clear advantages to using the host environment as an *in vivo* bioreactor. Grafts are composed of entirely autologous tissue, meaning no immunological mismatch is possible and no prior infection is present. The approaches to creating an *in vivo* TEBV require relatively simple polymer implants as opposed to any type of *in vitro* fabricated vascular construct. This means that this method has the potential of being cost effective and widely available.

Clear disadvantages of this method of course also exist. The grafts are limited in their applicability for acute procedures, due to their biological incubation time that generally requires several weeks. When utilizing in situ engineered blood vessels, the length required for certain arterial bypass procedures may not be attainable. For instance, creating femoral-popliteal bypass grafts that cross the knee joint could be challenging, as this requires flexible implant material to allow bending during the growth of the TEBV. The state of the host environment can alter the response to an implant. Gender [88], age, and the presence of disease can alter the response to an implant, and therefore the composition of the generated tissue. End-stage renal failure can impact wound healing and impair bone marrow function [76,89]. Therefore, the applicability of the grafts in these patient populations must be assessed in detail. As seen in some of the methods discussed in this review, there is a potentially large biological variation that must be assessed and controlled before these techniques can be considered for clinical translation.

Animal models

Ideally, an animal model is cheap, quickly develops a desired pathology, is readily available and mimics vascular setting of humans as closely as possible (i.e. thrombogenicity, vessel size, immune response). Clearly no animal model exists that fulfills all these requirements. In the development of a medical device and its translation to the clinic, animal models are indispensable. However, due to fundamental differences in the molecular pathways and pathologies between animal models and humans, animal studies do not translate well to the clinical phases of development [90].

For studying molecular pathways, mice are an ideal model, due to the large number of research tools and knockout animals available. However, their small size makes vascular grafting studies suboptimal. The slightly larger size of rats and rabbits make these more suitable for early proof of concept studies. Limitations here are the poor translation to the clinic, as well as the relative lack of knock-out animals compared to mice.

The vessels of larger animals are more representative of humans and are more appropriate to mimic human vascular conditions, as the size of the vasculature is determinant for the sheer

stress exacted on the vessel wall. Numerous sheep and goat models have been described in vascular studies [91-93]. However, high variability in response to anticoagulant and antiplatelet therapy in these animals is known to occur and must be taken into account [94]. Dogs have comparable vasculature to humans, are easy to handle due to their familiarity with humans, and readily available. Yet, dogs have however been found to be hypercoagulant in an *ex vivo* analysis [95]. However, synthetic grafts have shown high patency rates in dogs, with little sign of intimal hyperplasia, raising the question if dogs are a stringent enough model for vascular applications [96]. The vasculature of non-human primates is most similar to humans, making it the most accurate animal model available. However, due to high costs, special housing requirements, and stringent ethical concerns, the use of this model is limited.

Arguably, the 'aggressiveness' of the vasculature is the most important factor in assessing the long-term patency of vascular grafts, with intimal hyperplasia being the most common cause of TEBV failure. Different animal models vary greatly in the speed at which they develop intimal hyperplasia [97]. One of the most commonly used models in cardiovascular research today is the pig model [97]. The responsive nature of the vasculature of the pig makes it ideal for short-term pathophysiology studies. However, it is known that pigs for develop stenotic lesions up to six times faster than humans [98], making them less appropriate, arguably too stringent, for long term patency studies.

Clinical perspective

There are factors to take into account in the translation of TEBV methods to a clinical setting. Firstly, a TEBV derived from the FBR would require an additional implantation procedure, besides the grafting of the TEBV into the vasculature. Aside from this additional intervention, the use of these TEBVs may be somewhat surgically challenging, as the TEBV must first be removed from the implant before it can be grafted to the vasculature. The procedure would therefore require additional training by a surgeon to carry out properly. Following a surgical intervention, such as the implantation of a medical device, a patient may be given immune suppressants. Immune suppression may intervene with the FBR, and therefore TEBV development, which would need to be accounted for when considering FBR based TEBV methods in a clinical setting. Before a FBR mediated TEBV method is to be considered for a clinical setting, the variation between patients must be well known, and accounted for, to assure all TEBVs meet all requirements set for vascular grafts. It is known that for example CKD [89] and diabetes [99] can impair wound healing. Therefore, it is vital to show that the formation of the TEBVs in these patient populations occurs as expected, and with acceptable variation between patients.

In a pioneering clinical trial, the group of Shinoka successfully employed TEBVs, designed from bone marrow mononuclear cells, with no graft related mortality, indicating the potential of TEBVs in a clinical setting [100]. In a more recent clinical trial, the group of Niklason employed human acellular vessels as vascular access in 60 patients with ESKD, showing a primary patency of 28%, and secondary patency of 89% at 12 months post implantation, underlining the potential of TEBV approaches as potential vessel replacement [77]. VA sites offer an interesting target for the clinical implementation of TEBVs. The occlusion of an

arterial bypass is potentially fatal, while the occlusion of an arteriovenous conduit may render the VA site unusable, but is less dangerous for the patient, minimizing the risk of the trial. As was also stated earlier, the current options for creating a functioning arteriovenous graft are poor. Implementing a TEBV as an arteriovenous graft can be seen as a stringent model to assess TEBV functionality and patency, due to the harsh hemodynamic conditions, and frequent stenosis seen in arteriovenous conduits. Until now no FBR mediated TEBV approach has been assessed in a clinical phase.

Conclusion and future perspectives

In this review we have illustrated and summarized the potential of a body as a bioreactor for the generation of autologous tissue engineered blood vessels *in vivo*, including the mechanisms of the foreign body response that can result in new tissues, research groups that have attempted to utilize this approach, and the difficulties and limitations of developing such methods. The potential of autologous *in vivo* made grafts is clear through the promising pre-clinical studies that have been carried out. With a continued understanding of the FBR, and the common factors leading to graft failure, we foresee more fine-tuned approaches to the generation of TEBVs will be assessed. The limitation of the approach has been elucidated and must be overcome for the methods to ultimately be successful. The translation of not only these, but all vascular tissue engineering approaches remains difficult, and suitable animal models must be chosen to allow for successful clinical translation. In conclusion, autologous *in vivo* TEBVs show great potential as cell rich vascular grafts capable of remodeling in the vasculature.

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Indirect Burst Pressure Measurements for the Mechanical Assessment of Biological Vessels

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In the evaluation of tissue-engineered blood vessels (TEBVs), the utilization of the correct mechanical test to assess the burst pressure is pivotal for translation to a clinical setting. The ISO 7198 standard outlines various methods that may be implemented to evaluate the mechanical characteristics of vascular prosthetics. The gold standard is the direct measurement of the pressurized burst pressure. Two alternative indirect methods are the circumferential tensile strength (CTS) and the probe burst pressure. There are limited data validating the use of the indirect methods for their predictive capacity of the pressurized burst pressure in single biological vessel samples. We assess the two indirect methods compared with the direct pressurized burst pressure measurement, for their correlation within single biological samples, using methods presently used in literature and as they are proposed by the ISO 7198. The CTS, the probe burst pressure, and the pressurized burst pressure correlated very well (All $R^2 > 0.89$) when silicone samples were assessed, although the indirect methods resulted in a large overestimation of the burst pressure. The correlation between the three mechanical tests was poor (all $R^2 < 0.18$) when arterial and venous samples were investigated. Freezing and subsequent thawing before testing had no impact on the mechanical properties of the vessels. Strain rates within the strain rate window provided by the ISO 7198 (50–200 mm/min), likewise, had no impact on the outcome of the tests. Neither the CTS nor the probe burst pressure is predictive of the pressurized burst pressure of the biological vascular tissue. Unless explicitly validated in a testing system on a range of biological tissues, the derived methods should not be utilized for the evaluation of the burst pressure of biological TEBVs for clinical purposes.

Keywords: *mechanical testing, tissue engineering, vascular biology*

Impact Statement

Vascular tissue engineering (VTE) is a rapidly expanding field, with numerous approaches being explored both in preclinical and clinical settings. A pivotal factor in the development of VTE techniques is patient safety, notably with respect to the mechanical properties of the vessels. Of the mechanical properties, the bursting strength, representing the ability of a vessel to withstand the forces exerted on it by blood pressure, is the most important. The burst pressure is commonly assessed using one of the methods proposed by the ISO 7198. In this study, we evaluate the three burst pressure assessment methods exactly as they are presently used in the field of VTE. We show that the indirect assessment methods, as they are presently used, provide inconsistent and therefore inaccurate estimates of the true yield stress of a vessel.

Introduction

TISSUE-ENGINEERED BLOOD VESSELS (TEBVs) have great potential to offer a better alternative for synthetic grafts that are presently used in vascular surgery.[1] One of the most vital aspects of TEBVs is the ability to withstand the mechanical forces induced by arterial blood pressure. Therefore, it is vital to evaluate the mechanical properties of an engineered vessel before clinical use. The ISO 7198 provides an overview of tests to be carried out to evaluate the mechanical

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Chapter 3

Indirect burst pressure measurements for the mechanical assessment of biological vessels

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Abstract

In the evaluation of tissue engineered blood vessels, the utilization of the correct mechanical test to assess the burst pressure is pivotal for translation to a clinical setting. The ISO 7198 standard outlines various methods that may be implemented to evaluate the mechanical characteristics of vascular prosthetics. The gold standard is the direct measurement of the pressurized burst pressure. Two alternative, indirect methods are the circumferential tensile strength and the probe burst pressure. There is limited data validating the use of the indirect methods for their predictive capacity of the pressurized burst pressure in single biological vessel samples. We assess the two indirect methods as compared to the direct pressurized burst pressure measurement, for their correlation within single biological samples, using methods currently used in literature and as they are proposed by the ISO 7198. The circumferential tensile strength, the probe burst pressure and the pressurized burst pressure correlated very well (All R^2 >0.89) when silicone samples were assessed, although the indirect methods resulted in a large overestimation of the burst pressure. The correlation between the three mechanical tests was poor (all R^2<0.18) when arterial and venous samples were investigated. Freezing and subsequent thawing prior to testing had no impact on the mechanical properties of the vessels. Strain rates within the strain rate window provided by the ISO 7198 (50-200mm/min), likewise, had no impact on the outcome of the tests. The circumferential tensile strength nor the probe burst pressure are predictive of the pressurized burst pressure of biological vascular tissue. Unless explicitly validated in a testing system on a range of biological tissues, the derived methods should not be utilized for the evaluation of the burst pressure of biological tissue engineered blood vessels for clinical purposes.

Introduction

Tissue engineered blood vessels (TEBVs) have great potential to offer a better alternative for synthetic grafts that are currently used in vascular surgery [1]. One of the most vital aspects of TEBVs is the ability to withstand the mechanical forces induced by arterial blood pressure. Therefore, it is vital to evaluate the mechanical properties of an engineered vessel prior to clinical use. The ISO 7198 provides an overview of tests to be carried out to evaluate the mechanical properties of vascular prosthesis [2]. There are three methods listed in the ISO 7198 that could be utilized to assess the mechanical strength of a vessel; one direct method; the pressurized burst pressure, and two indirect methods; the circumferential tensile strength (CTS), and the probe burst pressure. The latter two methods require only a small piece of tissue (5mm, and 15mm respectively), which is a major advantage for in situ engineered vascular grafts, as the tissue size that can be excised for mechanical analysis is limited. The pressurized burst pressure is considered the gold standard, but this test requires a much longer tissue segment (>40mm). In testing of vascular prosthetics, the pressurized burst pressure, CTS, and probe methods are used interchangeably, and numerous studies provide mechanical testing data carried out according to the ISO 7198 [3-12]. However, the ISO 7198 was primarily developed for vascular prosthesis, and it is unclear to what extent its testing modalities carry over to biological tissue, due to the large variation to be inherent to such tissues.

It has previously been reported that the CTS is likely to overestimate the burst pressure of a given sample [3-5]. Previous studies have shown that with an adaptation of Barlow's formula (**Eq. 1**), the circumferential tensile strength of a grouped sample could be used to estimate of the burst pressure of a vessel [5]. Barlow's formula states, that the burst pressure of a cylindrical pipe may be computed from its wall thickness (t), its outside diameter (D_0) and its yield stress (σ_y) as

$$P = \frac{2\sigma_Y t}{D_0}. \quad (1)$$

However, it is still not known if there is an accurate correlation between the three testing modalities if a biological single sample were to be assessed. For applications such as mandrel based *in situ* TEBV, the burst pressure value must be evaluated precisely per patient using a small sample segment, as only a single sample is available [13-15].

The burst pressure may be influenced by the rate at which the sample is strained [16]. The ISO 7198 provides a window of strain rates that may be implemented. In this study, the impact of the strain rate on the measured burst pressure was evaluated. In addition, the impact of short-term freezing on the mechanical properties of the vessels was assessed.

The main objective of this study is to evaluate if the CTS and probe burst pressure methodologies currently used in literature provide an accurate and consistent estimation of

the pressurized burst pressure. Moreover, the validity of these methods for the assessment of biological samples is evaluated.

Methods

Study design and sample preparation

A custom system was built to analyze the pressurized burst pressure, CTS, and probe burst pressure according to the ISO 7198 (**Fig. 1**). Initially, the system was assessed using silicone tubing (J. Lindemann GmbH, Helmstedt, Germany), to determine the accuracy of the system. Silicone tubing with a wall thickness of 0.5, 1, 1.5, 2, and 3 mm were tested to assess the variance of the system. Different strain rates, within the window provided by the ISO 7198 guidelines, were used to assess the impact of strain rate on the measurement outcome. Following validation of the testing set-up using silicone tubing, bovine arteries and veins were evaluated. Carotid arteries and jugular veins of 8-month-old bovine calves were collected at the abattoir. Samples were at least 9cm in length and were cut using a custom-made cutting device (**suppl. Fig. 1**) to obtain standardized segments of 40 mm, 15 mm and 5 mm long, for the pressurized burst pressure, probe burst pressure and circumferential tensile strength test, respectively. Care was taken to remove only the peri-adventitial tissue from the samples in a uniform manner by a single technician. The samples were transported in DMEM medium supplemented with 1x penicillin/streptomycin. The samples were frozen at -20°C for a maximum of 7 days prior to mechanical testing. Two and a half hours before mechanical testing the samples were placed at 7°C for 2 hours, then at 37°C for 30 minutes as described by O'Leary et, al [17]. A subset of samples was not frozen to assess the impact of freezing on the mechanical tests. Care was taken to exclude any vessels with branches on the test location from the experiment. The number of samples used for each test can be seen in **figure 1**. During all tests the samples were kept at 37°C using a custom-made perfusion system. The samples were cleaned and tensioned to 60g before being cut using a custom designed device to allow all samples to be cut exactly equal. A 40mm segment was taken for the pressurized burst pressure, a segment with a length of 15 mm was taken for the probe burst pressure, and remaining 5mm segments were used for the CTS. Silicone control samples had an internal diameter of 6mm and a wall thickness of 0.5, 1, 1.5, 2, and 3mm. Finally, to verify freezing has no impact on the measurement outcome, segments of 8 samples were tested when unfrozen and frozen for 1, and 7 days.

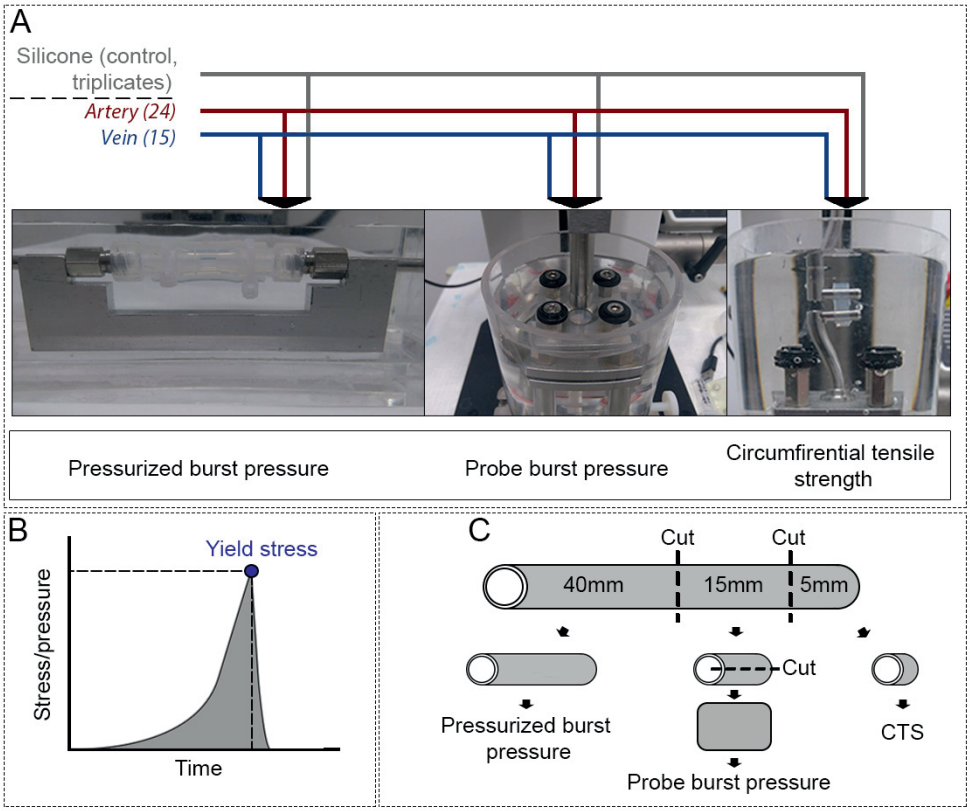

Figure 1. A) An overview of the samples and the tests done on the samples. B) graphical representation of the yield stress. C) The segmentation of the sample vessel used for the analysis.

Pressurized burst pressure

For the pressurized burst pressure, the vessel segment was placed between two Luer connectors and fastened using surgical vessel-loops and placed in a pressurized perfusion system (**Fig. 1a**). Phosphate buffered saline (PBS) was used to pressurize the vessel. Both the PBS surrounding the vessel and the PBS used for perfusion was kept at 37ºC at all times. The internal pressure was measured using a pressure gauge (Fluke, Washington, USA), and pressure could be induced using a 50ml syringe pump connected to a tensile tester (Mark-10, Washington, USA). The vessel length was tensioned to 60g during the tests, whereafter the pressure was increased in a steady state until vessel rupture. Care was taken to assure the rupture occurred away from the Luer connectors.

Circumferential tensile strength

The CTS was measured by inserting a small ring segment of tissue between two pins in a custom-made testing set-up (**Fig. 1b**). One of the pins is then moved away until sample failure. The ring segment was submerged in 37°C PBS during the entirety of the tests. The system was connected to a tensile tester, which could control pin movement and register

force using a load cell (Series-5, Mark-10, Washington, USA) capable of registering at 7000 Hz. The CTS is a means to determine the yield stress σ_y (in units of Pa), which is related to the load at failure (F_{CTS}) as **Eq. (2)**. **Eq. (1)** and **Eq. (2)** are then combined to give **Eq. (3)**, which can be used to estimate the yield stress. In this equation, *P* is the measured internal pressure in units of Pa, F_{CTS} is the force at failure, in units of N, during the test, L_0 is the initial length (m) of the sample and D_0 (m) is the initial diameter. , previously defined, is the wall thickness in m.

$$\sigma_y = \frac{F_{CTS}}{2L_0 t}, \tag{2}$$

$$P = \frac{F_{CTS}}{L_0 D_0}. \tag{3}$$

To investigate the impact of the initial elastic modulus on the strength of a vessel, the stress-strain curves, the Young's modulus (**Eq.4**) was calculated, whereupon the correlation between the 3 mechanical tests where adjusted for the Young's modulus.

$$E = \frac{FL_0}{A\Delta L}. \tag{4}$$

Probe burst pressure

The probe burst pressure was measured by cutting open a vessel segment and wedging it between two plates in a custom-made testing system (**Fig. 1a**). The plates have a central cavity through which a pin can protrude, exerting a strain on the vessel. The system was connected to a tensile tester which controlled the pin and could record the measured force. The central pin was pressed into the vessel until rupture. The yield stress was recorded, and the burst pressure was estimated using **Eq. (5)**, in which A_{sample} (m^2) is the initial surface area of the sample.

$$P = \frac{F}{A_{sample}} \tag{5}$$

Statistical analysis

The data are presented as mean ± standard deviation (SD). Correlation was assessed using linear regression analysis. Comparison of the burst pressure assessment modalities for arteries and veins were analysed using a repeated measured one-way ANOVA with Tukey

post-hoc analysis using GraphPad Prism 7.02. P-values < 0.05 were considered statistically significant.

Results

Method validation using silicone control samples

To validate the system, various thicknesses of silicone tubing were used to compare the testing modalities at different strain rates. The accuracy of the tests is evidenced by the low variation within the tests (**Fig. 2**). All three tests correlated well with each other as illustrated in **figure 3**. Although the probe burst pressure correlated well with the CTS and the pressurized burst pressure, both the probe burst pressure and CTS overestimated the burst pressure substantially when compared to the pressurized burst pressure. Using **Eq. (3)** and **Eq. (4)** to transform CTS and probe burst pressure respectively gives an overestimation of the pressurized burst pressure for both tests. A force of 100N would translate into a pressure of 2.5·10^4 and 4.9·10^3 mmHg for the CTS and probe burst pressure respectively.

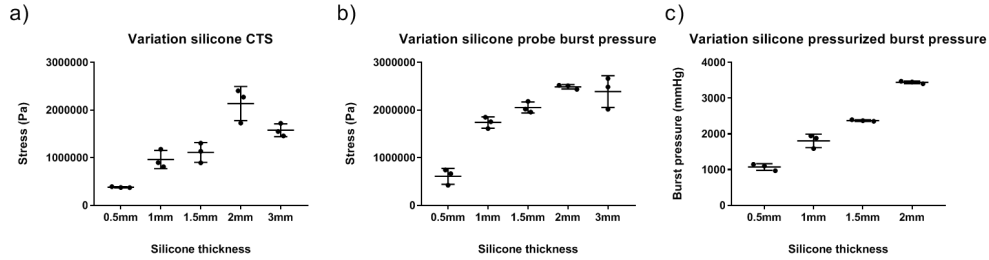

Figure 2. Variation in burst pressure of silicone tubing of different thickness using 3 testing modalities; a) CTS, b) Probe burst pressure, c) pressurized burst pressure. Only the 100mm/min strain speed is shown.

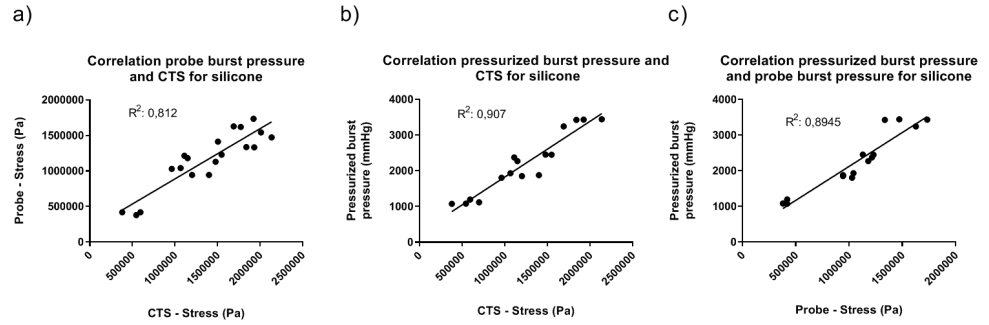

Figure 3. Correlation between the burst pressures of silicone tubing using the 3 testing modalities.

To assess if the strain rate within the window provided by the ISO 7198 (50-200mm/min) impacts the burst pressure, different rates were assessed and compared. We find, that the strain rate did not impact the measurements, and that the pressurized burst pressure measurements were particularly insensitive to changes in strain rate (**Fig. 4**).

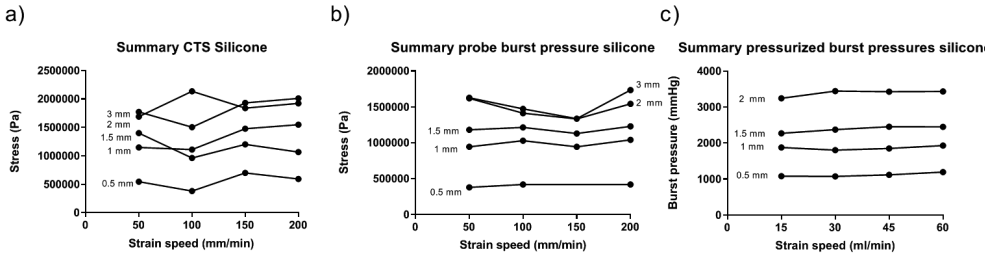

Figure 4. Impact of strain rate on burst pressure obtained by a) the CTS, b) the probe bust pressure, and c) the pressurized burst pressure test. The strain rate for the pressurized burst pressure has a different scale as the strain rate needed to be adjusted to the speed at which fluid was compressed.

Comparison of the tests using biological samples

To assess if these mechanical tests are also applicable for the assessment of biological tissue, bovine arterial and venous samples were tested using all three testing modalities (**Fig. 5**). The within-sample variation for the CTS and probe burst pressure for arterial samples was low (**Fig. 6**). The within-sample variation of the pressurized burst pressure could not be assessed due to insufficient sample length. There was no significant correlation between the tests for the arterial and venous samples (**Fig. 7**). For the arterial samples, the CTS overestimate the pressurized burst pressure. For the venous samples, both the CTS and probe burst pressure overestimate the directly determined pressurized burst pressure. Adjusting the data for the initial elastic modulus had no impact on the correlative capacity of the data (**suppl. Fig. 2**), suggesting that the elastic modulus of the does not explain the variability between tests.

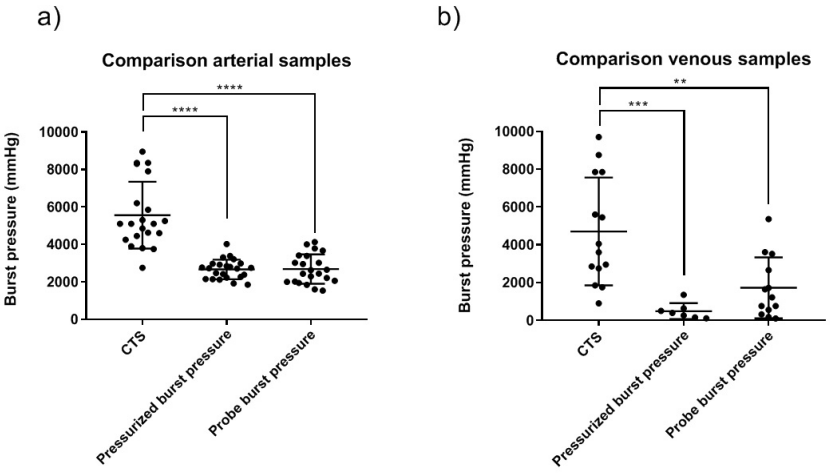

Figure 5. Burst pressure of a) arterial and b) venous samples using the three testing modalities. P < 0.005: **, P < 0.0005: ***, P < 0.0001: ****.

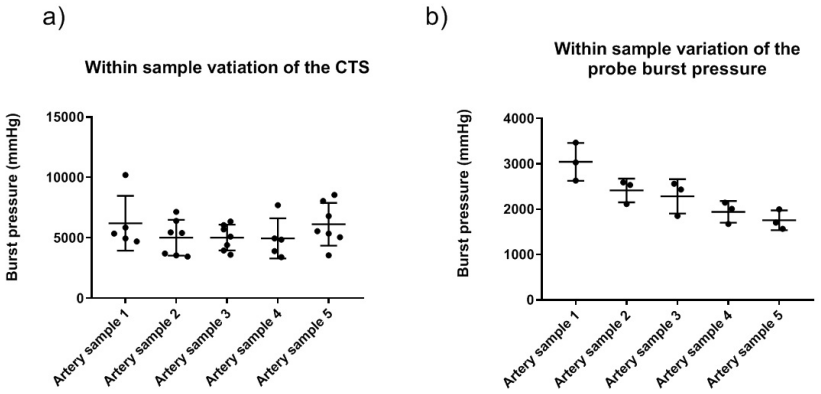

Figure 6. Variation in the CTS (a) and probe burst pressure (b) measurements using arterial samples. The variation of the pressurized burst pressure measurement could not be attained due to insufficient sample length.

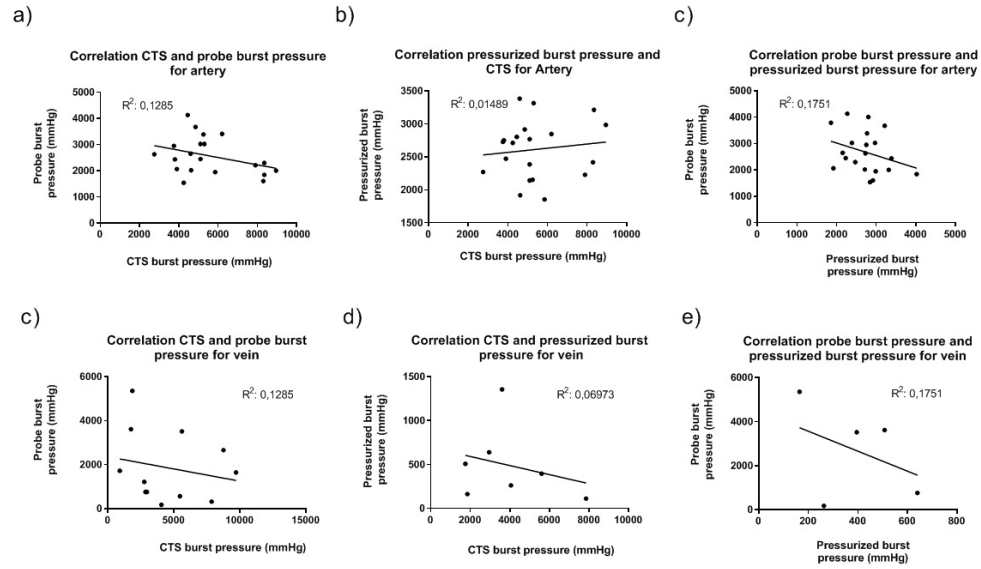

Figure 7. Correlation between testing modalities for arterial (a-c) and venous (d-f) samples. The correlation between the three mechanical tests was very poor.

The impact of freezing on biological samples

To confirm that short term freezing did not impact the results, various samples were assessed for the impact of freezing on the test outcome. The CTS was chosen to assess the impact of short term freezing as this would allow the most measurements to be done on a single sample. No difference between the groups was observed (**Suppl. Fig. 3**).

Discussion

The present study revealed that the CTS and probe burst pressure provide an overestimation of the pressurized burst pressure in both biological and synthetic material. In addition, the outcomes of the three test modalities evaluating the mechanical properties of different types of biological blood vessels correlate poorly with each other. The experiments using silicone tubing validated the experimental set-up with regard to all three tests as seen from the high correlation between the tests for all measurements and very low within sample variations. A low within sample variation is seen for all three tests, especially for the pressurized burst pressure, suggesting this is the most accurate of the tests.

For biological samples, the CTS significantly overestimated the pressurized burst pressure. When evaluating arterial and venous samples, CTS results resulted in a 2-fold and 10-fold higher estimated burst pressure when compared to the pressurized burst pressure respectively. Moreover, the burst pressures obtained with these two tests modalities did not correlate with each other, despite the low variation within the individual biological samples. The question arises why the different tests have such a poor correlation, whereas previous studies by Laterreur et al [5] revealed that the CTS was predictive of the pressurized burst pressure when using a grouped analysis, which is already not predictive for single sample comparison. Differences in collagen alignment between samples might explain the observed variation between samples. However, such difference does not necessarily explain the poor correlation between the 3 tests. In this respect, it is importance to mention that the pressurized burst pressure identifies the weakest spot of a vessel segment whereas both the CTS and probe burst strength measure the average yield stress of a vessel segment. Consequently, the poor correlation between the 3 tests might be explained by the fact that as they measure different aspects of the mechanical characteristics of blood vessels, the tests would be likely not to correlate with one and other. For the pressurized burst pressure, the forces are equally distributed along the vessel, yet a single week spot may incur higher forces. Barlow's formula assumes uniform circumferential stress in the vessel wall, yet soft cylinders often fail in a non-uniform manner, at a localized bulge that may experience far higher local stresses. Thus, the yield strength of the material is exceeded locally long before it would do so globally if stresses were distributed homogenously. Thus, the pressurized burst pressure is mainly a measure of the yield stress of a vessel at its weakest point. The yield stress of the probe burst pressure, on the other hand, assumes a flat sample and evenly distributed load, while neither assumption remains valid when the sample is close to failure. This effect, likewise, yields an inaccurate estimate of the burst pressure of a sample. Like the probe burst strength, the CTS assumes an evenly distributed load while not taking into account the weakest point in a vessel. Where the pressurized burst pressure measures the weakest point of a vessel, the CTS disregards this and measures the average yield stress. Thus, although the literature suggests that an estimation of the burst pressure can be made using the derived methods, both the CTS and probe burst strength are inherently likely to overestimate the yield stress of a vessel compared to the pressurized burst pressure if a sample is not perfectly homogeneous.

As seen in the silicone control, a perfectly homogeneous vessel provides a high correlation between all tests, and the indirect methods can indeed be used to predict the pressurized

burst pressure. Biological samples are inherently inhomogeneous, and the lack of correlation between the tests confirms this. Thus, a first conclusion that can be made is that the three tests correlate well and could be predictive with each other so long as perfect homogeneity can be assumed. This excludes the use of the indirect methods for biological tissue assumed to have any slight inhomogeneity.

Moreover, estimating to the pressurized burst pressure using the CTS or probe burst pressure with **Eq. (3)** and **Eq. (5)** respectively gave high overestimations for both silicone and biological samples. Therefore, despite homogeneity of the vessel, as in the silicone control, the indirect methods cannot be accurately recalculated to estimate the pressurized burst pressure using these formulas.

If an indirect method is implemented for the mechanical assessment of biological tissues, the correlative capacity between the indirect method and the direct pressurized burst pressure must first be assessed. When a high correlation is observed, subsequent measurements can be estimated. If no correlation is observed for the biological tissue, as in this study, the direct pressurized burst pressure must be used.

The ISO 7198 was meant for the mechanical assessment of vascular prosthetics. Often, two indirect methods of burst pressure assessment, the CTS and probe burst pressure, are implemented for the assessment of TEBVs. Vascular prosthesis are capable of exhibiting far greater homogeneity than biological vessels due to their composition. Most vascular grafts materials such as ePTFE are mainly composed of a single material. Being composed of numerous cell types, and extracellular components, biological vessels are inherently heterogeneous. Collagen is mainly responsible for the strength of the vessel[18]. It is known that the alignment of collagen is vital in determining the mechanical properties it elicits. In aneurysm formation, for example, the incorrect alignment of collagen leads to the integrity of the vessel wall being compromised[19]. Although TEBVs theoretically have the ability to be more homogeneous than biological tissues, biological variation will remain an issue.

The variation of the pressurized burst pressure for the arteries and veins is unknown due to a lack of sample length. Although the silicone data may indicate that the pressurized measurements have a low variation, this cannot be said for certain for the biological vessels.

Conclusion

The indirect methods can be used to estimate the pressurized burst pressure as long as perfect homogeneity of the sample can be assumed, and high correlation is validated. Transforming the CTS and probe burst pressure values to mmHg directly should not be done as this provides potentially large overestimations of the pressurized burst pressure, partly as these indirect methods are inherently prone to providing a higher yield stresses than the direct pressurized burst pressure. Indirect methods of burst pressure assessment should not be used for the clinical evaluation of TEBVs unless perfect homogeneity is assumed, and a high correlation is validated.

Acknowledgements

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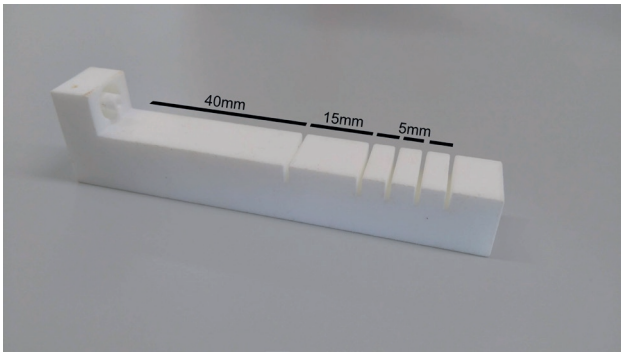
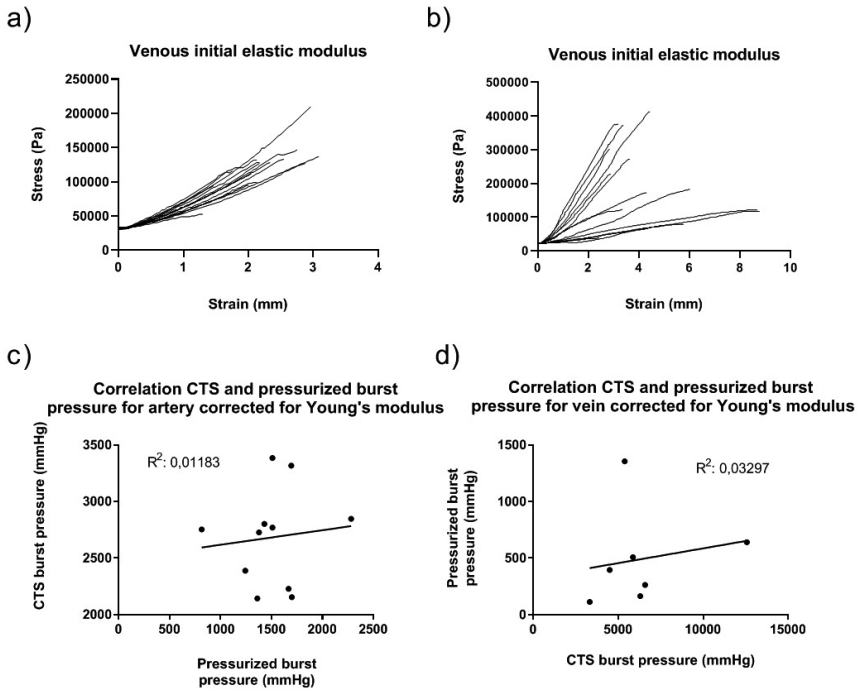
Supplementary materials



Supplemental figure 1. A custom made cutting device to assure each piece is cut precisely to the correct length



Supplemental figure 2. a + b) the initial elastic modulus of the stress-strain curves of the arterial and venous samples. c + d) the adapted correlation between the CTS and pressurized burst pressure for arterial and venous samples.

A novel method for engineering autologous non-thrombogenic in si[illegible] tissue-engineered blood vessels for arteriovenous grafting

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ABSTRACT

The durability of prosthetic arteriovenous (AV) grafts for hemodialysis access is low, predominantly due to stenotic lesions in the venous outflow tract and infectious complications. Tissue engineered blood vessels (TEBVs) might offer a tailor-made autologous alternative for prosthetic grafts. We have designed a method in which TEBVs are grown *in vivo*, by utilizing the foreign body response to subcutaneously implanted polymeric rods in goats, resulting in the formation of an autologous fibrocellular tissue capsule (TC). One month after implantation, the polymeric rod is extracted, whereupon TCs (length 6 cm, diameter 6.8 mm) were grafted as arteriovenous conduit between the carotid artery and jugular vein of the same goats. At time of grafting, the TCs were shown to have sufficient mechanical strength in terms of bursting pressure (2382 ± 729 mmHg), and suture retention strength (SRS: 1.97 ± 0.49 N). The AV grafts were harvested at 1 or 2 months after grafting. In an *ex vivo* whole blood perfusion system, the lumen of the vascular grafts was shown to be less thrombogenic compared to the initial TCs and ePTFE grafts. At 8 weeks after grafting, the entire graft was covered with an endothelial layer and abundant elastin expression was present throughout the graft. Patency at 1 and 2 months was comparable with ePTFE AV-grafts. In conclusion, we demonstrate the remodeling capacity of cellularized *in vivo* engineered TEBVs, and their potential as autologous alternative for prosthetic vascular grafts.

1. Statement of significance

Tissue engineered blood vessels are considered a promising alternative for prosthetic vascular grafts as arteriovenous vascular access for hemodialysis. In a goat model of arteriovenous grafting, we have demonstrated sufficient mechanical characteristics of in situ engineered vascular grafts to allow safe vascular grafting with similar patency rates compared to ePTFE AV-grafts. This indicates that in situ TEBVs are a robust vascular graft, with the potential to be used in as arteriovenous vascular access for hemodialysis.

2. Introduction

The high[illegible] of vascular access sites contributes to the rising cost of renal replacement [illegible] a growing issue when combined with the increasing prevalence of end-[illegible] disease [3]. Autologous vessels offer the best patency rates with regard to [illegible] grafting. However, preexisting disease, previous harvesting, and unsuitability for a vascular procedures limits their use [4]. Synthetic options however, are prone to infection, intimal hyperplasia (IH), and/or thrombosis [5,6]. Thus, tailor-made tissue-engineered blood vessels (TEBVs) may serve as a viable alternative to synthetic grafts.

A wide range of tissue-engineering approaches have been suggested for replacing synthetic grafts [7]. One such method involves the so-called mandril-based approach, which relies on the foreign body response (FBR) driving the development of an autologous *in vivo* cellularized vascular substitute surrounding an implanted medical device [8]. Using both *in vivo* [9,10] and *in vitro* [11] approaches, we previously reported that the implant's surface characteristics can influence the resulting FBR, thereby affecting the composition of the tissue that forms around the implant. Based on these studies, we found that subcutaneously implanted polymeric rods can elicit an FBR, resulting in the encapsulation of the rods with a fibrocellular tissue capsule that can [illegible] grafted into the vasculature as a carotid interposition [illegible]

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Chapter 4

A novel method for engineering autologous non-thrombogenic *in situ* tissue-engineered blood vessels for arteriovenous grafting

W.J. Geelhoed, K.E.A. van der Bogt, T.C. Rothuizen, F.F.R. Damanik, J.F. Hamming, C.D. Mota, M.S. van Agen, H.C. de Boer, M. Tobón Restrepo, B. Hinz, A. Kislaya, C. Poelma, A.J. van Zonneveld, T.J. Rabelink, L. Moroni and J.I. Rotmans

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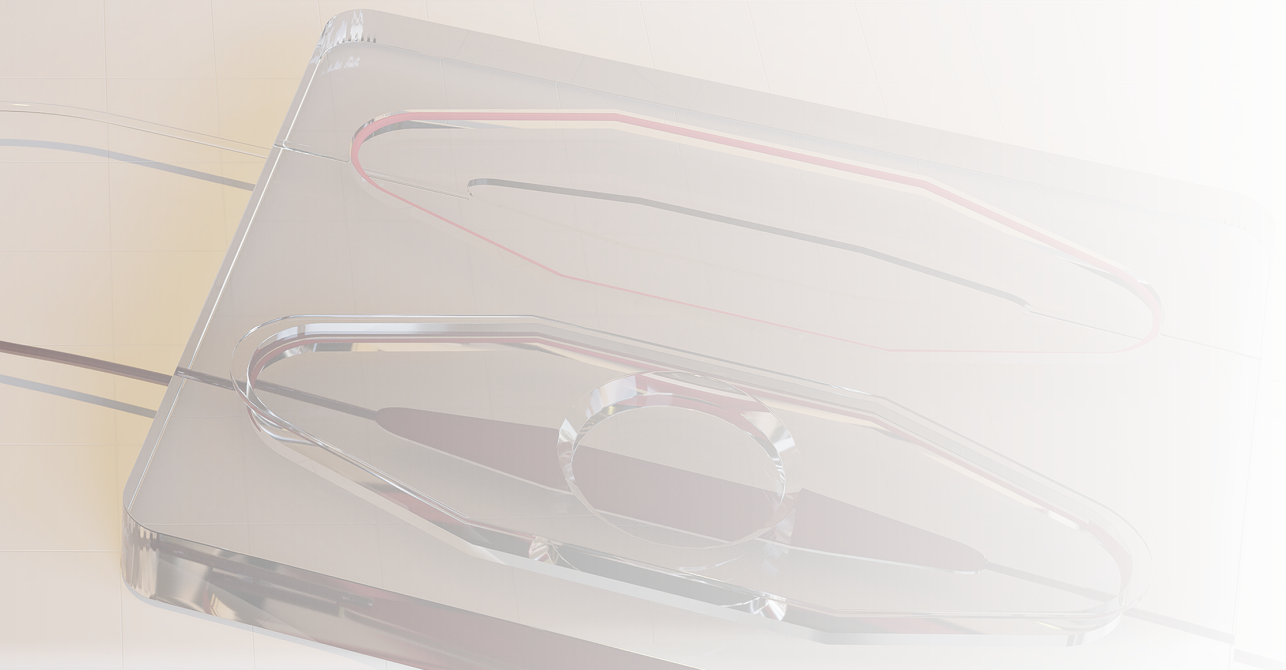

Abstract

The durability of prosthetic arteriovenous (AV) grafts for hemodialysis access is low, predominantly due to stenotic lesions in the venous outflow tract and infectious complications. Tissue engineered blood vessels (TEBVs) might offer a tailor-made autologous alternative for prosthetic grafts. We have designed a method in which TEBVs are grown in vivo, by utilizing the foreign body response to subcutaneously implanted polymeric rods in goats, resulting in the formation of an autologous fibrocellular tissue capsule (TC). One month after implantation, the polymeric rod is extracted, whereupon TCs (length 6 cm, diameter 6.8 mm) were grafted as arteriovenous conduit between the carotid artery and jugular vein of the same goats. At time of grafting, the TCs were shown to have sufficient mechanical strength in terms of bursting pressure (2382 ± 129 mmHg), and suture retention strength (SRS: 1.97 ± 0.49 N). The AV grafts were harvested at 1 or 2 months after grafting. In an ex vivo whole blood perfusion system, the lumen of the vascular grafts was shown to be less thrombogenic compared to the initial TCs and ePTFE grafts. At 8 weeks after grafting, the entire graft was covered with an endothelial layer and abundant elastin expression was present throughout the graft. Patency at 1 and 2 months was comparable with ePTFE AV-grafts. In conclusion, we demonstrate the remodeling capacity of cellularized in vivo engineered TEBVs, and their potential as autologous alternative for prosthetic vascular grafts.

Introduction

The high failure rate of vascular access sites contributes to the rising cost of renal replacement therapy[1,2], a growing issue when combined with the increasing prevalence of end-stage renal disease[3]. Autologous vessels offer the best patency rates with regard to vascular grafting. However, preexisting disease, previous harvesting, and unsuitability for a vascular procedures limits their use[4]. Synthetic options however, are prone to infection, intimal hyperplasia (IH), and/or thrombosis[5,6]. Thus, tailor-made tissue-engineered blood vessels (TEBVs) may serve as a viable alternative to synthetic grafts.

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Here, we report the generation of functional TEBVs using a goat model of vascular grafting, and we describe the dynamic properties by which grafted TEBVs remodel to produce a suitable vascular phenotype. The arteriovenous grafting model was chosen as it allows the functional assessment of TEBVs in terms of vascular remodeling and patency in a rigorous setting.

Methods

Study design

All experiments were approved by the Animal Care and Use Committee of the University Medical Center Utrecht and were performed in accordance with Dutch and European legislation. The study included 17 adult female Dutch milk goats that were randomly divided into a TEBV group (n=8) and an ePTFE control group (n=9). Each animal received nadroparin (15,000 IU SID) starting the day after vascular grafting and clopidogrel (150 mg SID) after a loading dose of 300 mg beginning 7 days prior to grafting and continuing until the animal was sacrificed. One day prior to surgery, the goats received a buprenorphine patch (5 μg/hr delivery rate) and were fasted overnight. For the TEBV group, two cylindrical-shaped polymer rods were implanted subcutaneously in the neck. The neck was chosen as an implantation site as this allowed good surgical access to the carotid artery and jugular vein, from which an arteriovenous conduit could be grafted. In addition, implantation in the neck provided the option to check patency of the arteriovenous graft manually. One month later, the rods with tissue capsules grown around them were harvested, and one tissue capsule

was grafted as an arteriovenous conduit between the carotid artery and jugular vein in the same goat. The cylindrical shaped tissue that forms around the implant is considered a tissue capsule, while it is considered a TEBV as soon as it is grafted into the vasculature. The other tissue capsule was used for histology, mechanical testing, thrombogenicity assays, and cell culture experiments. In three animals, an additional rod was implanted during the grafting operation to ensure that one month after grafting a TEBV, TC, and artery could be harvested for use in the thrombogenicity assay (see **Fig. 1**).

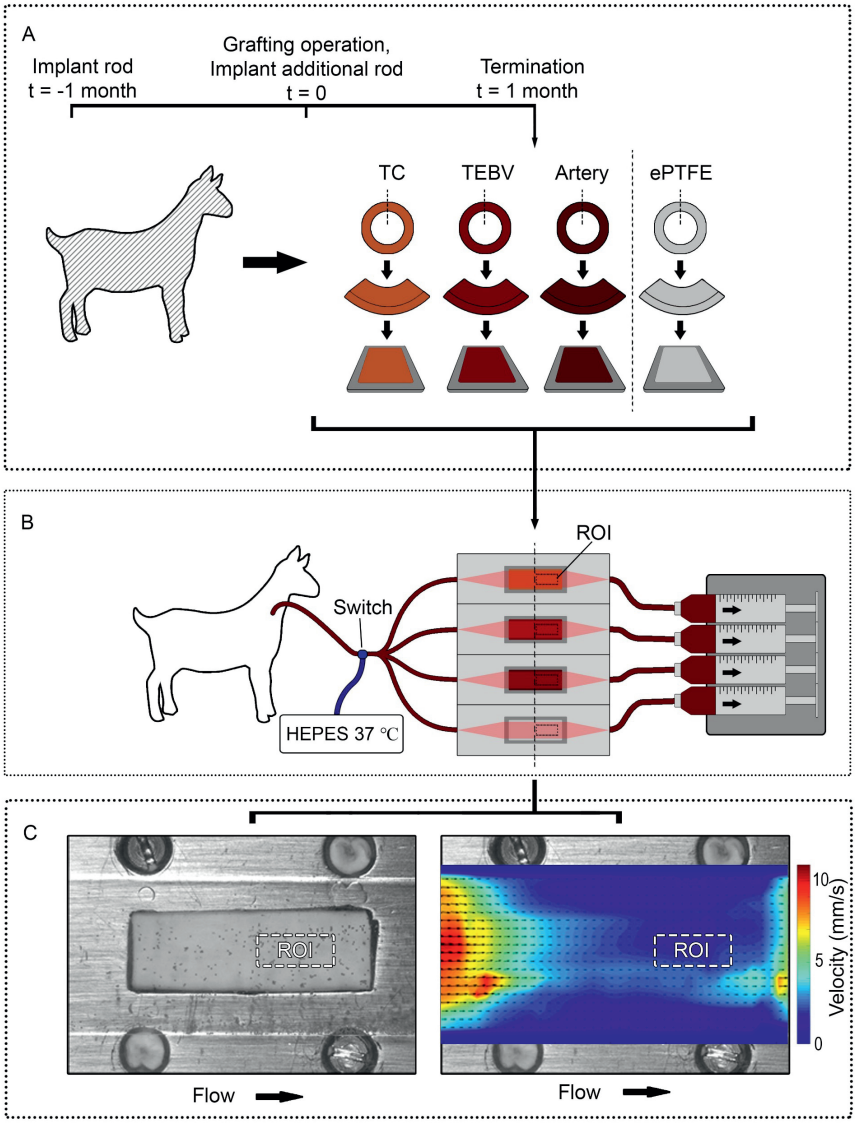

Figure 1. Schematic overview of the experimental setup used for the thrombogenicity assay performed on explanted samples. **A)** In 3 animals, an additional rod was implanted during the grafting surgery (t=0); at t=1 month a TEBV, TC, and artery were harvested from a single animal. A sterile new piece of ePTFE was used as a control. The vessel samples were cut open and placed longitudinally in a sample holder chip with the sample's lumen exposed. The samples were immersed in HEPES buffer at all times during handling. **B)** The sample holders were placed in parallel in a specially designed ex vivo perfusion chambers. Blood from a second animal was perfused in parallel over the ROI in the chamber at a rate of 12.5 ml/min, with blood being drawn from the animal into and into the syringes. After a total volume of 6.25 ml had perfused over the chamber, 37°C HEPES was automatically perfused over the samples at 12.5 ml/min to wash the samples. **C)** Left: Image of tracer particles flowing through the chamber for particle image velocimetry. Right: Pseudocolor image showing the velocity fields generated by the samples; note that the flow is homogeneous at the ROI.

Patency of the graft was monitored weekly using ultrasonography with a CX50 ultrasound machine (Philips, Eindhoven, the Netherlands). One and two months after grafting, 5 and 3 animals, respectively, were euthanized and the grafts were removed for histological analysis, thrombogenicity assays, and cell culture experiments. Pre-grafted tissue capsules were compared to post-grafted tissue capsules for their ability to remodel within the vasculature. In contrast, the control group received a 6-mm ePTFE graft placed between the carotid artery and jugular vein; this graft was left in place for one or two months (n=6 and n=3, respectively), after which the animals were euthanized and the grafts were removed for histological analysis and thrombogenicity assays.

Production of polymer rods

Cylindrical solid rods (6 mm in diameter and 6 cm in length) were fabricated using a specially designed mold at the MERLN Institute for Technology-Inspired Regenerative Medicine, Maastricht, the Netherlands. The rods were composed of the co-polymer poly(ethylene oxide terephthalate)–poly(butylene terephthalate) (PEOT/PBT; PolyVation, Groningen, the Netherlands) with a weight ratio of PEOT/PBT of 55/45 and an initial molecular weight of 300 g/mol. In brief, polymer pellets were heated to 165°C, injected into the mold, and allowed to gradually cool before removing the rods. The rods were then cleaned with 70% ethanol, the tips were rounded in order to facilitate implantation, and the surface of the rods was modified as described previously[9,10]. The rods were briefly submerged in chloroform, washed, and then dried under nitrogen gas. The surface topography of the rods was confirmed using SEM analysis. The rods could not be broken with the application of manual pressure. Due to a single faulty polymer batch, rod breaking was an issue in a subset of implants, these rods were excluded from the analysis performed here.

Surgical procedures

Two polymeric rods were implanted in the neck of each goat by making a small incision and bluntly preparing a subcutaneous pocket approximately 9 cm in length. For the animals used in the thrombogenicity assay (n=3), an additional rod was implanted during the vascular grafting surgery. Each rod was secured to the surrounding subcutaneous tissue using 2-0 Prolene sutures at both ends. One month after implantation, the neck was opened via a midline incision and the polymer rods—including the surrounding tissue capsules—were removed by gentle dissection, after which the rod was extruded easily from the tissue. The integrity of the tissue capsule was confirmed by inflation with saline, and the tissue capsule was then implanted as an end-to-side arteriovenous conduit between the carotid artery and the jugular vein using running 6-0 Prolene sutures. Flow was measured at the time of grafting and at termination using a perivascular probe. In a subset of animals, an angiogram

was performed using an Allura Xper FD20 angiograph (Phillips, Eindhoven, The Netherlands) in order to confirm post-surgical positioning of the TEBV.

Mechanical testing of tissue capsules

All mechanical tests were performed in accordance with ISO-7198-1998 guidelines[13]. To measure suture retention strength, a suture was placed at three locations at a depth of 2 mm in the tissue using 5-0 Vicryl (Ethicon, Johnson & Johnson) and pulled at a steady rate of 5.0 mm/min; the maximum force applied was recorded using a Kern 5K5N hanging scale (Kern, Balingen, Germany) with a resolution of 0.05 N. Prior to grafting, dynamic compliance was measured for a tissue capsule 6 cm in length, which was strained with a load of 0.5 N and placed in a specially designed compliance measurement system at 37±1°C; the sample was then subjected to a pulsatile pressure of 120/80 mmHg at 1 Hz. Pulsations were recorded using a high-definition microscope camera with an accuracy of ±0.02 mm, while simultaneously using a TruWave pressure transducer (Edwards Lifesciences, Irvine, CA) and a model 700G pressure gauge (Fluke, Eindhoven, the Netherlands). The temperature was recorded using a model PCE-779N infrared thermometer (PCE instruments, Enschede, The Netherlands). Compliance of each vessel was determined by combining the high-definition images with the corresponding pressure data. To determine the vessel's bursting pressure, a 5-cm tissue capsule was perfused with saline at 37±1°C with steadily increasing pressure until the graft ruptured (**Suppl. Video S1**). Methylene blue was added to the saline solution in order to accurately determine the burst location and to confirm that the rupture did not occur at the vessel connectors. All ruptures occurred away from the connectors.

Sample preparation and histology

For histology and immunohistochemistry, tissues were fixed in 4% formaldehyde, processed, and embedded in paraffin. Cross-sections (5 μm thickness) were cut from the tissue capsule before grafting, 1 month after grafting, and 2 months after grafting; these sections were used for thrombogenicity assays. Picrosirius red–stained sections were analyzed for the presence of collagen. A hematoxylin phloxine saffron (HPS) stain was used to assess cellular alignment. To examine cellular composition, immunohistochemistry was performed using the following antibodies: α-smooth muscle actin (α–SMA, Dako, Amstelveen, the Netherlands; 1:1000) for myofibroblasts; vimentin (Immunologic, Duiven, the Netherlands; 1:100) for fibroblasts; desmin (Immunologic; 1:100), myosin (smooth muscle) heavy chain (Alfa Aesar, Karlsruhe, Germany; 1:200) for contractile vascular smooth muscle cells (VSMCs); Calprotectin (Bio-Rad, Munich, Germany; 1:100) for macrophages; and eNOS (BD, Vianen, the Netherlands; 1:2500), and Elastin (Sigma-Aldrich, Houten, the Netherlands; 1:2500) for endothelial cells. Each antibody was visualized using 3,3'-diaminobenzadine (DAB), and the sections were counterstained with hematoxylin. Where necessary, antigen retrieval was performed using heat-induced 0.1% trypsin. Fluorescence and bright-field images of the stained sections were obtained using a panoramic slide scanner (3DHistec, Budapest, Hungary). All stained sections were compared to their respective isotype controls and control tissues. To quantify the staining, the percentage of DAB positivity relative to the total area (for fibroblasts, myofibroblasts, and smooth muscle cells) was quantified using ImageJ software with three histological sections per sample. For each sample, a minimum of three histological sections (HPS staining) near the venous anastomosis were used to assess stenosis, in which the lumen surface area was compared (as a percentage) to the area of the original lumen.

Contractility assay

Primary VSMCs from a carotid artery, VSMC-like cells from a TEBV, and fibroblasts/myofibroblasts from a tissue capsule were isolated from two animals. If present, the endothelial monolayer was detached by gently scraping with sterile surgical forceps. Care was taken to ensure that the tissue from the tunica media of the artery and the luminal tissues of the TEBV and tissue capsule were isolated. The samples were dissected into small pieces and plated on 60-cm Petri dishes pre-coated with collagen (Purecol, 10 μg/ml). After 14 days in culture with DMEM/F12 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin, the cells were trypsinized and re-plated on 10-cm Petri dishes and cultured for an additional 7 days. At approximately 80% confluence, the cells were trypsinized and used for the contractility assay. The HFF1 cell line was used as a control and was cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For each sample, 20,000 cells were plated in triplicate on silicone elastomer dishes containing DMEM/F12 medium supplemented with 1% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Deformable silicone elastomer (5 kPa) 24x24 mm Petri dishes were coated overnight with collagen in serum-free DMEM/F12 prior to use. Twenty-four hours after the cells were plated, bright-field microscopy images were taken of ten random fields of view at 100x magnification. Sample contractility was determined by counting the number of contractile cells per field of view and is expressed relative to the total number of cells, as described previously[14]. Cell contractility was determined by the presence of visible deformations in the elastomer beneath the cells.

Thrombogenicity assay

One month after grafting, the animal was sacrificed and a 2-cm mid-graft section of the TEBV, the un-operated contralateral carotid artery, and an ungrafted tissue capsule were removed (n=3). Using great care, the samples were directly placed in 37°C HEPES buffer, and the tissue rings were cut open and placed into sample holders. Care was taken to ensure that the samples were not directly exposed to air. The sample holders were placed in a specially designed *ex vivo* perfusion chamber (**Fig. 1A**). A sterile new piece of ePTFE was used as a control. Blood was drawn from the jugular vein of a control animal that had received no therapeutics and was perfused at a rate of 12.5 ml/min over all samples in parallel using a syringe pump with a total volume of 6.25 ml (**Fig. 1B**). The flow system was designed to ensure that all samples received exactly the same volume of blood and flow rate. Moreover, this flow rate allowed a stable shear rate of 20 dyn/cm^2 to be attained in all chambers. After 30 s of perfusion, the samples were washed with 37°C HEPES buffer. The flow chamber was then opened, and each sample was divided into two sections. One section was washed in 37°C HEPES and fixed in 1% paraformaldehyde for confocal microscopy. The other section was washed in 37°C HEPES and fixed in glutaraldehyde for 2 days for SEM analysis. To examine luminal adhesiveness, SEM imaging was performed using an ROI in which the flow rate and shear stress were stable between samples based on our characterization experiments (**Fig. 1C**); thus, the same imaging location was used for all samples.

To investigate the flow characteristics of the *ex vivo* flow chamber, particle image velocimetry (PIV) was performed in separate experiments[15]. These measurements were performed using an *ex vivo* flow chamber that contained an ePTFE graft in the sample holder. A water-glycerol solution (with a density of 1100 kg/m^3 to mimic the viscosity of

whole blood) was perfused at 37° though the system to generate pressure-driven flow with a constant flow rate of 12.5 ml/min using an neMESYS pump (Cetoni, Korbussen, Germany). The solution was seeded with monodispersed polyethylene microspheres (106-125 μm diameter; Cospheric, Santa Barbara, CA). Images were recorded using a LaVision Imager sCMOS camera (sCMOS, 16-bit, 2560 x 2160 pixels, pixel pitch 6.5 μm). Image acquisition and PIV processing were performed using LaVision DaVis version 8.3 to create a flow field derived from the setup (**Fig. 1C**).

Statistical analysis

All summary data are presented as the mean ± the standard error of the mean. Data for the mechanical tests were analyzed using the Mann-Whitney *U* test using GraphPad Prism version 7.00. Differences with a *P*-value <0.05 were considered statistically significant.

Results

Formation of the TEBVs

The surface properties of the implanted rods were analyzed using scanning electron microscopy (SEM). Gamma irradiation did not affect the surface topography of the rods (data not shown). One month after implantation, a thick tissue capsule had formed around each implanted rod (**Fig. 2A**). This tissue capsule was easily removed from the rod, was easy to handle for surgical procedures, and was suitable for use in forming an arteriovenous conduit (**Fig. 2B**).

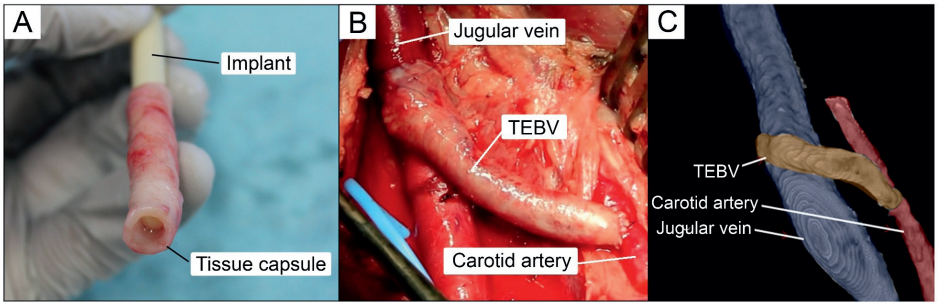

Figure 2. A) Example image of a tissue capsule grown around an implanted rod prior to grafting. B) A tissue capsule implanted as an arteriovenous conduit to form a TEBV. C) Example angiogram used to assess the position of the TEBV.

Mechanical properties of the TEBVs

The burst pressure, suture retention strength, and dynamic compliance of the TEBVs are summarized in **Fig. S1**. The burst pressure was 2382 ± 129 mmHg; although this is lower than the burst pressure of a native human artery, it is higher than the burst pressure of a native human vein and far exceeds physiological blood pressure levels[16]. The suture retention strength of our TEBVs was 1.97 ± 0.49 N, with little variance between samples;

this strength exceeds the requirements for safe grafting, which are generally defined by the mechanical properties of the saphenous vein[7,16].

Tissue capsule formation and remodeling to form a vascular phenotype

Prior to grafting, the tissue capsule is composed of extracellular matrix components (**Fig. 3**), fibroblasts, and myofibroblasts (**Fig. 4A-C**), with several macrophages lining the inner layer (**Fig. 3C**). Collagen is also abundant in the TC, as shown using picrosirius red staining (**Fig. 3D**). In contrast, the tissue capsule does not contain elastin (**Fig. 3A**) or an endothelial lining (**Fig. 3B**) prior to grafting. The eNOS-positive cells in the pre-grafted tissue capsule are attributed primarily to capillaries, confirming that the tissue capsule is well-vascularized (**Fig. 3**). Extremely few foreign body giant cells were observed; however, the presence of calprotectin-positive cells (**Fig. 3C**) indicates the presence of macrophages in the luminal region. The majority of cells present in the tissue capsule are vimentin-positive, α-SMA–positive, desmin-positive fibroblasts and myofibroblasts (**Fig. 4A-C**); however, at this stage the cells lack the features of contractile smooth muscle cells (e.g., they do not express myosin heavy chain (HC)) (**Fig. 4D**).

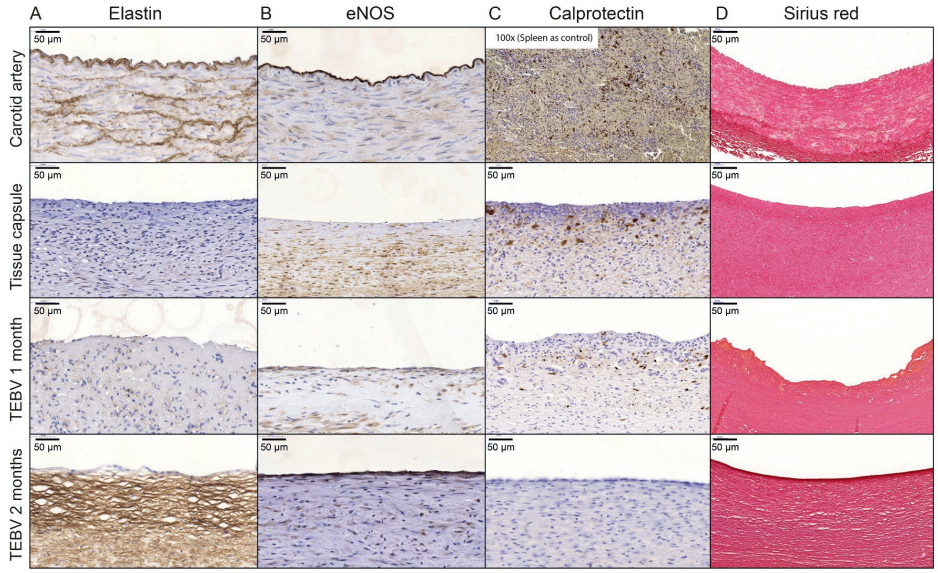

Figure 3. Mid-graft cross-sections showing a notable increase in elastin expression (A), a clearly defined endothelial monolayer (B), an absence of inflammatory cells two months after grafting (C), and an abundance of collagen (D).

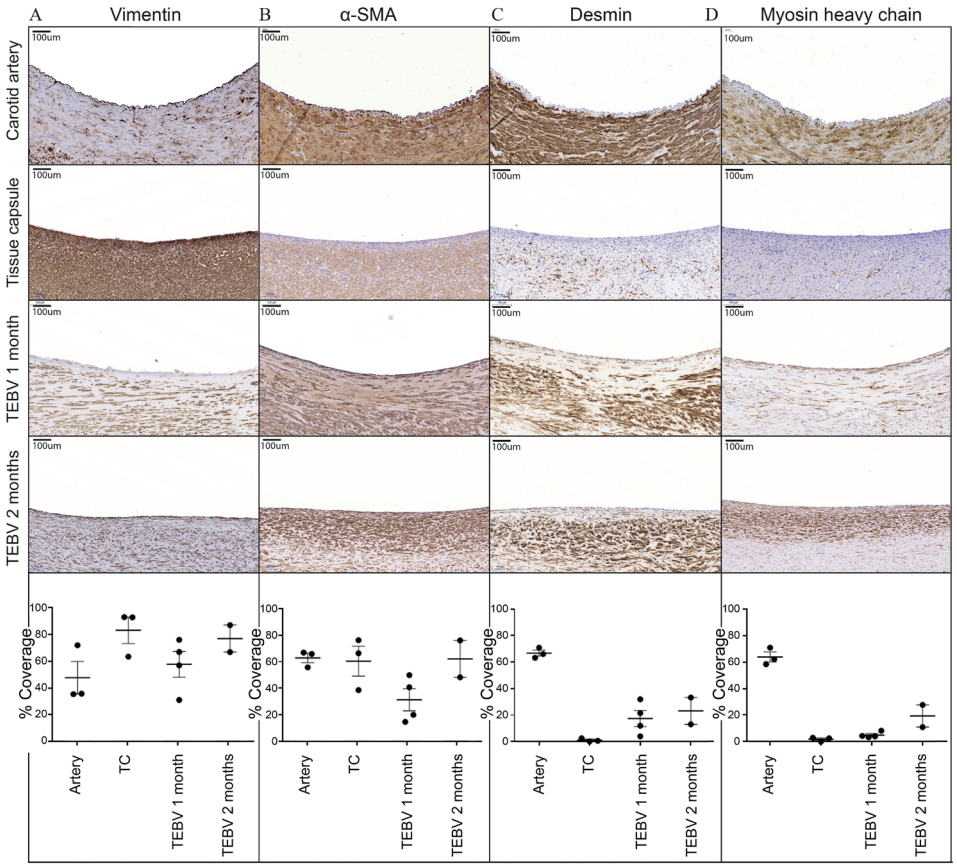

Figure 4. Immunohistochemistry showing the cellular composition of native arteries, tissue capsules, TEBVs at 1 month, and TEBVs at 2 months.

After grafting, the tissue capsule underwent remodeling, forming a thick-walled graft. One month after grafting, the majority of cells in the inner lining of the TEBV were vimentin-positive, α-SMA–positive, and desmin-positive (**Fig. 4A-D**), consistent with smooth muscle cell-like properties; interestingly, however, some of the cells were also myosin-HC–positive. Two months after grafting, nearly all of the cells in the inner lining of the graft were strongly myosin-HC–positive (**Fig. 4D**). Moreover, at 1 month, the tissue capsules lacked elastin; in contrast, the tissue capsule expressed high levels of elastin 2 months after grafting (**Fig. 3**). Of note, the distribution of elastin varied throughout the vessel (**Fig. S2**), consistent with the putative contribution of elastin formation to the TEBVs hemodynamic properties. In addition, the VSMC (vascular smooth muscle cell)-like cells are circumferentially aligned (**Fig. S3B**), closely mimicking the morphology of mature arterial cells (**Fig. S3C**).

One month after grafting, isolated and cultured luminal cells have a notable increase in contractility compared to cells isolated prior to grafting (**Fig. 5A-B**). This finding supports the histologically identified contractile phenotype.

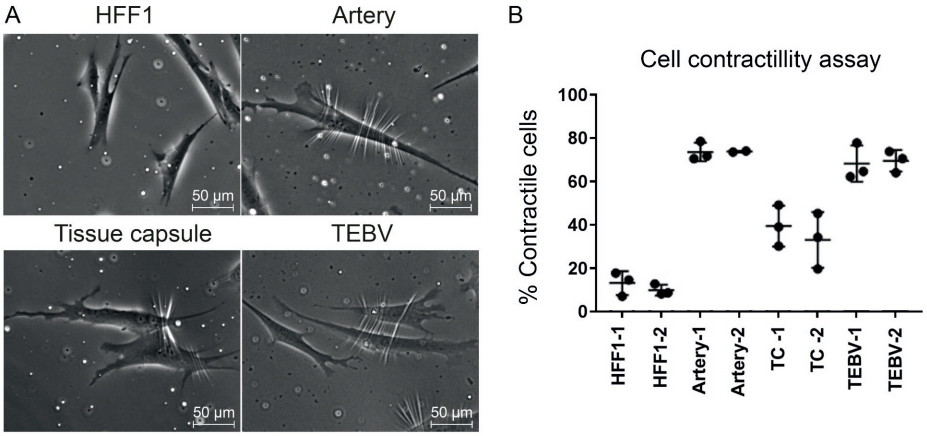

Figure 5. A) Bright-field images of the cells used in the contractility assay. B) Quantification of contractile cells isolated from two arteries, two tissue capsules (TC), and two TEBVs showing an increase in contractility between the tissue capsules and TEBVs measured 1 month after grafting. The HFF1 cell line was used as a control.

Thrombogenicity assay and characterization of the *ex vivo* whole blood perfusion chamber

Next, we performed an *ex vivo* thrombogenicity assay in order to determine whether the endothelium present in the TEBVs (see **Fig. 3**) confers anti-thrombotic properties to the vessel's surface. The perfusion chambers used in the assay were capable of perfusing four samples in parallel, with a constant flow rate and equal blood volume (**Fig. 1B**), while using a small (10 mm x 5 mm) patch of tissue. The tissue samples exposed to whole blood flow were then compared to separate samples that were not perfused.

We then examined the flow characteristics of the *ex vivo* flow chamber using particle image velocimetry (PIV) and found that the flow was stable in the region of interest (ROI) with minimal fluctuations in flow velocity. The mean velocity in the ROI was 2 mm/s, and the corresponding Reynolds number was 0.733; thus, the flow is laminar, steady, and reproducible. We used a standardized flow rate of 12.5 ml/min in the system, generating an average wall shear stress in the ROI of 1.44 N/m^2 (14.4 dyn/cm^2); this is comparable to the time-averaged arterial shear stress levels (**Fig. 1C**). SEM analysis revealed that *ex vivo* perfusion of our samples caused a notable decrease in the adhesive capacity of the tissue capsule's luminal surface after it had been grafted and exposed to the circulation for one month, as compared to the non-grafted tissue capsules which had not been exposed to the circulation. Exposing pre-grafted tissue capsules to non-anticoagulated whole blood drawn

directly from the jugular vein of a goat caused the complete coverage of the tissue with thrombocytes and fibrin; no adhesion of fibrin and thrombocytes occurred when exposing carotid artery samples to exposed to blood (**Fig. 6A**). In contrast, no blood components adhered to TEBVs obtained 1 month after vascular grafting (**Fig. 6A-B**), confirming that the eNOS-positive endothelial lining on the TEBVs luminal surface is functional (*Fig. S5*). Importantly, two ePTFE samples did not have adherence, although a protein/fibrin-rich structure adhered to a third ePTFE sample (**Fig. 6A** and $\mathbf{C_3}$). Our analysis revealed that primarily erythrocytes were present in the artery sample (**Fig. 6C$_1$**).

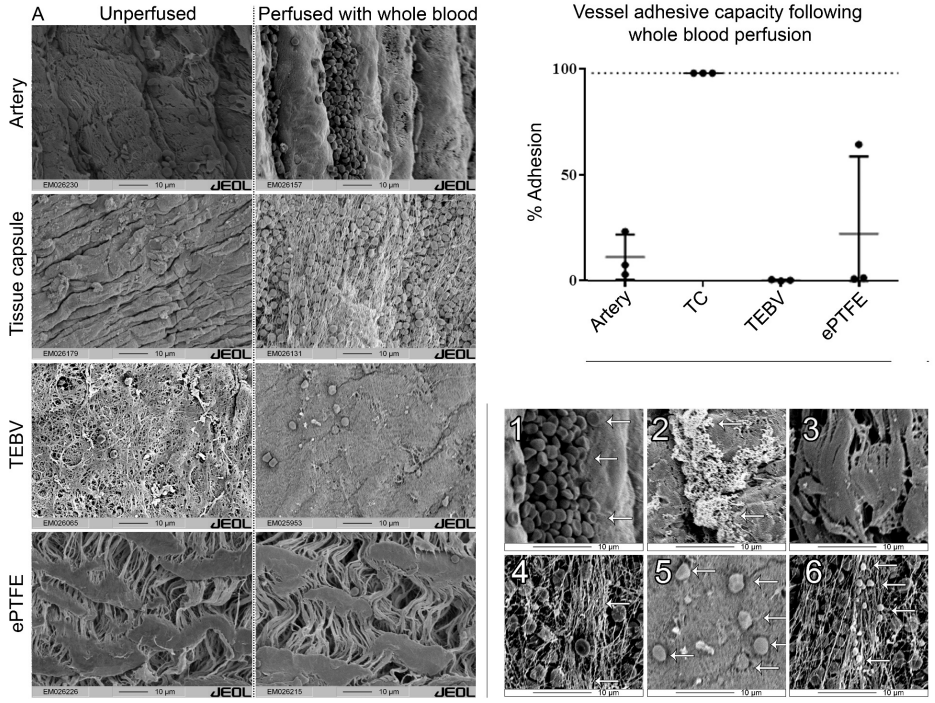

Figure 6. A) Example SEM images of the indicated tissues before (left column) and after (right column) laminar whole blood perfusion at arterial shear. After perfusion, the arterial sample contains only erythrocytes gathered in the folds of the artery. The perfused tissue capsule sample is completely covered in shear-aligned fibrin fibers with numerous erythrocytes and some thrombocytes. The TEBV sample has only minor adhesion of compounds. Finally, the ePTFE sample has extremely little adhesion of compounds. B) Quantification of adhesion before and after flow. Note the drastic decrease in the adhesive capacity of the TEBV one month after grafting. C) Detailed images of adherent structures (arrows) observed after the various tissues were perfused. C1: The arterial tissue has erythrocytes adhered to it. C2: Example ePTFE covered with fibrin fibers that were possibly degraded by fibrinolytic enzymes. C3: Example of an ePTFE sample containing non-descript adherent structures. C4: A tissue capsule sample showing shear-aligned fibrin structures that have captured red blood cells. C5: Example TEBV showing adherent non-erythrocyte cells, which were possibly monocytes or endothelial progenitor cells. C6: A tissue capsule sample with attached thrombocytes.

Patency of TEBV grafts and ePTFE grafts

Lastly, we compared patency between our TEBV grafts and synthetic ePTFE grafts; the results are summarized in **Table 1**. One month after vascular grafting, 4 of the 5 TEBV grafts and 5 of the 6 ePTFE grafts remained patent; two months after grafting, 2 out of 3 TEBV grafts and 1 out of 3 ePTFE grafts were still patent. At 1 month, stenosis in the non-occluded (i.e., patent) TEBV grafts was higher than in the non-occluded ePTFE grafts (52.22 ± 29.10% versus 4.17 ± 1.95%, respectively); in contrast, at 2 months stenosis in the TEBV grafts was lower than in the ePTFE grafts (12.94 ± 16.19% versus 85.61%, respectively) (**Table. 1**). The stenoses in both the TEBV and ePTFE grafts formed primarily near the venous anastomosis, and in the venous outflow tract. The majority of cells in the stenotic lesions near the venous anastomosis were vimentin- and α-SMA–positive fibroblasts and calprotectin-positive macrophages (**Fig. 7** and **Fig. S6**). Moreover, none of the TEBV or ePTFE grafts developed calcification (**Fig. S7**).

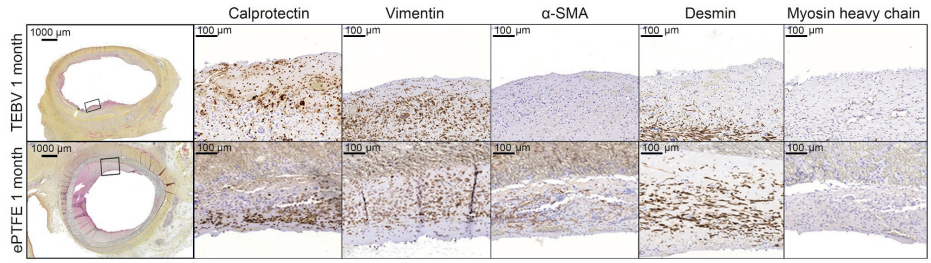

Figure 7. Example cross-sections of stenotic lesions in a TEBV and ePTFE obtained one month after grafting, with immunohistochemistry to stain various cell types present in the lesion. Overall, the cellular composition was similar between the two lesions.

Table 1. Summary of the patency and stenosis rates of TEBVs and ePTFE grafts harvested one or two months after grafting.

	Number of grafts	Patent	% Stenosis* (mean ± Standard error of the mean)
TEBV 1 month	5	4	52.2 ± 14.6
TEBV 2 months	3	2	12.9 ± 11.5
ePTFE 1 month	6	4	4.2 ± 1.2
ePTFE 2 months	3	1	85.6

* Only the patent grafts were included in this analysis. SEM, standard error of the mean.

Discussion

Here, we report that engineered TEBVs can be generated by *in vivo* remodeling of autologous fibrocellular tissue capsules. Notably, our TEBVs have low thrombogenicity and are composed of cells with a functional contractile phenotype. Moreover, the pre-grafted tissue capsules exhibit sufficient mechanical properties to restrain arterial pressure. At this stage, the tissue capsules are composed primarily of fibrocellular tissue with very little elastin; therefore, the dynamic compliance of the tissue capsules is lower than that of a native artery. Importantly,

the patency rate was similar between TEBV grafts and synthetic ePTFE grafts both one and two months after vascular grafting. Growing the tissue capsule within the subcutaneous space over a 1-month period facilitated the growth of an autologous, cellularized TEBV that has sufficient mechanical strength and can be successfully grafted into the vasculature. In a previous study we have already demonstrated the capacity of the TEBV to be successfully cannulated, with a time to hemostasis of only 3 minutes[9]. Moreover, these TEBV can remodel to form a vascular phenotype with VSMC-like properties and a functional endothelial layer, which were confirmed using histology and a novel thrombogenicity assay.

Prior to vascular grafting, the tissue capsules were composed primarily of fibroblasts, myofibroblasts, and collagen. Fibroblasts are characterized by remarkable plasticity[17-19], with the ability to differentiate into VSMCs[20]. Even though the initial tissue capsule was composed almost entirely of fibroblasts and numerous luminal macrophages, within a month of vascular grafting the tissue capsules began to show a notable increase in desmin-positive VSMC-like cells, as well as a notable decrease in macrophages. Two months after grafting, the number of myosin-HC–positive cells had increased further, and a clearly defined endothelial lining with confirmed anti-thrombogenic properties was present. Importantly, virtually no macrophages were observed at this stage. Based on functionality, protein expression, and the cell types present, the densely cellularized nature of the tissue capsule clearly resulted in the capsule's differentiation into a vascular phenotype upon exposure to hemodynamic factors. Whether the VSMC-like cells originated from infiltrating cells or differentiated from fibroblasts/myofibroblasts present in the tissue capsule is currently unclear. Similarly, the origin of the endothelial cells is unclear, as they may have been derived from endothelial progenitor cells (EPCs)[21] or via ingrowth from the artery and/or vein[22]. Pre-seeding the luminal surface of a vascular graft with endothelial cells is both labor-intensive and costly and requires access to a well-equipped facility[23]. In this respect, an autologous, cellularized graft can be reendothelialized more easily, thereby providing long-term patency. Moreover, our finding that the cells are distributed uniformly two months after grafting indicates a morphological shift from the initial cells in the tissue capsule to VSMC-like cells. Finally, we found no signs of calcification in either the TEBV or ePTFE grafts.

In native blood vessels, the vascular endothelium plays an essential role in preventing the formation of intimal hyperplasia (IH), thrombosis, and calcification[23]. At the time of vascular grafting, the tissue capsules lacked an endothelial lining of the luminal surface. However, because the surface of the tissue capsule is rich in collagen, tissue factors, and ECM components, it is intrinsically thrombogenic material. Using a pig model of arterial interposition grafting, we previously reported that the tissue capsule becomes endothelialized following vascular grafting[9]. Using a parallel *ex vivo* flow experiment under controlled standardized circumstances, we now report that the TEBV has reduced adhesive properties after vascular grafting compared to the initial, pre-grafted tissue capsule. This change may be attributed to the formation of an endothelial layer monolayer that closely mimics a native artery within two months of grafting.

Compelling evidence suggests that both fibrin and platelets are essential for the endothelialization that occurs in our model. After exposure to whole blood, a structure formed that was based on the direction of flow and consisted of aligned fibrin, erythrocytes,

and platelets. Importantly, this process did not result in acute thrombus formation in the grafted TEBVs, despite starting low molecular-weight heparin (LMWH) therapy only the day after surgery. The animals had however received clopidogrel prior to surgery. This indicates the possibility that the thrombogenic response of the tissue capsule's surface may be stabilized in an acute setting. Using a model of vascular injury, fibrin and platelets were found to play an essential role in recruiting endothelial progenitor cells (EPCs) and promoting their differentiation into mature endothelial cells[24,25]. Thus, the initial thrombogenic layer observed in the tissue capsules may be essential for either promoting the future endothelialization of the lumen and/or recruiting circulating cells to the TEBV [26].

Despite having similar patency, venous stenosis remained an issue in both TEBV and ePTFE grafts. In both groups, the stenotic lesions occurred either near the venous anastomosis or directly at the venous outflow tract. Moreover, the lesions in both groups contained randomly aligned fibroblasts, myofibroblasts, VSMC-like cells, and macrophages, all of which are characteristic of IH[27]. A decrease in the stenotic lesion in the TEBV at the 2-month time point compared to the 1-month time point was observed. This decrease in stenotic lesion area is not completely understood however it may be due to the progressive remodeling of the tissue over time. In the context of an arteriovenous fistula, wall shear stress increases considerably; when combined with turbulent flow, this can cause damage to endothelial cells and initiate the formation of IH[28-30]. Wall shear stress is an important factor in the development of IH[31-33]; therefore, the presence of a functional endothelium may reduce the risk of IH.

In addition to compliance mismatch[34] and turbulent flow[29,35-37], the inflammatory foreign body response caused by ePTFE is considered a contributing factor to graft failure[38,39]. On the other hand, removal of possible foreign body inflammatory factors—as in our TEBV method—does not entirely eliminate IH, underscoring the greater importance that compliance and hemodynamic factors play in graft occlusion compared to inflammation, at least in the setting of vascular access for hemodialysis. Thus, innovative new strategies to modulate flow and/or turbulence at the vascular access site, as well as anti-proliferative strategies, may help improve the long-term durability of the graft.

A possible limitation of using goats as a model is their tendency for relatively rapid stenosis formation and graft occlusion. Furthermore, the high degree of inter-individual variation among goats and sheep with respect to thrombogenicity[40] underscores the importance of directly comparing patency with an ePTFE-based vascular graft or other relevant control. The variable degree of thrombogenicity of the vasculature, as well as the lack of response to acetylsalicylic acid, was a reason that LMWH was administered to the animals, providing additional evidence of the dissimilarity between patients and conventional animal models with respect to the vasculature. In contrast, both dogs and baboons are less prone to developing IH[41]. Therefore, the characteristics of the animal model, the study outcomes, and the corresponding control group must be taken into consideration when attempting to translate the results to the broader research field. Moreover, although no chronic kidney failure was induced in this study, we have recently reported that the presence of uremia does not impact the formation of the tissue capsules[12].

In the late 1960s, Sparks published the first attempt to use an autologous FBR-based tissue graft grown around a Dacron mesh substrate[42]. However, incomplete understanding of the FBR, together with the incorporation of foreign body materials into the engineered tissue, led to the occurrence of graft rupture and aneurysm formation[43]. Later, Campbell further developed this concept by using the peritoneal cavity as the *in vivo* bioreactor[44]. As the knowledge regarding modulation of the FBR increased, this so-called mandrel-based method has been fine-tuned and strengthened[8].

The advantage of the approach used in our study compared to other tissue-engineering techniques is that autologous cellularized tissue is generated entirely *in vivo*, without the need for expensive and time-consuming *in vitro* steps, the need for host cell invasion of decellularized scaffolds, or patient variability with respect to replacement of a degradable scaffold. We show that our autologous TEBVs can undergo remodeling within the vasculature, resulting in endothelialization and the production of VSMC-like cells. Moreover, our method does not require a maturation period, as the TEBVs can be implanted with predetermined dimensions tailor-made to the needs of the individual patient. Despite its promise, the patient's age[45], gender[46], and health status[47] can affect the FBR, and these factors must be taken into consideration when investigating the clinical relevance of this technique.

In conclusion, our results provide compelling evidence that our *in vivo* engineered TEBVs can be implemented as arteriovenous vascular access. Using a robust *ex vivo* perfusion model, we confirmed that the newly formed endothelium is non-thrombogenic. Moreover, we show that our TEBVs undergo cellular reorganization to produce a vascular morphology with elastin formation, and we show that the cells differentiate into a VSMC-like phenotype. Despite the presence of stenosis in the TEBV grafts, the patency rate was similar to ePTFE grafts, indicating that our *in vivo* engineered TEBVs may serve as a viable arteriovenous conduit, or arterial bypass grafts.

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Supplementary materials

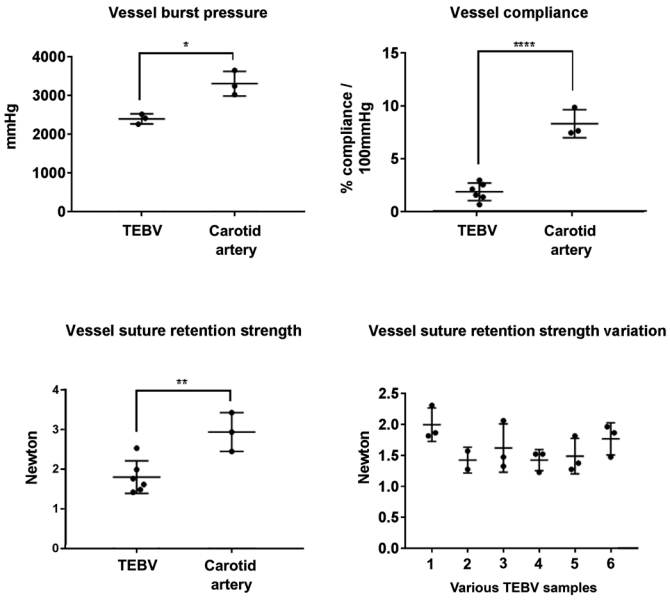
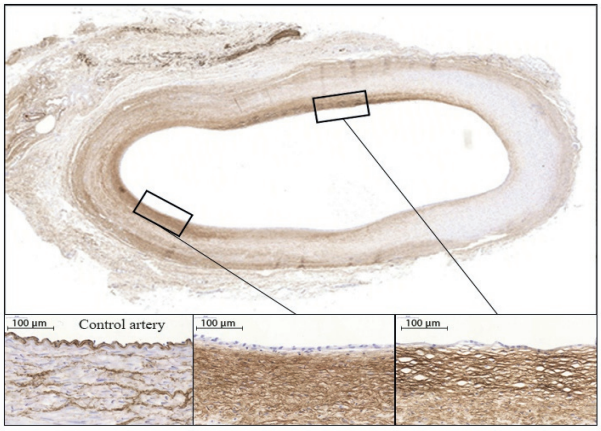
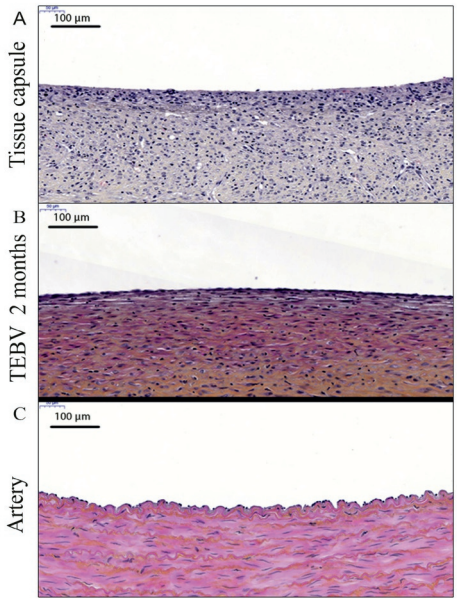


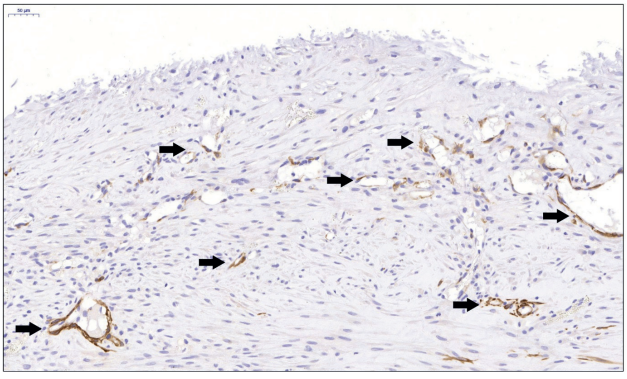
Figure S1. Summary of the mechanical testing results for pre-grafted TEBVs prior to grafting and a native carotid artery.



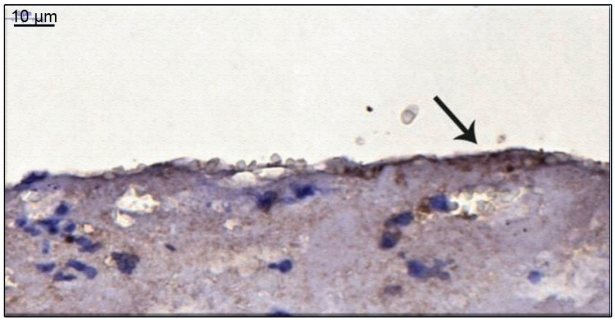
Suppl. Figure S2. Immunohistochemical staining of elastin in a TEBV after 2 months. A native artery was included as a control. Note that the distribution of elastin varied throughout the TEBV.



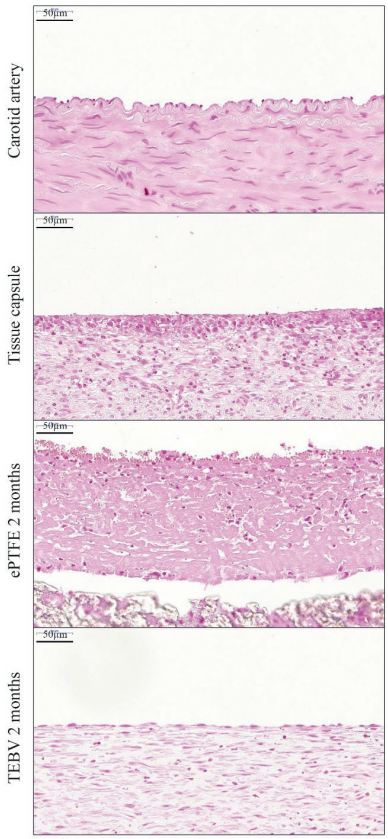
Suppl. Figure S3. Mid-graft cross-sections stained with hematoxylin phloxine saffron, showing the change in cellular morphology from a tissue capsule (A) to a TEBV (B) 2 months after grafting; for comparison, a native artery is also shown (C).



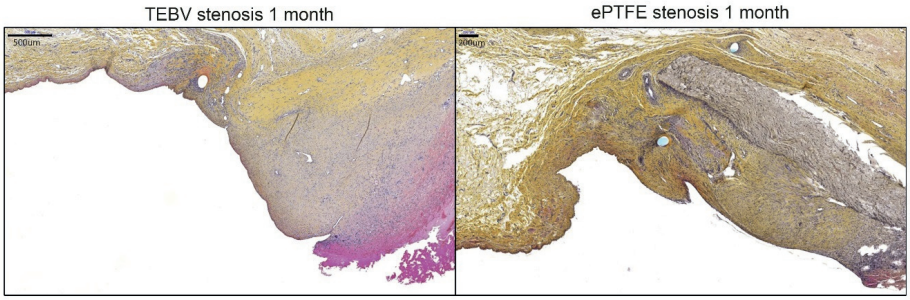
Suppl. Figure S4. Immunohistochemical staining of elastin in a TEBV after 1 month. Arrows indicate the Myosin heavy chain stain, showing the capillary network within the TEBV.



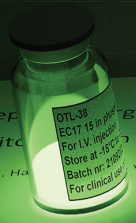
Suppl. Figure S5. eNOS-positive luminal cells (arrow) on the surface of a TEBV sample perfused with arterial shear whole blood in a thrombogenicity assay.



Suppl. Figure. S6. Hematoxylin phloxine saffron stain showing stenosis present at the venous anastomosis in a TEBV (left) and ePTFE (right) sample one month after grafting.



Suppl. Figure S7. Von Kossa calcium stain of mid-graft cross-sections of a carotid artery, tissue capsule, ePTFE at 1 month, and TEBV at 2 months. Note the lack of calcification in the TEBV, ePTFE, artery, and tissue capsule.



Folate recep geted near-infrared fluorescence imaging to monito of in situ engineered tissue capsules

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Abstract

*Tissue engineered blood vessels (TEBV) have been proposed as a suitable alternative to prosthetic arteriovenous (AV) grafts, mainly due to the high rate of complications in the latter. One method of generating TEBV, known as the mandril-based approach, aims to utilize the foreign body response (FBR) to biomaterials to create autologous *in-situ* blood vessels, suitable for AV grafting. In this method, the subcutaneous implantation of a polymeric rod results in the formation of a fibrous tissue capsule (TC) around the rod. Upon removal of the rod several weeks after implantation, the remaining TC can be grafted into the vasculature as AV-shunt. Currently, there is no non-invasive procedure by which TC maturation can be assessed. In this study a folate analog conjugated to indocyanine green known as OTL-38 was assessed for its capability to allow transcutaneous quantification of TC inflammation, an indicator of TC maturation as the contribution of inflammatory cells in the TCs decreases over time. Polymeric rods were implanted subcutaneously in the abdomen of 13 rats at t=0, T=21, and t=35 days. At t=42 days 0.4mg/kg OTL-38 was intravenously administered and the animals were imaged using the Artemis and PEARL imaging systems. An increase in near-infrared (NIR) signal was observed over time for both the Artemis and PEARL imaging systems. Staining for CD68+cells showed that TC inflammation decreased over time. Immunofluorescence combined with NIR microscopy showed no localization of OTL-38 in the TC, while PEARL imaging confirmed that OTL-38 accumulated exclusively in the skin and not the TC. In conclusion, NIR imaging may be an effective tool in the assessment of transcutaneous processes. However, OTL-38 is not suitable for the in vivo monitoring of TC maturation, as its expression and imaging does not correlate with the inflammatory processes in the TC.*

Chapter 5

Folate receptor-α targeted near-infrared fluorescence imaging to monitor the growth of *in situ* engineered tissue capsules

Manuscript in preparation

Abstract

Tissue engineered blood vessels (TEBVs) have been proposed as a suitable alternative to prosthetic grafts, mainly due to the high rate of complications in the latter. One method of generating TEBVs, known as the mandrel-based approach, aims to utilize the foreign body response (FBR) to biomaterials to create autologous *in-situ* blood vessels, suitable for AV grafting. In this method, the subcutaneous implantation of a polymeric rod results in the formation of a fibrous tissue capsule (TC) around the rod. Upon removal of the rod several weeks after implantation, the remaining TC can be grafted into the vasculature as AV-shunt. Currently, there is no non-invasive procedure by which TC maturation can be assessed. In this study a folate analog conjugated to indocyanine green known as OTL-38 was assessed for its capability to allow transcutaneous quantification of TC inflammation, an indicator of TC maturation as the contribution of inflammatory cells in the TCs decreases over time. Polymeric rods were implanted subcutaneously in the abdomen of 13 rats at t=0, T=21, and t=35 days. At t=42 days 0.4mg/kg OTL-38 was intravenously administered and the animals were imaged using the Artemis and PEARL imaging systems. An increase in near-infrared (NIR) signal was observed over time for both the Artemis and PEARL imaging systems. Staining for CD68+cells showed that TC inflammation decreased over time. Immunofluorescence combined with NIR microscopy showed no localization of OTL-38 in the TC, while PEARL imaging confirmed that OTL-38 accumulated exclusively in the skin and not the TC. In conclusion, NIR imaging may be an effective tool in the assessment of transcutaneous processes. However, OTL-38 is not suitable for the in vivo monitoring of TC maturation, as its expression and imaging does not correlate with the inflammatory processes in the TC.

Introduction

The increase in cardiovascular diseases is a driving force behind the ever-increasing need for vascular grafts for vessel replacement surgeries [1]. Although native vessels offer the best patency rates with regard to vascular grafting, their use is limited due to preexisting disease, previous harvesting, and unsuitability for vascular procedures. Synthetic alternatives such as expanded-polytetrafluoroethylene (ePTFE) on the other hand are prone to infection, intimal hyperplasia, and thrombosis [2-4]. Thus, novel methods for creating a vascular access site are constantly being explored. The field of vascular tissue engineering (VTE) for example, aims to generate new blood vessels using concepts from regenerative medicine, in which cells and tissues are regenerated or replaced using biological replacements to restore normal function [5]. Tissue engineered blood vessels (TEBV) have previously been implemented as vascular access in numerous preclinical, and clinical trials [6,7]. One method of generating TEBV, known as the mandrel-based approach, aims to utilize the foreign body response (FBR) to biomaterials to create autologous *in-situ* blood vessels for vascular access [8]. In this method, a polymeric rod is implanted under the skin eliciting a FBR, resulting in a thick fibrous tissue capsule (TC) forming around the polymer implant. The first step in this FBR process is the formation of a provisional matrix, and monolayer of macrophages. These macrophages aim to phagocytose the material. Failure to do so (in the case of a large foreign body) results in the body trying to encapsulate the foreign body in a fibrous capsule. Fibroblasts are recruited to the wound site and, differentiate to myofibroblast and create extracellular matrix components to encapsulate the foreign body. Ultimately, the foreign body is encapsulated by a thick layer of myofibroblasts, extracellular matrix (ECM), and a monolayer of macrophages. Thus, consensus is therefore that the degree of inflammation decreases over time as the encapsulation process stabilizes [9]. The polymer implant is then removed, leaving the fibrous tissue capsule (TC) in place to be grafted into the vasculature. This fibrous capsule then remodels over time in response to hemodynamic factors to more closely mimic a blood vessel [8]. One of the fundamental questions in this method is however at what time point the polymeric rod must be removed to allow for the optimal tissue composition for grafting. As the FBR is known to vary amongst individuals [10], and especially amongst ESKD patients [11], it is vital to be able to track the maturation of the tissue capsule in order to determine the optimal grafting moment. One method may be the transcutaneous, non-invasive imaging and quantification of inflammation and the implant site. A promising target to quantify inflammation over time is the novel folate-receptor (FR) agonist OTL-38. This compound has been conjugated with a near infrared (NIR) fluorophore resembling Indocyanine green (ICG) and has previously been used in a clinical setting. OTL-38 is rapidly cleared from the circulation following intravenous administration and has a terminal half-life of 5.2 hours in rates. OTL-38 is thought to bind to cell-surface FR- σ, where after it is internalized into the cell. The folate receptor family includes the FR-σ, FR-β, FR-γ, and FR-δ, which all bind folate with a high affinity [12]. OTL-38 has originally been developed for the non-invasive imagining of tumor cells, as the FR- σ is known to be overexpressed in several malignant tissue variants.

Clinical experimentation revealed that lymph nodes gave a false positive signal for malignant tumor tissue, where the signal was shown to originate from activated FR-β positive macrophages in the lymph node [13]. As OTL-38 also has a binding affinity for the FR-

β, activated macrophages may thus also be visualized using OTL-38. We hypothesized that as the FBR aimed at generating our TEBV was driven primarily by a macrophage induced inflammatory process, OTL-38 imaging may be used as a non-invasive method of quantifying TEBV development over time. The decrease in inflammatory signal could then be correlated to TC generation over time. Thus, the aim of this study was to aim to quantify OTL-38 fluorescence and TC inflammation over time and assess the predictive capacity of OTL-38 for TC maturation.

Methods

Study design

All experiments were approved by the Animal Care and Use Committee of the Leiden University Medical Center and were performed in accordance to Dutch legislation. 13-week old male Lewis rats (n=13, Charles Rives, France) were housed in standard cages with *ad libitum* access to food and water intake. All operations were performed under isoflurane inhalation anesthesia. Polymeric implants composed of the elastomeric co-polymer poly(ethylene oxideterephthalate)–poly(butylene terephthalate) (PEOT/PBT) were surface treated using chloroform etching as previously performed [14]. Per animal, one rod was implanted subcutaneously in the abdomen at t=0, t-=21, and t=35 days using a small (0.5cm) incision. This allows for TCs to be matured for 1, 3 and 6 weeks to be present at t=42 days, when imaging is performed. Prior to the imaging procedure the animals received 0.4mg/kg bodyweight of OTL-38 (On-Target Laboratories, The United States) via intravenous tail vein injection. NIR Imaging was performed using the Artemis (Quest Medical Imaging, The Netherlands), and PEARL (LI-COR, The United States) imaging systems. The Artemis imaging system allows non-invasive in-vivo imaging of the rats and is a clinically available NIR camera system. The PEARL system allows higher resolution imaging than the Artemis system, but is however not clinically available. Moreover, only tissue segments could be imaged using the PEARL system due to size constraints. Artemis imaging was performed prior to OTL-injection, and at 1- and 4-hours post injection. The system was placed at 30 cm from the abdomen of the animal, with an exposure time of 25ms. Artemis quantification was performed using ImageJ software. PEARL imaging was performed following surgical resection of the animal due to space constraints of the system. The abdomen, including muscle layers, and relevant tissues (kidneys, liver and spleen) were pinned down and placed in the PEARL system. An exposure time of 30ms was used, which was automatically determined by the system. PEARL quantification was performed using built in software. Following PEARL imaging the tissues were fixed in 4% paraformaldehyde.

Histology

Following fixation in 4% paraformaldehyde and storage in 70% ethanol the rods within the TCs were removed, where after the TCs were processed and embedded in paraffin. Serial cross-sections of 5µm of three parts of each tissue capsule were made for histological, immunohistochemical and morphometrical analysis. The macrophage population within the tissue capsules was characterized using CD68 (Abcam, The UK, 1:100, heat-induced 0.1% trypsin antigen retrieval) and the FR-β was characterized using a custom-made antibody

(1:100, On target laboratories, The United States, 1:100, heat-induced 0.1% trypsin antigen retrieval). Quantification was performed using ImageJ software.

Statistics

Data are presented as mean ± standard deviation. Data was analyzed using one-way paired ANOVA with Tukey post-hoc analysis was using Graphpad (Prism, The United States). Statistical significance values are indicated as follows: *= P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001

Results

Imaging

Following the injection OTL-38, the NIR signal could clearly be seen both by the Artemis and PEARL imaging systems (Figure 1). PEARL analysis of various tissues showed a low OTL-38 signal in the spleen and liver, and a high concentration in the skin and kidneys. A high concentration in the kidneys was expected due to the renal clearance of OTL-38.

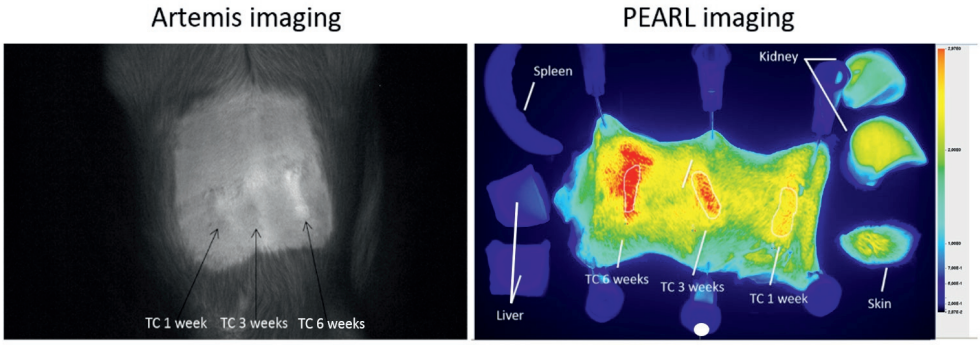

Figure 1. Left: Artemis imaging of the TCs in a rat, showing the TCs implanted at 1, 3 and 6 weeks. Right: PEARL imaging of the rat abdomen with TCs implanted at 1, 3, and 6 weeks, spleen, liver, kidney, and skin. Due to the nature of the PEARL imaging system live imaging was not possible as the animals were too large.

It was hypothesized that NIR signal in the TCs would decrease over time due to a known decrease in inflammatory cell presence over time in TC formation. Contrary to this hypothesis, Artemis and PEARL imaging both showed an increasing trend in NIR signal over time (Figure 2). As expected, CD68 quantification of the TC showed a decrease in inflammatory cell presence over time (Figure 3).

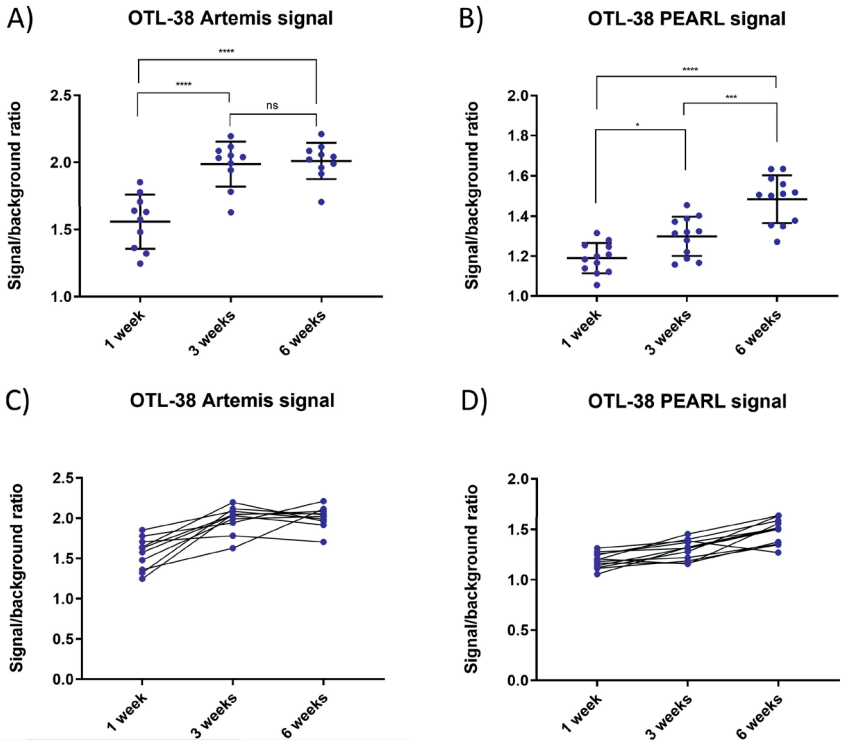

Figure 2. A) Quantification of Artemis measurements. B) Quantification of PEARL measurements. C) Paired data showing increase in Artemis signal within-individuals over time. D) Paired data showing increase in PEARL signal within-individuals over time.

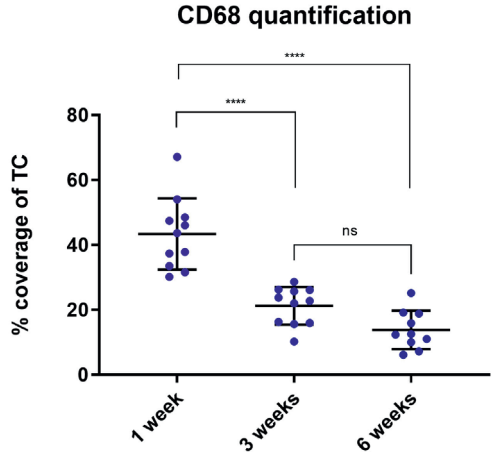

Figure 3. The histological quantification of the CD68 staining.

To assess the true localization of the OTL-38 compound, immunofluorescence coupled with a NIR microscopy was performed. Here it became evident that the OTL-38 did not accumulate in the TC macrophages as was expected (Figure 4). Within the TC the CD68 positive macrophage monolayer was positive for FR-β as hypothesized, however these cells were OTL-38 negative. Background signal was visible in erythrocytes, however erythrocytes or circulating OTL-38 was not responsible for the observed Artemis and PEARL signals as a blood sample taken from the rats did not exhibit an IR signal. Background OTL-38 signal was higher in the skin samples than the TC samples, indicating the OTL-38 accumulated more in the skin. Moreover, CD68 positive cells in the skin sample coincided positive OTL-38 signal in the dermis (Figure 4). There were however very few CD68 positive cells present in each sample (~20 per sample), it was therefore highly unlikely that these CD68 positive cells were responsible for the NIR signal on a whole tissue level.

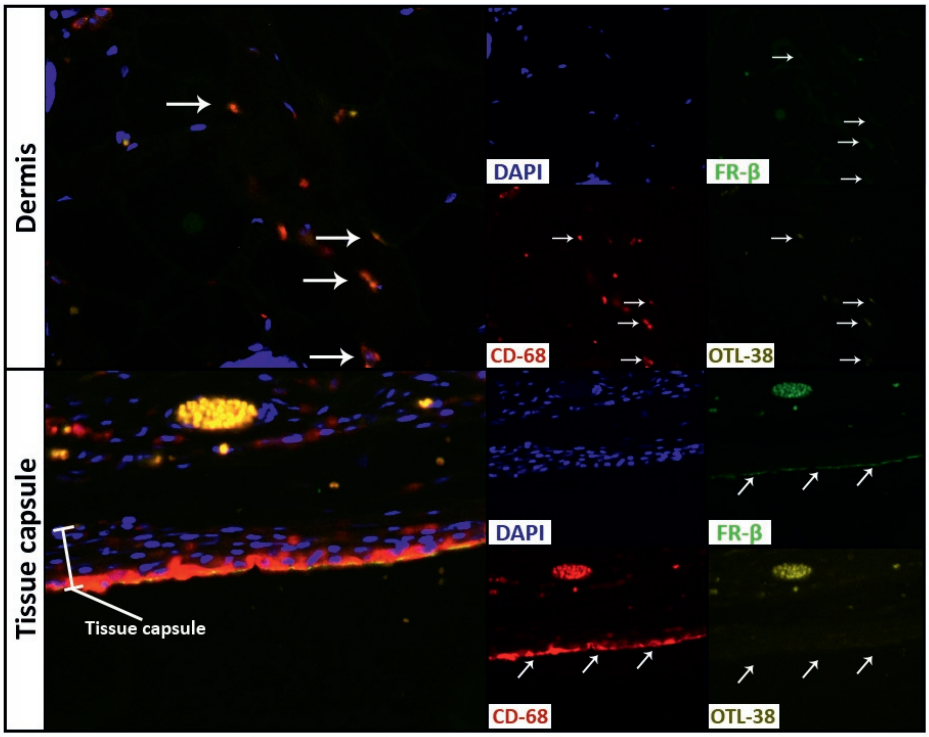

Figure 4. Showing immunofluorescence combined with NIR microscopy of tissue samples. Top: the dermis of a rat showing a coinciding OTL-38, FR-β and CD68 signal (arrows). Bottom: the tissue capsule showing a coinciding FR-β and CD68 signal of the macrophage monolayer of the TC. OTL-38 is shown to be completely absent in the TC.

Following the immunofluorescence and NIR microscopic observation that the OTL-38 did not accumulate in the TC but rather in the skin (Figure 4), PEARL imaging confirmed that the

NIR signal indeed originated from the skin (Figure 5). This demonstrated that the OTL-38 was not directly representative of TC processes.

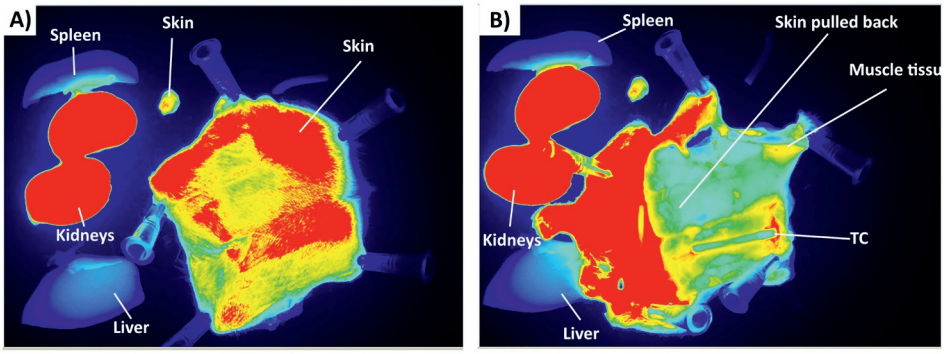

Figure 5. A) Rat abdomen viewed through a PEARL camera following OTL-38 administration with the skin in place and a clearly visible NIR signal on the abdomen. B) Rat abdomen with the skin pulled away from the subcutaneous muscle tissue revealing that the OTL-38 NIR signal originates almost exclusively from the skin, and not from the tissue capsule.

Discussion

OTL-38 is a fluorescent folate analog, and therefore thought to bind exclusively to the FR. As the FR-β is known to be highly expressed in macrophages, it was hypothesized that macrophage driven inflammation could be visualized and quantified using NIR imaging. Previous data indicates that inflammation decreases over time following encapsulation of a foreign body in the foreign body response to a long-term implanted biomaterial. However, contrary to the hypothesis the NIR imaging of both the Artemis and PEARL systems shows an increasing trend of fluorescence levels over time. Further analysis showed that the OTL-38 did not accumulate in the macrophages in the tissue capsules. An explanation for this is that during the encapsulation response of the FBR, the body aims to isolate the foreign body, and prevent access to systemic circulation. It does this through the creation of a fibrous capsule with a monolayer of macrophages lining the foreign body [9]. Although the fibrous capsule is relatively well perfused in order to oxygenate the fibroblasts that compose it, the macrophage monolayer appears relatively isolated from the systemic circulation. Thus, in combination with its rapid clearance from circulation, the compound likely never reached the TC macrophages. Macrophages in the skin did however show positivity for OTL-38. As there were few CD68 positive cells in the skin, macrophages are likely not responsible for this high signal. It was observed that the background signal in the skin was relatively higher than that of the TCs. With a 1414 kDa molecular weight OTL-38 is too large to spontaneously extravasate through healthy blood vessels. Healthy blood vessels allow the spontaneous extravasation of molecules smaller than ~70kDa. In the presence of vascular inflammation however, induced hyperpermeability may allow molecules of up to 2000kDa to leak from blood vessels [15,16]. OTL-38 may thus extravasate into tissues at a higher rate at a location of inflammation, a potential reason for the high NIR signal at the implantation wound site. However, during an unpublished model development experiment using unconjugated ICG,

no NIR signal was observed at the wound site. The potential hyperpermeability of the blood vessel at the wound site therefore does not account for the OTL-38 NIR signal, indicating the NIR signal observed with the Artemis and PEARL systems were a targeted folate receptor mediated response. Moreover, the OTL-38 signal was relatively high throughout the entire skin, not only at the implantation site where hyperpermeability of the blood vessels would occur.

The aim of this study was to assess the potential of OTL-38 for the direct quantification of TC maturation. The OTL-38 signal did not however originate from the TCs. As the OTL-38 signal did not directly reflect TC processes it was concluded that OTL-38 was not an ideal candidate to monitor TC maturation over time. One argument may be that if the skin OTL-38 signal correlates with TC maturation it may yet be representative of TC maturation. However, the use of a derivative method to quantify TC maturation was deemed insufficient to be practical in a clinical setting.

Although the mechanism by which OTL-38 accumulated in the skin above the implanted TC in our model is not completely understood, it is likely the result of a wound healing process, which currently lies beyond the scope of this research. We can however hypothesize on potential mechanisms by which OTL-38 accumulation occurs. In a recent study, folate supplementation was shown to improve wound healing in diabetic mice, potentially through the removal of reactive oxygen species (ROS) [17]. This underlines the potential role of folic acid in the process of wound healing. It is known that the implantation of a polymeric rod results in an increased proliferation of fibroblasts and macrophages surrounding the implant. Subcutaneous foreign body implantation also damages surrounding skin cells, requiring them to proliferate and repair as they might in a wound healing response. Folic acid is a key carbon donor during nucleotide biosynthesis and therefore vital for cell proliferation [18]. The proliferation required for wound healing may therefore result in an upregulation of the FR-α and FR-β in cells involved in the wound healing process. As OTL-38 binds to both the FR-α and FR-β one hypothesis is that the upregulation of the FR's in a wound healing process, as occurs with the implantation of a foreign body, would result in the accumulation of OTL-38 in the skin at the site of implantation. In this model, OTL-38 imaging may therefore be a marker of wound healing above the implanted polymeric rod.

Further investigation can be made into other means of quantifying TC maturation over time for example using other NIR probes. Although OTL-38 did not target the TC, the imaging method worked very well and NIR imaging may yet be a valuable tool in the quantification of a subcutaneous FBR. Moreover, the role OTL-38 plays in wound healing can be investigated further. The current study underlines the potential of OTL-38 as a non-invasive marker for the transcutaneous imaging of folate and folate analogues. The most abundant cell type in the skin is the keratinocyte [19], and the role it plays in folate uptake in a wound healing process could be investigated further. Quantification and correlation of the FR-α and FR-β with OTL-38 may elucidate its role in wound healing. As there is emerging evidence into the importance of folate in wound healing, the use of the imaging agent OTL-38 may be of additive value in researching the physiological role of folate *in vivo*.

Conclusion

In this study we have shown the potential of NIR imaging for the quantification of transcutaneous processes. Both the Artemis and PEARL systems were able to accurately measure NIR signal from an intravenously administered infrared probe. OTL-38 was however deemed unsuitable for the direct quantification of TEBV maturation as OTL-38 accumulated in the skin above the implanted foreign body and did not directly reflect the status of the TC.

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A proof-of-principle study of the design and optimization of a novel fluid driven automated retracting needle system

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Abstract

The cannulation of blood vessels is one of the most basic and essential interventions in medical practice. A common adverse event of this procedure is miscannulation with infiltration of the second part of the vessel wall, often resulting in a perivascular hematoma. In hemodialysis patients, surgically created arteriovenous conduits are cannulated 3-4 times per week to provide sufficient blood supply to the hemodialysis machine. However, the high blood flow and pressure in these vascular access sites increase the risk of complications upon miscannulation. A novel needle system that allows for rapid automatic retraction of the needle in response to contact with blood after positioning the cannula in the blood vessel, was developed to reduce the risk of miscannulation. The device can easily be incorporated into existing needle designs. The mechanical functionality of the device was validated by testing prototypes in an ex vivo system. Optimization of the needle system was performed to enhance retraction speed and piston shape. A final prototype design was manufactured and validated. The optimal membrane composition and piston shape were determined, which resulted in a needle retraction speed of 40ms upon contact with fluid at a pressure of 100mmHg (arterial pressure). Here, we have successfully designed, mechanically validated, and tested a novel automated retraction system that allows incorporation into existing needle systems. This ... notably decrease the difficulty of vessel cannulation and the prevalence of ...ation.

Chapter 6

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Submitted

Abstract

The cannulation of blood vessels is one of the most basic and essential interventions in medical practice. A common adverse event of this procedure is miscannulation with infiltration of the second part of the vessel wall, often resulting in a perivascular hematoma. In hemodialysis patients, surgically created arteriovenous conduits are cannulated 3-4 times per week to provide sufficient blood supply to the hemodialysis machine. However, the high blood flow and pressure in these vascular access sites increase the risk of complications upon miscannulation. A novel needle system that allows for rapid automatic retraction of the needle in response to contact with blood after positioning the cannula in the blood vessel, was developed to reduce the risk of miscannulation. The device can easily be incorporated into existing needle designs. The mechanical functionality of the device was validated by testing prototypes in an ex vivo system. Optimization of the needle system was performed to enhance retraction speed and piston shape. A final prototype design was manufactured and validated. The optimal membrane composition and piston shape were determined, which resulted in a needle retraction speed of 40ms upon contact with fluid at a pressure of 100mmHg (arterial pressure). Here, we have successfully designed**,** mechanically validated, and tested a novel automated rapid needle retraction system that allows incorporation into existing needle systems. This device could notably decrease the difficulty of vessel cannulation and the prevalence of hematoma formation.

Introduction

The cannulation of blood vessels is one of the most basic and essential interventions in medical practice. A common adverse event of this procedure is miscannulation with infiltration of the second part of the vessel wall, often resulting in a hematoma [1]. Although hematoma formation always results in a burden for patients, its effects on end stage kidney disease (ESKD) patients requiring hemodialysis can be particularly detrimental [2]. Patients on hemodialysis require a long-term vascular access site in the form of an arteriovenous fistula (AVF), or arteriovenous graft (AVG), in order to dialyze efficiently with a high volume of blood being purified by the dialysis machine per minute. These conduits allow the cannulation of a patient's blood stream 3-4 times per week using two dialysis needles per session and are accordingly referred to as a patient's "lifeline" [3]. A vessel is usually cannulated at an angle of ~25-35° with the aim of placing the needle and cannula directly in the middle of the blood vessel. However, when the dorsal- or lateral part of the vessel wall is also penetrated by the needle, a hematoma can occur (Figure 1). Due to the high blood flow going through a vascular access site (often > 1000ml/min) [4] miscannulation and subsequent hematoma formation carries added risks for hemodialysis patients. Indeed, the occurrence of a hematoma can hinder the dialysis procedure as the vascular access may not be suitable for cannulation. In such a case an alternative vascular access must be found, or the dialysis procedure may be discontinued. In the worst case the hematoma can even lead to the vascular access site being lost [2].

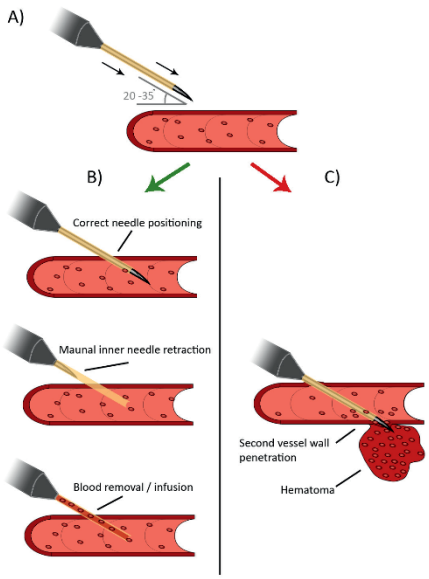

Figure 1: showing A) the cannulation of blood vessel the correct way, (B) and with back wall penetration leading to hematoma formation (C).

A 2006 study by Lee and coworkers found that the prevalence of major needle infiltration, where a fistula is unusable for at least a week, occurs with a prevalence of 5.2% in the United States [2]. As many as 31% of patients experience miscannulation within the first 6 months after a new vascular access has been placed, with a higher complication rate in AVF than AVG [1]. Moreover, patients in which major needle miscannulation occurs, require 2.4 additional procedures per patient for vascular access salvage (e.g. percutaneous transluminal angioplasty). This is associated with pain and discomfort for the patient, not to mention the fear for future cannulation procedures [5]. Moreover, 0.4% of all hemodialysis deaths are due to bleeding complications at the vascular access site [2], making miscannulation for hemodialysis a very serious problem. As such, the creation of a novel needle system that would make the cannulation procedure easier and decrease the risk of hematoma formation could improve the patients' quality of life (QOL) and hemodialysis efficacy.

Currently there are two types of cannulas for use in dialysis patients. Metal cannulas use a single large (commonly 16 gauge) cutting edge to cannulate the vessel. Plastic cannulas use an inner needle surrounded by a plastic sheath. The metal inner needle is used to place the sheath in the vessel, after which the inner metal needle can be retracted manually leaving only the sheath in the vessel. The plastic cannulas have been shown to have a favorable patient outcome compared to metal needles [6]. However, neither needle type allows automated retraction of a needle in response to blood vessel cannulation and both rely on manual user retraction. Currently, no known commercially available needle design allows for automated retraction upon cannulation of a fluid vessel. The aim of the current study was to conceive, design, and validate a novel needle that would decrease the probability of hematoma formation through the automated retraction of the needle into the sheet following vessel cannulation and blood contact.

Methods

Design requirements

The design requirements set for the system were that it should **i**) automatically retract in response to a user penetrating a first vessel wall, **ii**) automatically retract by a fluid (blood) driven mechanism, **iii**) retract fast enough to prevent the second vessel wall being penetrated, **iv**) only retract when the cannulation sheet is safely in the vessel, **v**) allow incorporation in any existing needle design, and **vi**) be simplistic enough to allow mass manufacturing at a competitive price, An unknown factor for the design of the system is the speed by which vascular access conduits are normally cannulated, as there are currently no data available regarding this parameter. Therefore, a range of needling speeds is tested in this study in which the system may be effectively used. In order to determine a minimal retraction speed threshold for vascular access cannulation several aspects must be known. The blood pressure, blood flow, vessel diameter, and cannulation speed must all be known. Moreover, there is no worldwide consensus on the speed at which dialysis needles are inserted. Based in the retraction speed determined in this study, a maximum cannulation speed may be determined. The aim here is therefore to attain the lowest possible retraction speed.

Core concept

The proposed retracting needle system is composed of a standard inner needle and outer cannula, which have been adapted to include: i) a piston, ii) a membrane, and iii) a spring. The core concept is based on the instant loss of tensile strength of a cellulose membrane material following fluid contact (Figure 2). When the needle system penetrates a vessel, blood enters the needle and travels through the lumen of the needle to reach the membrane component. Once fluid contacts the membrane, the membrane loses its tensile strength instantly, allowing the spring to overcome the restraining force of the membrane, and retract the inner needle into the cannula. The position of the hole at the side of the needle (instead of the conventional location at the tip) results in a preferred delay of needle retraction in order to prevent retraction of the needle before the cannula is safely positioned inside the vessel.

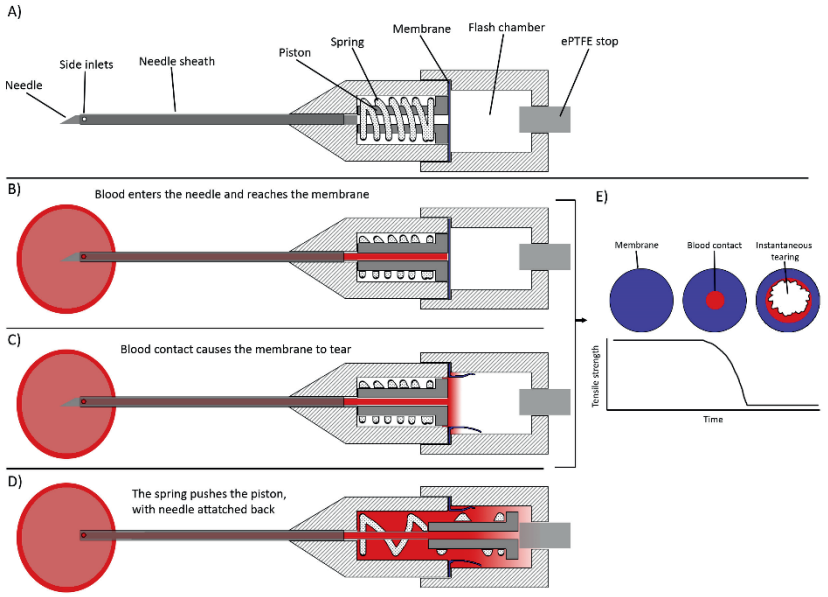

Figure 2. The concept of the system. A, B) Blood enters the needle through the hole in the tip of the sheath and moves through the needle towards the membrane. C) The membrane breaks due to fluid contact. D) the rupturing of the membrane allows the spring to expand and the needle to retract. E) Rupturing of the membrane is caused by an instant loss of tensile strength of the membrane materials upon fluid contact.

When a vessel is pressurized (as in an arteriovenous vascular access) the retraction will prevent the user from puncturing the second vessel wall and causing a hematoma. Also, it allows the user to perform a single-handed cannulation of a vessel, as no additional act is required to retract the needle. The system can be modified in order to alter **i**) the speed of

retraction, **ii**) the distance the needle retracts, iii) the force the membrane can restrain prior to retraction, and **iv**) the location of the system within existing needle systems.

Piston shape assessment

To assess the impact of the curvature of the piston, the effect of three different piston shapes on the tensile strength of the membrane was studied. The piston shape may have a considerable impact on the retraction speed as the piston conveys the force from the spring to the membrane. A custom-made probe burst pressure set-up was designed and fabricated (Figure 3). This set-up consisted of stainless-steel plates with a cavity of 6mm in diameter in the center, between which a membrane could be placed. Pistons with curvatures with a radius of 0, 1, and 2mm were placed into the pressure sensor of a Mark-10 tensile tester (Mark-10, United States) and driven through the membrane with a constant speed of 50mm. min. The force exerted on the membrane was recorded using MESUR gauge plus software (MESUR Version 2.0.5, Mark-10, United States) at 50hz.

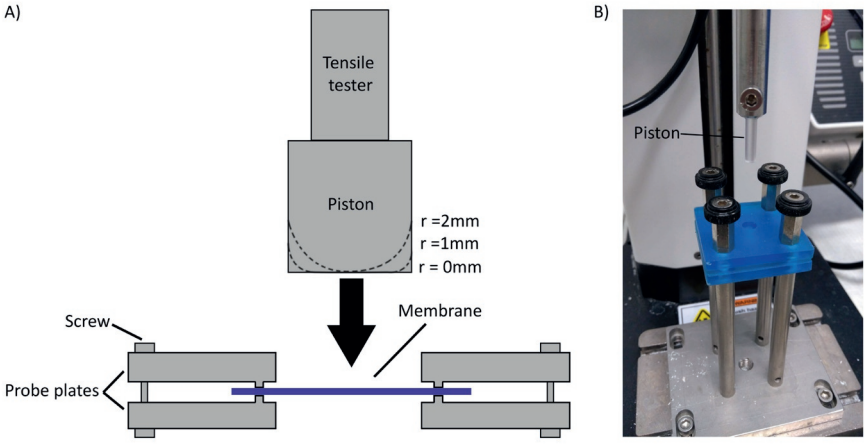

Figure 3. The experimental set-up to assess the impact of the curvature of the piston. A) showing a schematic of the system. B) showing an image of the experimental set-up with the tensile tester.

Optimization of the cellulose membranes

Ten variations of cellulose-based membranes were assessed for their applicability in the system. Ultrathin cellulose membranes were purchased from SWM medical (SWM international, United States) and tested for their effect on retraction speed (Table 1). Ultrathin cellulose membranes were chosen due to their high dry tensile strength yet express a rapid loss of tensile strength when exposed to fluid. The tensile strength of the membranes was assessed in their dry state by driving a piston with an edge radius of zero through a sample at 50mm.min using a custom-made probe burst pressure set-up. The tensile test was then repeated with the same membrane type after addition of 10ul of PBS

with glycerol to attain a 3×10−3Pa.s solution with a similar viscosity to blood. All tests were carried out in triplicates.

Table 1. An overview of the composition of the tested membrane materials.

Designation	Company paper code	Density	Porosity	Calcium carbonate concentration
M1	*EP140*	++	++++	++
M2	*EP80*	++	+++	++
M3	*1.1C*	++	++	++
M4	*16-17.5*	+	+	+
M5	*10.18*	+	+	-
M6	*10.23.1*	++	-	+
M7	*10.23.2*	++	-	+
M8	*10.23.3*	++	-	+
M9	*0.8C*	+++	++	++++
M10	*0.35C*	+++	+	+++

Retraction speed of membrane compositions

The retraction speed of the needle was assessed with various compositions of cellulose membranes (Figure 4) in order to determine the optimal membrane composition. A custom-made needle retraction device (Figure 5) was placed in the pressure sensor of the tensile tester. The needle was primed using a membrane and driven through a shore A60 silicone tube (J. Lindemann GmbH, Germany, wt: 3mm, ID: 6mm) at a constant speed of 50mm.min. ShoreA60 silicone was selected as this is provides a robust model of tissue cannulation. A thickness of 3mm was chosen as this allowed for optimal cannulation in the retraction speed set-up. As this experiment only evaluated the retraction speed upon puncture of the fluid vessel lumen itself, the wall thickness of the silicone. By comparison, the shore value of skin of the human forearm is approximately 30 [7]. A wall thickness of 3mm was used as this assured all fluid entered the needle and did not leak past the cannulation site. The start coordinates of the system were recorded. The silicone lumen was filled with PBS with glycerol up to a pressure of 100 mmHg. The needle was allowed to puncture the silicone tubing, causing fluid to enter the needle and induce retraction of the needle. The tensile tester software recorded the increase in pressure as the needle entered the silicone, as well as the drop-in resistance following retraction, allowing the retraction speed to be determined. After the optimal membrane composition was ascertained, the retraction speed of the optimal membrane was assessed at varying pressures (10-100mmHg) in the same experimental set-up in order to determine the impact of pressure on the needle.

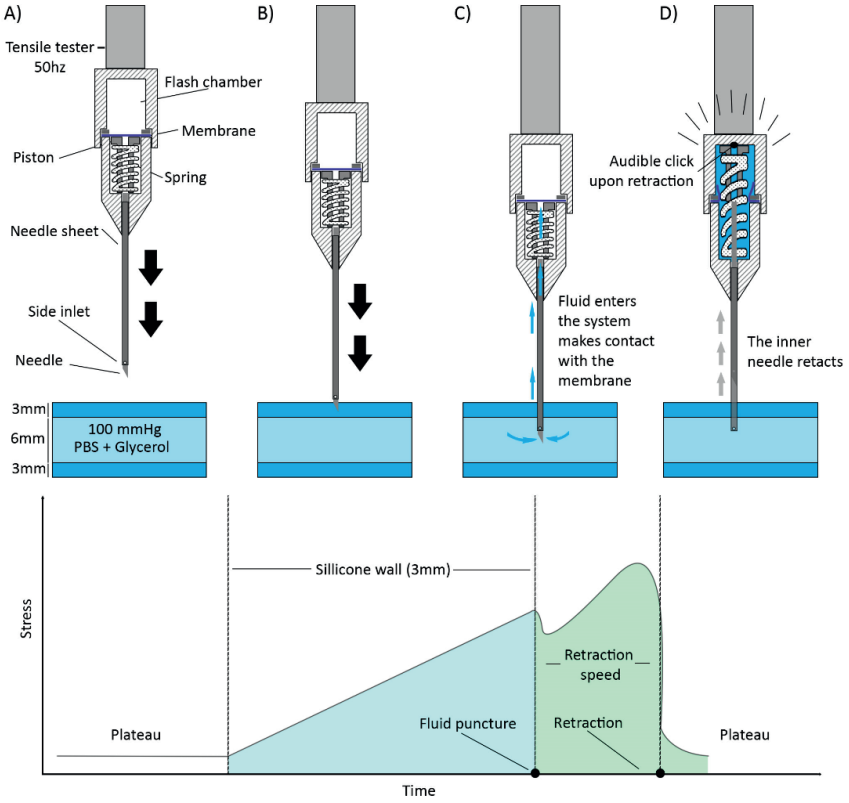

Figure 4. The experimental retraction speed set-up where a) the loaded system is driven through the silicon, a) the needle begins to penetrate the silicone wall, increasing the recorded stress, c) the first silicone vessel wall is penetrated, which provides a momentary decrease in stress, and d) fluid enters the needle, and makes contact with the membrane causing the needle to retract

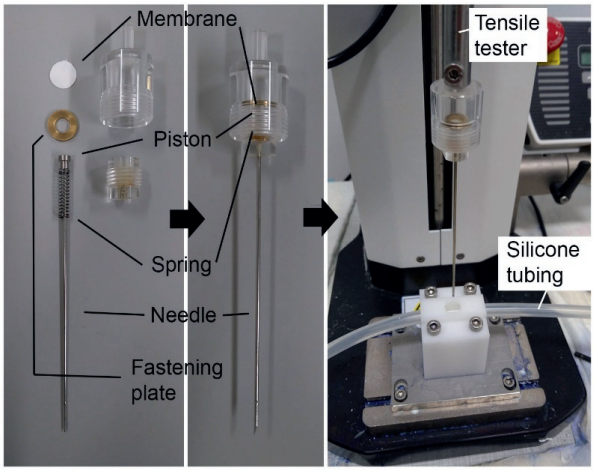

Figure 5. Showing the experimental setup of the retraction speed device. For the retraction speed device, a fastening plate was used to keep the membrane in place to allow high-throughput measurements.

Results

Optimal shape of the needle piston

To determine the optimal shape of the piston, the impact of curvature of the piston was assessed. Various curvatures of the piston were assessed with a fixed membrane diameter (Figure 6). The piston with a radius close to 0 displayed the largest difference in strength required to break the membrane in dry versus wet state. This highest piston surface area relative to the membrane was therefore desirable.

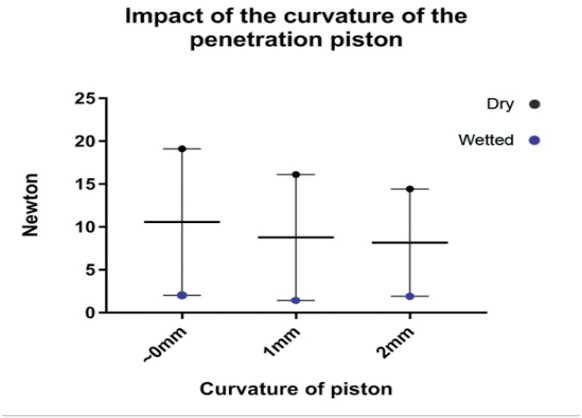

Figure 6. The impact of the curvature of the piston on dry and wet tensile strength, performed in triplicates.

Optimization of the cellulose membrane

The optimal membrane material was determined by comparing dry and wet tensile strength of the materials (Figure 7). The samples varied based on their density, porosity, and calcium carbonate levels (Table 1). Sample M10 was used as a control as this was composed of a cellulose material with a hydrophobic coating, as demonstrated by the identical tensile strength in dry and wetted state. It was concluded that features of sample M7-8 were optimal in this test as the delta between the dry and wetted state were the largest, and the wetted state had a low yield strength.

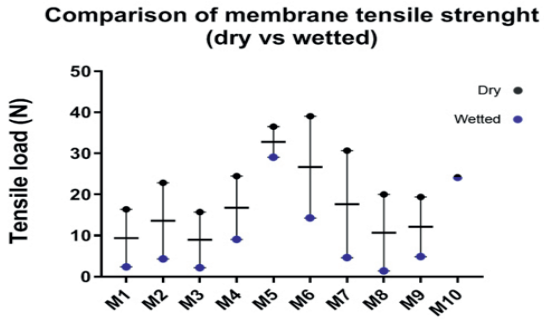

Figure 7. Comparison of the dry and wetted state of various membrane compositions. All measurements were performed in triplicates.

Retraction speed of membrane compositions

To assess the optimal material for the system, membrane types M1-10 were assessed for their retraction speeds (Figure 8). To assess the optimal membrane composition, samples were selected with the highest delta tensile strength when wetted, in combination with the lowest reaction time. Sample M8 was shown to have the highest delta tensile strength when wetted and fastest retraction speed at 100mmHg.

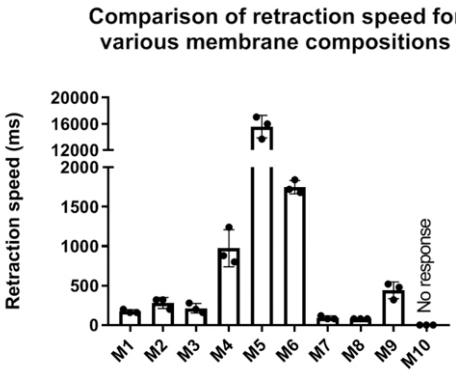

Figure 8. The retraction speed of the various membrane compositions. Sample M10 was hydrophobic and therefore had no response.

Next, retraction speed of the optimized membrane (M8) was assessed over a pressure range from a static pressure system. An increase in retraction time is observed with a decrease in pressure (Figure 9). Thus, assuming a static pressure system, the retraction speed of the needle is determined by the speed at which fluid moves towards the membrane component. The determining factor for retraction speed is the speed at which fluid enters the needle and contacts the membrane.

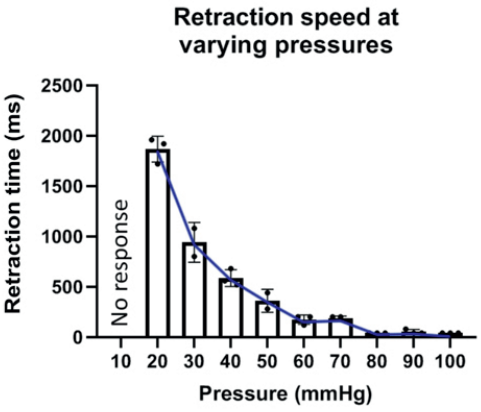

Figure 9. The retraction times of the optimized membrane system. An increase in retraction speed is observed when pressure in the static fluid compartment is decreased.

Discussion

Here, we have devised a method that allows a needle system to automatically retract upon contact with blood. The inner needle is connected to a spring system that is tensioned against a membrane. Once blood reaches this membrane, it instantaneously loses tensile strength and ruptures allowing the needle to retract safely into the flash chamber. In order to avoid the needle activating prior to the sheath having penetrated the blood vessel, a delaying mechanism is incorporated by positioning the blood inlets on the sides of the sheet, thus only allowing needle retraction if both the needle and the sheath are within the blood vessel. Benchtop optimization of the system showed that a low curvature of the piston, and a greater piston surface area is desired. Various membrane compositions were assessed for their applicability in the system. Automated retraction of the needle occurred within 40ms after puncturing a silicone tube containing PBS with glycerol at a pressure of 100 mmHg. This would indicate that the activation of the system (membrane rupture and spring activation) occurs within 40ms following fluid contact. An increase in retraction speed was observed with a decreasing pressure from a static fluid vessel. The primed needle with the M8 membrane was able to withstand a 20N load.

Owing to the high dry tensile strength of our membrane material, and the rapid loss of tensile strength upon fluid contact, ultrathin cellulose membranes were found to have the ideal characteristics for our application. Cellulose is a naturally occurring polysaccharide [8], and cellulose-based materials have previously been proposed as humidity sensors [9]. Through the process of water sorption an increase in the water content of the material results in the stiffness, tensile strength, and compression strength being affected adversely [10].

The pressure within an AVF decreases along a gradient from the high pressure arterial system (~120mmHg) to the low pressure venous system (~40mmHg) [11]. The high blood flow through the AVF will likely impact the speed at which blood enters and passes through the cannulation needle. This must be taken into account as the cannulation site may therefore also be determinant for the retraction speed. Assuming a retraction speed of 40ms at 100mmHg with a matured AVF vessel lumen diameter of 6mm a maximum cannulation speed of 15cm/s could be attained before the second vessel wall is punctured.

Few studies have been performed documenting the miscannulation rate in dialysis patients [1,2]. The conclusion is however clear that the rate of miscannulation is high, and that this can have serious implications for patient welfare. As stated earlier, plastic cannulation needles are preferred over metal needles [6]. Ultrasound guidance is often used to aid the cannulation of AVF. This however requires the user to multitask using one hand to operate the ultrasound device, and the other hand to cannulate the vessel. Regardless, the use of ultrasound has been shown to improve the success rate of venous cannulation procedures [12]. Currently, no other needle system exists that allows the fluid driven automated retraction of a needle upon blood vessel cannulation.

A limitation of this experiment was that the system was only assessed in response to pressure in a static system. In an AVF, blood flow is high (~1000ml.min), and the cannulation of a high flow system may lead to blood entering the needle faster than in a static system,

which would be favorable for our application. Although the cellulose membranes were commercially attained, only global characteristics of the membranes was received. And although current study outlines and validates the proof-o-principle of the automatic retraction concept, concurrent studies would require a more detailed examination of materials used. Moreover, in the current study cellulose membranes are focused on. Cellulose may induce complement activation which may be detrimental for the cannulation procedure [13]. Although the design of the needle system is such that following retraction the cellulose membrane is shielded form blood circulation this does need to be considered in future design iterations. Alternative membrane compositions such as methyl cellulose may be explored as potential alternatives. Moreover, silicone tubing was used in this study to allow for a highly controlled experimental set-up for concept validation. Ideally, a material more closely mimicking human skin and subcutaneous tissue would be assessed as well.

An alternative application for our needle design is the cannulation of arterial vessels, such as peripheral atrial cannulation that is a frequently performed procedure [14]. As mean arterial pressure is generally 90 mmHg, retraction time would approximately be 40ms. Therefore, our needle system may be of use for both the cannulation of AVF, and arterial cannulation procedures. Another alternative use of this needle system is venous cannulation. Currently, nursing staff requires both hands to cannulate a vein for an intravenous line. The needle system proposed here could be utilized to allow for single hand cannulation, freeing the other hand of the nursing staff (e.g. for ultrasound guidance). Although retraction times may be suboptimal for small veins due to the low pressure, the additive value of allowing single hand cannulation may be worth investigating.

Before this needle system can be introduced in the clinic, several steps need to be taken. Initially, the needle must be incorporated into either an existing needle system or designed to comply with medical device regulations. Further benchtop assessment of the system should be carried out to further optimize the needle design. The system should be assessed in a benchtop model mimicking the flow in an arteriovenous fistula, and a material may be used that more closely mimics human skin. Finally, animal studies must be performed in order to validate the needle system *in vivo* with real blood vessels prior to the start of clinical studies.

In conclusion, we have successfully designed, mechanically validated, and optimized a novel automated rapid needle retraction system that allows incorporation into existing cannulation designs. This device may notably decrease the difficulty of vessel cannulation and the prevalence of hematoma formation.

Acknowledgements

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A proof-of-principle
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Wouter Jan Geelhoed, MSc,[1,2] Resh
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Wouter J. Geelhoed[1,2] · Loren

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2. Introduction

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Chapter 7

Summary and discussion

Summary

Worldwide, the incidence of chronic kidney disease (CKD) and end stage renal kidney (ESKD) is increasing each year, and with it the number of patients reliant on dialysis for survival[1]. The most common form of dialysis is hemodialysis (HD), where the blood is filtered by an extracorporeal dialysis machine. Without a suitable vascular access (VA) site, an efficient HD procedure cannot be performed. Therefore, an arteriovenous fistula (AVF) or arteriovenous graft (AVG) is used to create long-term VA sites. While AVF are prone to maturation failure, AVG are prone to infection, while both are prone to the formation of stenotic lesions [2-6]. An alternative to AVF and AVG are tissue engineered blood vessels (TEBVs)[7]. One method of producing TEBVs by utilizing the foreign body response (FBR) to biomaterials to generate *in vivo* TEBVs has been discussed in this thesis (**Chapter 2**). The subcutaneous implantation of a foreign body results in the formation of a fibrous tissue capsule (TC). This TC can then be grafted in the vasculature to form a TEBVs.

One of the most vital aspects of TEBVs is their ability to withstand the mechanical forces induced by arterial blood pressure, thus ensuring patient safety[7,8]. In **chapter 3**, we compare three methods of evaluating the mechanical strength of biological vessels and discuss their suitability for use with TEBVs. We show that the derived methods known as the circumferential tensile strength and probe burst pressure methods provide an overestimation of the pressurized burst pressure in both biological and synthetic material. Moreover, the outcomes of the three test modalities evaluating the mechanical properties of different types of biological blood vessels correlate poorly with each other. Indirect methods of burst pressure assessment should thus not be used for the evaluation of TEBVs unless perfect homogeneity of the sample is assumed, and a high correlation is validated.

In **chapter 4**, we evaluate the remodeling capacity of autologous *in vivo* TEBVs for arteriovenous grafting in a large animal model. Histology showed that the initially implanted TCs were primarily composed of fibroblasts, myofibroblasts, and collagen. Within a month of vascular grafting, the TCs showed a notable increase in desmin-positive VSMC-like cells, as well as a notable decrease in macrophages. Two months after grafting, the number of myosin-HC–positive cells had increased further, and a clearly defined endothelial lining was present. One of the main experiments of this study were the functionality tests of the endothelial monolayer of the TEBVs. A custom-built *ex vivo* microfluidic perfusion chamber was specially designed to house large vascular tissue sections that could subsequently be perfused in parallel with blood drawn directly from a living animal. The system ensured that all samples were exposed to identical volumes of blood and shear stress levels. TEBV samples were shown to have less adherent blood components than TC samples. This indicated that the initial lack of endothelial cell coverage of the TCs does not result in acute thrombosis and that the observed endothelial monolayer of the TEBVs that developed in the 8 weeks after grafting was indeed non-thrombogenic *in vivo*. The burst pressure and suture retention strengths of the TEBVs exceeded the requirements for safe grafting, which are generally defined by the mechanical properties of the human saphenous vein [8,9]. A contractility assay revealed an increase in contractility of TEBVs at one-month post grafting compared to TC. This coincides with the relative increase in histological contractility markers in the TEBVs. One month after vascular grafting, 4 of the 5 TEBV grafts and 5 of the 6 ePTFE grafts remained

patent; two months after grafting, 2 out of 3 TEBV grafts and 1 out of 3 ePTFE grafts were still patent. At 1 month, stenosis in the non-occluded (i.e., patent) TEBV grafts was higher than in the non-occluded ePTFE grafts (52.22 ± 29.10% versus 4.17 ± 1.95%, respectively); in contrast, at 2 months stenosis in the TEBV grafts was lower than in the ePTFE grafts (12.94 ± 16.19% versus 85.61%, respectively).This study underlines the remodeling capacity of autologous TEBV *in vivo.* Venous stenosis however remains a problem for both ePTFE and TEBV grafts, a limitation which will need to be monitored during the clinical implementation of autologous TEBV.

Important in the clinical implementation of subcutaneously grown autologous *in vivo* TEBVs is the development of a non-invasive method to quantify TEBV maturation. Following implantation of the TC a layer of macrophages forms around the implant that drives the FBR. As the number macrophages decrease, the collagen content of the TC is known to increase. In **Chapter 5** we evaluate a novel folate-receptor-α (FR-α) agonist conjugated with the NIR fluorophore Indocyanine green (ICG) in order to quantify these macrophages over time. A decreased NIR signal was expected in accordance with a decrease in the number of macrophages. Contrary to the hypothesis, the NIR imaging revealed an increasing trend of fluorescence levels over time. Moreover, no NIR signal was observed to originate from the TC or from TC macrophages. Further analysis revealed the NIR signal originated exclusively from the skin layer above the TC. As the signal did not directly reflect TC processes it was concluded that this fluorophore was not an ideal candidate to monitor TC maturation. The FBR to biomaterials intrinsically aims to isolate an implant from the body through its encapsulation in a fibrous capsule. Due to its low circulation half-life the fluorophore likely did not reach the isolated TC macrophages. The fluorophore did however show potential for the non-invasive transcutaneous quantification of folate.

A common adverse event of this blood vessel cannulation is miscannulation with infiltration of the second part of the vessel wall, often resulting in a hematoma [10]. Although hematoma formation is always a burden for patients, its effects on ESKD patients requiring hemodialysis can be particularly detrimental due to the high blood flow of the VA [11]. Current dialysis needles types do not allow automated retraction of a needle in response to blood vessel cannulation and rely on manual user retraction. **Chapter 6** describes the design and proof-of-principle assessment of a novel needle design that rapidly automatically retracts in response to fluid contact. The system is based on the loss of tensile strength of a cellulose membrane upon fluid contact. The system was successfully optimized and showed automated retraction within 40ms upon cannulating a vessel pressurized to 100mmHg. As this study was a proof-of-principle assessment of the concept, further work is needed. Prior to this needle system being introduced in the clinic it must be incorporated into either an existing needle system or designed to comply with medical device regulations. Further benchtop assessment of the system should be carried out to further optimize the needle design.

Discussion and future perspectives

VA is essential for the survival of HD patients, where the gold standard intervention is the AVF [12]. Yet, due to its numerous downsides the AVF can also be viewed as a surgically induced

pathological condition. The placement of an AVF increases the risk of heart failure, damages blood vessels resulting in poor patency rates. In addition, AVF cannulation is painful and aesthetically unpleasant due to aneurysm formation, thereby causing a substantial burden for patients [12-14]. It may therefore be viewed as a necessary evil for HD patients.

The need for innovation in field of HD and VA is clear. The incidence of ESKD is increasing and will result in more patients requiring HD and a VA [1]. The healthcare costs associated with VA totaled to a staggering $3 billion in 2016 in the US, a number expected to be a third higher if private care costs are included [15].Various innovations attempting to improve VA outcomes are in development. Examples are the endoAVF systems, where a catheter-based device uses heat and pressure to create an AVF [16]. The endoAVF system is however limited to certain vascular anatomies. Fist Assist devices aim to improve AVF outcomes through venous dilation prior to AVF surgery, and although training of the forearm prior to AVF surgery is promising, data supporting these devices themselves are currently insufficient[17-19]. The improvement of AVF maturation and patency through pharmacological intervention is also under development[20]. Although as AVF pathology is immensely complex and not entirely understood, therapeutic intervention is complicated. Evidence of this may be the outcome of the phase 3 PATENCY-2 clinical study which failed to meet its primary endpoint. In this trial a recombinant elastase was administered to AVF during surgery, in the hope to stimulate outward remodeling [21]. An incomplete understanding of the exact pathophysiology of AVF failure may have, among other factors, contributed to the failure of this investigational compound. VA for HD is also an interesting field for vascular tissue engineering (VTE) [7]. VA for HD provides a stringent test case for any TEBV technology due to the high failure rate of both AVF and AVG. A long patency of a TEBV as VA will likely translate to a long patency as other vascular indications such as arterial bypass interventions. The risk to patients is relatively low, as the occlusion of a TEBV as arteriovenous conduit will restore normal arterial and venous flow. In contrast, the failure and occlusion of a TEBV as arterial bypass would result in loss of arterial blood flow and the risk of peripheral ischemia, making this application less suitable for a first-in-man study using TEBVs. In the field of VTE, the company Humacyte is currently conducting a phase 3 clinical trial of acellular TEBVs as arteriovenous grafts[22]. The success or failure of their technology may have large implications for the field of VTE. Data from their phase 2 study showed promising results although the 28% primary patency of the TEBV at 12 months was not superior to conventional ePTFE grafts [23].

The field of VTE itself is relatively new, which is evident by the lack of clear regulations specific for TEBVs regarding their functional assessment. Arguably the most vital characteristic of a TEBV is its ability to be safely grafted into the vasculature of a patient without mechanical failure (rupture due to pressure, suture rupture, aneurysm formation) [7,8]. Yet no consensus has been reached on what method for the mechanical assessment of TEBVs is optimal. The ISO7198 for the assessment of prosthetic grafts is often used as a starting point[24]. This document was however made for synthetic grafts and does not account for the variation that naturally occurs in biosocial tissues and vessels. The mechanical assessment of TEBVs has been performed differently in numerous studies[8,25-29]. Researchers often use derived methods of the gold standard pressurized burst pressure test, such as the circumferential tensile strength and probe burst pressure, to estimate TEBV burst pressure. It is tempting

to use a derived method, as these require smaller samples, while long segments of TEBVs needed for pressurized tests are often valuable samples. Moreover, studies frequently lack a detailed methodology, making it hard to deduce how the test was precisely performed. As the derived methods are based on a theoretical estimation of the real burst pressure, and do not consider biological variation, based on **chapter 3** of this thesis it is likely to assume that derived burst pressure estimations are often overestimated in literature.

Chapter 4 of this thesis describes the evaluation of autologous *in vivo* TEBV. The main competitor for these autologous TEBV are AVGs, as autologous vessels remain the ideal tissue for a vascular procedure. The TEBV displayed a cellular remodeling capacity towards a vascular phenotype through histology and contractility and attained a functionally non-thrombogenic endothelial monolayer. Moreover, as this was autologous tissue no rejection reaction is observed. AVG grafts on the other hand are available instantly and off-the-shelf, while these TEBVs require at least a month of subcutaneous maturation, excluding them from use in an acute setting. AVG are available in any required length, while the TEBVs are restricted to segments of max ~20cm.

Prior to the clinical application of the TEBV numerous steps must also be taken. The risk of aneurysm formation and vessel rupture must be taken extremely seriously, and prior to the clinical grafting of an *in vivo* TEBV several issues must be tackled. The manufacturing method of the implants must be standardized, and implants using a new production method must be validated *in vivo* in a large animal model. Changes in both the manufacturing method and polymer composition may have an unfavorable impact on the mechanical qualities of the implants and thus the composition of the formed TC. Therefore, TCs derived from polymeric implants intended for clinical use should be assessed for their pressurized burst pressure, suture retention strength, and long-term grafting safety if any change in the manufacturing method occurs. Changes in the manufacturing method may include a new manufacturing location, variations in the polymer composition or supplier, or other deviations from previously implemented productions protocols. The production method of the polymer implants presented in this thesis are reliant on chloroform-based surface modifications. These surface modifications are required to improve TC formation[30]. Chloroform exposure is associated with numerous health risks [31]. Solvent based etching of a polymer may lead to swelling of the polymer matrix, thus allowing the solvent to penetrate into the polymer [32]. Washing of the polymer implant following chloroform etching may remove the majority of surface chloroform, yet chloroform may be present within the polymer matrix itself. Post-implantation degradation or cracking of the polymer implant could therefore release chloroform into the body. The polymer implants should therefore be assessed for any chloroform residue both on the surface and within the polymer matrix structure prior to any clinical implantation. This may for example be done using nuclear magnetic resonance assessment utilizing the correct polymer solvents. Moreover, a study must be performed to assess the mechanical properties of the clinical implant as the subcutaneously implanted polymer must be able to withstand both the mechanical forces acting on it, such as bending of nearby tissue, and a potential external force of impact.

If the above requirements are met an initial clinical may be initiated to assess the mechanical properties of the *in vivo* TEBVs in humans. This clinical trial would not involve a vascular

grafting operation and only assess the growth of the subcutaneous TCS. It is vital that the mechanical properties of the TCs are extensively characterized in humans. Translation of animal study data to a clinical setting is notoriously unpredictable [33]. Data from the large animal study presented in this thesis should thus by no means be extrapolated to estimate the mechanical properties of similar TCs in humans. The mechanical quality of the TCs must be clinically validated using at least the SRS and pressurized burst pressure, as derived methods result in an overestimation of the results and should in never be used for the validation of biological tissue for a clinical study. Moreover, depending on the clinical application, a suitable trial population should be selected. For arteriovenous vascular access grafting a CKD patient population should be used in an initial trial. Although animal studies have indicated that CKD does not impact TC maturation [34], this must be confirmed in patients. The trial population should be sufficient to take into account patient variation with regard to TC maturation. This variation may arise due to CKD severity, or comorbidities such as diabetes or obesity. It is crucial for the *in vivo* TEBV method that optimal mechanical properties are attained upon vascular grafting. Although a method to non-invasively quantify TC maturation *in vivo* was attempted in **chapter 5** of this thesis, no applicable method currently exists. Prior to the initiation of a clinical trial involving TEBV grafting, a method should be developed and validated to accurately monitor TEBV maturation with regard to its mechanical properties. Such a method may be assessed in a first clinical evaluation of the TCs. Data from the first clinical trial should report positively report on the mechanical properties of the polymer implant, provide sufficient data regarding the variation within the intended patient population, and validate the non-invasive quantification of TC maturation. Subsequently, a clinical trial may be initiated to evaluate the safety and efficacy of *in vivo* TEBV as arteriovenous vascular access grafts. Aneurysm formation, anastomotic rupture, graft infection/inflammation should be closely monitored, and graft patency should be compared to that of AVF as standard of care.

Besides the problems associated with the patency of long term VA options, miscannulation during dialysis procedures has shown to be an underreported yet pressing issue[10,35]. Currently, there is no literature available that accurately describes the incidence of miscannulation events in dialysis patients in the Netherlands. In an effort to improve upon current VA cannulation procedures, proof-of-principle studies of a novel automated needle design has been outlined in this thesis (**chapter 6**). This validated the proposed concept as the base mechanism functioned reliably and well. The use of such a needle system may decrease the risk of miscannulation of VA sites, notably for new and less experienced personnel. Although, training and guidance of dialysis personnel, as well as ultrasound guided cannulation may be enough to significantly reduce the risk of miscannulation.

The increase in incidence of CKD and ESKD may in part be attributed to a sedentary lifestyle [1,36]. The prevention of a pathology is desirable over its incidence and treatment. Besides the focus on the downstream complications of CKD such as HD VA, a societal emphasis should be placed on promoting an active lifestyle in combination with dietary considerations such as a low sodium diet.

One way to improve VA for HD may be through the implementation of an adjustable anastomosis. Most VA associated complications arise through the change in vascular

hemodynamics. AVF and AVG placement results in an increased cardiac output, increasing the cardiac burden and risk of cardiac events[13]. Turbulent hemodynamics and high flow at the anastomosis have been associated with shunt stenosis, and AVF and AVG can both lead to VA steal syndrome[14,37]. Thus, ideally the anastomosis itself may be adjustable to allow for control of shunt flow. A thin synthetic graft section may be connected to the artery and vein in a side-to-side manner, thus normalizing blood flow when the graft is shut in a vertical manner. Such a system would allow the formation of an arteriovenous conduit only during dialysis procedures, potentially decreasing complications associated with permanent high arteriovenous flow.

To conclude, there is a clear societal need to improve upon current VA options, and numerous innovative paths are being explored by the field. Owing to the verified cellular remodeling capacity of TEBVs, the field of VTE may offer promising new VA options. To truly innovate VA for HD now and in the future, a multidisciplinary approach is essential to bringing about innovation, and progressing those innovations from mere ideas, to lifesaving treatments.

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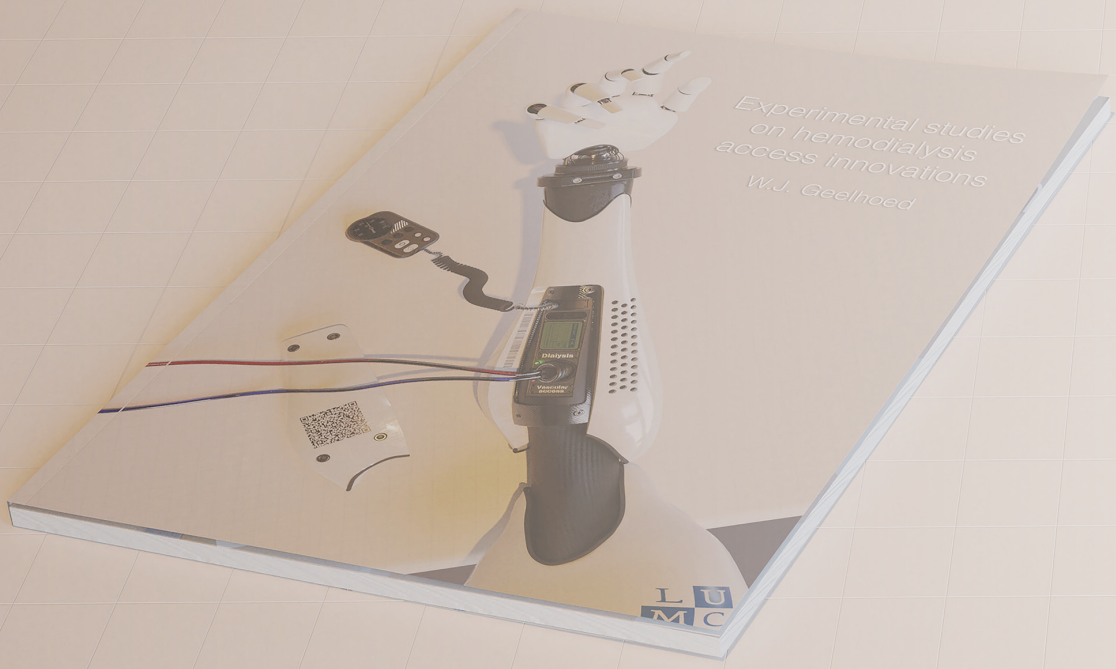
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Chapter 8

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Samenvatting in het Nederlands

Wereldwijd zijn er steeds meer mensen met chronisch nierfalen. Dit heeft als gevolg dat er steeds meer mensen afhankelijk worden van dialyse voor hun overleving. De meest voorkomende vorm van dialyse is hemodialyse, waarbij het bloed van de patiënt buiten het lichaam gefilterd wordt door een dialyse apparaat. Voor een efficiënte hemodialyse procedure is een vaattoegang nodig. De twee meest gebruikte vaattoegang methodes zijn de arterioveneuze fistel, en synthetische arterioveneuze graft. Bij een arterioveneuze fistel wordt een ader direct aan een arterie geplaats. Bij een synthetische arterioveneuze graft worden de ader en arterie met elkaar verbonden door middel van een synthetisch vat. Deze twee vaattoegang vormen zijn echter allesbehalve ideaal. De arterioveneuze fistel is vatbaar voor maturatie falen en de synthetische arterioveneuze graft is vatbaar voor infectie. Ook zijn beide methodes vatbaar voor het dichtgroeien van het vat in de vorm van stenose. Een alternatief voor deze vaattoegangsvormen zijn tissue-engineered bloedvaten, of TEBV.

Een methode om TEBV te ontwikkelen maakt gebruik van de reactie van het lichaam om een lichaamsvreemd materiaal, deze methode heet *in vivo* TEBV. **Hoofdstuk 2** beschrijft de manier waarop het lichaam met behulp van dit lichaamsvreemd materiaal, een weefsel kan vormen wat potentieel gebruikt kan worden als bloedvat. De subcutane implantatie van een lichaamsvreemd object leidt tot de vorming van een fibrotische weefselcapsule over het object heen. Deze weefselcapsule kan gebruikt worden als vervangend stuk bloedvat.

Het meest belangrijke aspect van een TEBV zijn de mechanische eigenschappen. Een TEBV moet namelijk dermate sterk zijn dat het geen gevaar vormt voor de patiënt. Data van dierproefstudies laten zich zelden echt accuraat vertalen naar de mens. Het is daarom belangrijk dat, voordat deze bloedvaten klinisch worden toegepast, er ruim voldoende data beschikbaar is over de mechanische eigenschappen van de *in vivo* TEBV in mensen. In **hoofdstuk 3** worden de drie meest gebruikte methodes getest voor het bepalen van de barstdruk van biologische vaten. Twee methodes hiervan zijn afgeleide methodes, wat wil zeggen dat een klein stukje weefsel wordt gebruikt om, door middel van een kleine proef én enkele formules, een schatting te geven van de echte barstdruk van het vat. De derde methode die bekend staat als de 'pressurized' barstdruk meting, is een standaardmethode waarbij meer weefsel gebruikt wordt om een realistischer beeld te krijgen van de barstdruk van een vat. Voor deze test is een speciale opstelling gebouwd en gevalideerd, om te zorgen dat de metingen goed verlopen. Beide afgeleide methodes gaven voor biologische én synthetische vaten een grote overschatting van de barstdruk ten opzichte van de standaardmethode. Bovendien was er geen correlatie tussen metingen van verschillende methodes die gedaan werden met dezelfde bloedvaten. De conclusie is dat afgeleide methodes niet gebruikt kunnen worden om de barstdruk van biologische vaten te testen en dat de standaard 'pressurized' methode nodig is om een beeld te krijgen van de mechanische eigenschappen een biologisch vat.

Hoofdstuk 4 beschrijft hoe de *in vivo* TEBV wordt geremodelleerd als deze als bloedvaten in de bloedsomloop worden geplaatst. Deze studie werd uitgevoerd in een groot diermodel, waarbij de *in vivo* TEBV één of twee maanden in de bloedomloop zaten als arterioveneuze

verbinding tussen een ader en een arterie. Als controle werd ook een synthetisch ePTFE vat als arterioveneuze fistel geplaatst.

Aanvankelijk bestaat de TEBV uit fibrotisch weefsel. Door middel van histologie werd aangetoond dat de cellen in de TEBV na implantatie in de circulatie steeds meer gingen lijken op cellen van een echt bloedvat. Een gezond bloedvat heeft een laag endotheel. Een van de taken van het endotheel is om te zorgen dat niets plakt aan de wand van het bloedvat. Eén van de hoofd experimenten van deze studie was het uitvoeren van testen om de functionaliteit van het endotheel van de TEBV te bepalen. Een op maat gemaakte opstelling werd gebouwd om delen weefsel van de TEBV én controle weefsel, in een microfluidisch systeem bloot te stellen aan identieke volumes bloed bij een constante schuifspanning. Dit experiment gaf aan dat het endotheel van de TEBV functioneel anti-trombogeen is. De barstdruk en hechtingretentiekracht van het weefsel waren boven de vereiste waardes voor veilige plaatsing in de bloedbaan. Aan het eind van één en twee maanden waren een vergelijkbaar aantal TEBV en ePTFE nog open. De TEBV hadden echter meer last van stenose. Deze studie liet zien dat als de *in vivo* TEBV in de bloedsomploop werd geplaatst, deze remodelleert om meer op een echt bloedvat te gaan lijken. Voordat deze TEBV methode in mensen worden toegepast moet er nog veel onderzoek worden gedaan naar de veiligheid van *in vivo* TEBV in mensen.

In vivo TEBV worden gemaakt door middel van de reactie van het lichaam om een lichaamsvreemd materiaal. Er is een maturatie tijd nodig voordat deze TEBV klaar zijn om als bloedvat gebruikt te worden. In **hoofdstuk 5** wordt er gekeken naar een methode om de maturatie van de TEBV non-invasief te kwantificeren. Folaat is een stof die onder andere belangrijk is voor de celdeling, deze hoopt zich onder andere op in macrofagen. Een folaat receptor agonist (een stof die chemisch gezien op folaat lijkt) werd gekoppeld aan de fluorofoor Indocyanine groen (een infrarood lichtgevende stof), om de stof genaamd OTL-38 te maken. Deze werd gebruikt om inflammatie in de weefselcapsule aan te kleuren. Deze kleuring kon gekwantificeerd worden door infraroodcamera's. Met de korte halfwaardetijd van OTL-38 en het afgesloten karakter van fibrotisch weefsel, bereikte de OTL-38 de weefsel capsule echter niet. Wel werd bevestigd dat OTL-38 een ideaal middel is voor de non-invasieve transcutane accumulatie van folaat.

Fout aanprikken van bloedvaten is een veel voorkomend probleem bij dialyse patiënten. Als de tweede vaatwand van een bloedvat wordt doorbroken kan er een hematoom ontstaan. Met name voor dialyse patiënten, die veel aangeprikt worden en een hoge bloedstroom hebben door hun vaattoegang, kan een hematoom een groot probleem zijn. Denk bijvoorbeeld aan pijn, herstellende operaties, of (tijdelijk) verlies van de vaattoegang. Een conventionele plastic dialyse naald bestaat uit twee hoofdcomponenten: de binnenste metalen naald en een buitenste plastic buis. De metalen naald wordt gebruikt om aan te prikken en wordt vervolgens via de buitenste plastic buis verwijderd, zodat alleen de plastic buis in het vat van de patiënt blijft. Huidige naaldtypes hebben geen automatisch terugtrek mechanisme en zijn afhankelijk van de gebruiker om de naald volledig te bedienen. In **hoofdstuk 6** beschrijven we het ontwerp en de proof-of-principle beoordeling van een nieuw ontwerp naald, die automatisch en snel terugtrekt bij vloeistof contact. Het systeem is gebaseerd op het verlies van de treksterkte van een cellulose of cellulose achtig membraan.

Het nieuwe ontwerp werd geoptimaliseerd en liet een automatische intrekking van de naald zien binnen 40ms bij 100mmHg. Gezien dit een proof-of-principle experiment betreft, zijn verdere optimalisatie experimenten noodzakelijk voordat dit systeem zijn intrede kan doen in de kliniek. Het systeem moet geïncorporeerd worden in bestaande naaldvairanten, of los ontwikkeld worden, om te voldoen aan de regelgeving voor medische hulpmiddelen.

Ten slotte geeft **hoofdstuk 7** een beschouwing en discussie van de studies die in dit proefschrift zijn beschreven én bediscussieert zij het onderzoek.

About the author

Wouter Jan Geelhoed was born on the 14th of November 1989 in Jakarta, Indonesia. He completed his high school education at the YCIS in Shanghai in 2008 and began studying bio-pharmaceutical sciences at Leiden University in 2009. During his bachelor's studies he focused on organic chemistry and performed an internship at the department of analytical chemistry at the LACDR. In 2012 Wouter began his master's degree in bio-pharmaceutical sciences. His first internship was performed at the CHDR in Leiden where he aided in the execution of clinical trial. A second internship was performed under the supervision of T. Bezhaeva at the Leiden University Medical Center investigating the impact of CKD on TEBV formation. During his master's degree Wouter followed numerous science-based business courses, underlining his interest in the commercial aspect of science. Mid-2015 he received his master's degree Cum-Laude in biopharmaceutical sciences with a specialization in pharmacology.

In 2015 Wouter began his PhD under the supervision of J.I.Rotmans at the department of internal medicine at the Leiden University Medical Center. The results of his research are published in peer-reviewed international scientific journals and presented in this thesis. Besides this, he has presented his research at several (inter)national congresses including the 2018 American Society of Nephrology congress and 11th Congress of the Vascular Access Society in 2019 (for which he won the best oral abstract award). He was the Leiden winner of the 2016 FameLab presentation competition and 2018 Kolff innovation challenge. He is co-inventor on two patents for medical devices in the field of dialysis vascular access and was the recipient of an NWO-take off grant for one of his innovations.

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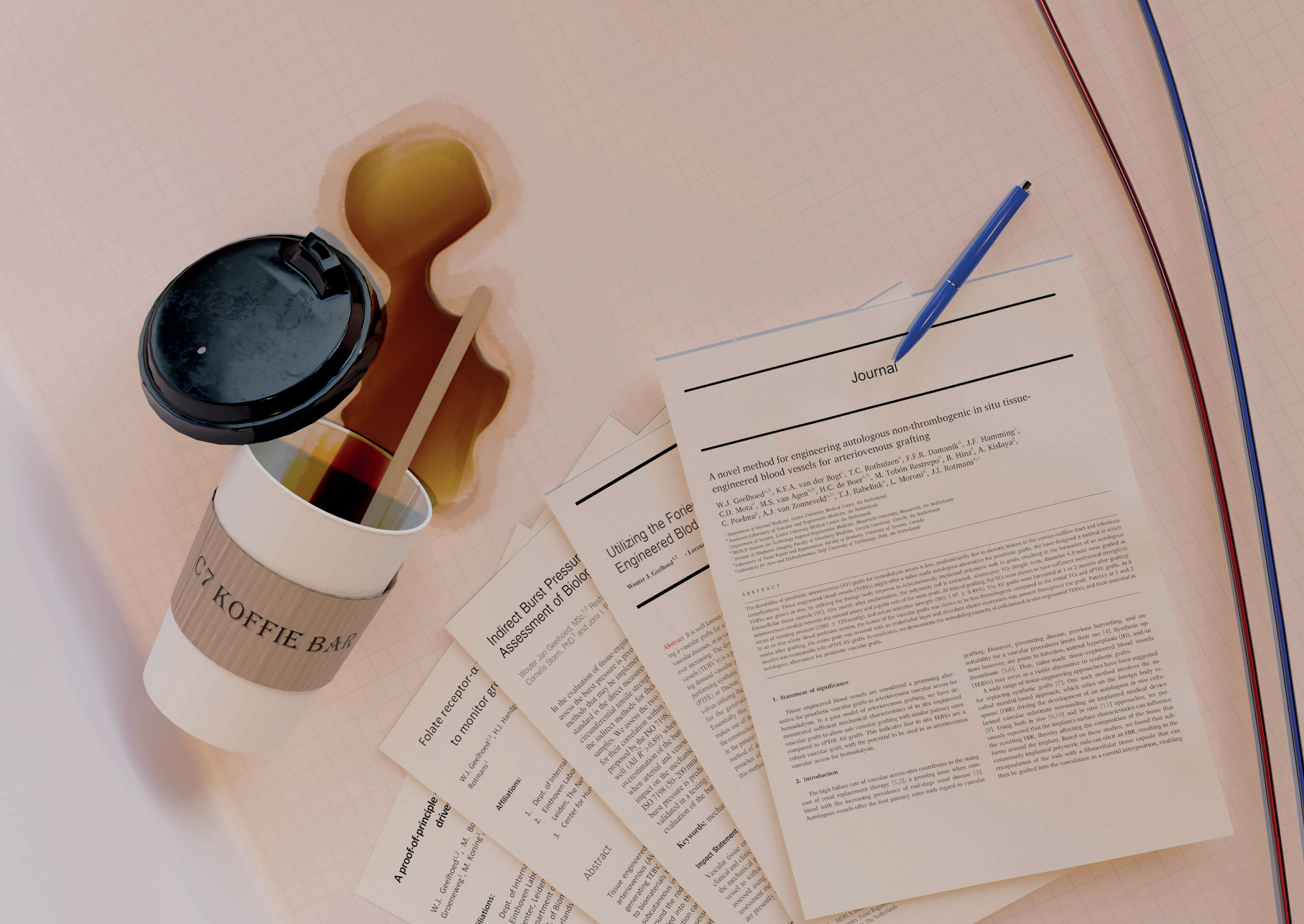
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Journal
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