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Development and evaluation of *in vivo* tissue engineered blood vessels in a porcine model

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ABSTRACT

Background: There's a large clinical need for novel vascular grafts. Tissue engineered blood vessels (TEBVs) have great potential to improve the outcome of vascular grafting procedures. Here, we present a novel approach to generate autologous TEBV *in vivo*. Polymer rods were engineered and implanted, evoking an inflammatory response that culminates in encapsulation by a fibrocellular capsule. We hypothesized that, after extrusion of the rod, the fibrocellular capsule differentiates into an adequate vascular conduit once grafted into the vasculature.

Methods and results: Rods were implanted subcutaneously in pigs. After 4 weeks, rods with tissue capsules grown around it were harvested. Tissue capsules were grafted bilaterally as carotid artery interposition. One and 4-week patency were evaluated by angiography whereupon pigs were sacrificed. Tissue capsules before and after grafting were evaluated on tissue remodeling using immunohistochemistry, RNA profiling and mechanical testing. Rods were encapsulated by thick, well-vascularized tissue capsules, composed of circumferentially aligned fibroblasts, collagen and few leukocytes, with adequate mechanical strength. Patency was 100% after 1 week and 87.5% after 4 weeks. After grafting, tissue capsules remodeled towards a vascular phenotype. Gene profiles of TEBVs gained more similarity with carotid artery. Wall thickness and αSMA-positive area significantly increased. Interestingly, a substantial portion of (myo)fibroblasts present before grafting expressed smooth muscle cell markers. While leukocytes were hardly present anymore, the lumen was largely covered with endothelial cells. Burst pressure remained stable after grafting.

Conclusions: Autologous TEBVs were created *in vivo* with sufficient mechanical strength enabling vascular grafting. Grafts differentiated towards a vascular phenotype upon grafting.

1. Introduction

Small caliber vascular graft failure is still a frequent complication in vascular surgery, involving substantial morbidity [1] and health care costs [2]. Autologous vessels are used as golden standard as they show superior patency when compared with synthetic grafts in various vascular settings, such as peripheral bypass grafts [3,4] and arteriovenous grafts for hemodialysis access [5,6]. The inferiority of synthetic grafts is attributed to the lack of compliance, thrombogenic surface and continuous presence of synthetic material in the vasculature [1,7]. Unfortunately, native vessels are frequently unavailable or unsuitable for surgical procedures [8,9]

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due to pre-existent vascular disease [10–12].

Tailor-made tissue engineered blood vessels (TEBVs) could offer a better alternative for prosthetic grafts. Most vascular tissue engineering approaches aim to develop off-the-shelf vascular grafts consisting of extracellular matrix (ECM) that in some cases is pre-seeded with vascular cells before implantation into the vasculature [13–15]. Recently, we described a method for *in situ* vascular tissue engineering that allowed us to generate autologous TEBVs *in vivo* in only a few weeks [16,17]. This approach utilizes the foreign body response (FBR) directed to a subcutaneously implanted polymer rod that culminates into the formation of a fibrocellular tissue capsule. Upon extrusion of the rod several weeks after implantation, the remaining tissue capsule is grafted into the vasculature whereupon it could differentiate towards a vascular phenotype. Indeed, flow and wall tension can enhance matrix synthesis [18,19] and vessel wall thickening [20], (myo)fibroblast to vascular smooth muscle cell (VSMC) differentiation [21] and luminal caliber remodeling to adapt the tissue to the present hemodynamic situation [20,22,23].

In previous *in vitro* [16] and *in vivo* [17] studies, we showed that the implant material's surface largely dictates the morphology and composition of the formed tissue. Based on these studies, we selected the optimal polymer compound and surface to generate a tissue capsule that forms a suitable basis for a TEBV. Our previous *in vivo* rat study revealed that the tissue capsules generated with this method were well vascularized capsules largely composed of circumferentially aligned collagen and (myo)fibroblasts. Here, we describe the development of TEBVs in a porcine model and the remodeling towards a vascular phenotype upon grafting as autologous carotid interposition graft.

2. Materials and methods

2.1. Study design

Experiments were approved by the Animal Care Committee of the University Medical Center Utrecht and performed according to Dutch legislation, using 4 female Landrace pigs weighing approximately 40 kg. Cylindrical shaped polymer rods as described below more detailed were implanted subcutaneously. Four weeks later, rods with tissue capsules grown around it were harvested. Per pig, 2 tissue capsules were used as autologous vascular graft for bilateral carotid artery interposition. Thus, in total 8 tissue capsules were bilaterally grafted. Remaining tissue capsules were used for histology, RNA isolation and mechanical assessment. A biodegradable, elastic sheet was wrapped around the vascular graft and anastomosis for temporal external support during the first period of tissue capsule remodeling after exposure to flow. As standard antiplatelet therapy [24], pigs received 80 mg/d acetylsalicylic acid starting 6 days before vascular surgery until termination and 225 mg clopidogrel one day before vascular surgery, continued at 75 mg/d until termination. Patency was assessed by angiography 1 week and 4 weeks after vascular surgery whereupon pigs were sacrificed and tissue capsules were harvested. Tissue capsules before vascular grafting were compared to tissue capsules 4 weeks after vascular grafting to evaluate tissue remodeling after exposure to flow and strain.

2.2. Implant material

Solid cylindrical rods of 8 cm length and 4.2 mm diameter were fabricated using a stainless steel compression molding device. Rods were composed of the co-polymer poly(ethylene oxide terephthalate)–poly(butylene terephthalate) (PEOT/PBT, Polyvation, The Netherlands), with a PEOT/PBT weight percentage of 55/45 and 300 g/mol molecular weight of the initial polyethylene glycol used for the copolymer reaction. The implant surface was modified by etching with chloroform as previously described [16,17]. Surface topography of all modified rods was evaluated using SEM. In addition, surface roughness (Rq) of unmodified and chloroform etched rods was measured with AFM, using Tapping 1 Mode (PicoScan Controller 2500, Molecular Imaging, USA) with a sharp TESP cantilever: 42 N/m, 320 kHz, 2–5 nm ROC, No Coatings (Bruker AFM Probes). For unmodified and modified rods, 4 rods were analyzed, using 4 spots of 25 μm^2 per rod. Roughness was assessed with Scanning Probe Image Processor, SPIPTM, version 4.2.2.0 software.

External electrospun sheets were composed of poly-ε-caprolactone (PCL) (Purac Biomaterials, The Netherlands). Rods and sheets were sterilized using gamma-radiation of >25 kGy (Synergy Health, The Netherlands). The effect of gamma-radiation on the surface was evaluated using SEM.

2.3. Surgical procedures

Per pig, 6 rods were implanted subcutaneously in the abdominal area. A small incision was made and a pocket of 8 cm length was bluntly prepared. Rods were inserted in the pocket and attached to the abdominal wall using 4-0 prolene sutures. The skin was closed intracutaneously. Four weeks after insertion of the rods, tissue capsules with the rods were harvested. In short, a longitudinal incision lateral to the rod was made and the tissue capsule was gently removed from the surrounding tissue. After harvesting, rods could easily be extruded from the tissue capsule. Subsequently, tissue capsules were bilaterally inserted as autologous carotid artery interposition. Briefly, a midline incision in the neck was made and the carotid artery was dissected. After infusion of 100 IU/kg heparin intravenously, the carotid artery was clamped proximally and distally of the anastomosis site. A 3 cm segment of the carotid artery was excised and replaced by 4 cm tissue capsule using 2 end-to-end anastomoses. Subsequently, the flow was measured using a perivascular flow probe. Next, the external sheet was wrapped around the tissue capsule and covered 1 cm carotid artery adjacent to each anastomosis. After wrapping around the graft, the 2 neighboring ends of the sheet were interconnected using 6-0 sutures as illustrated in Fig. 1D. To assess patency, an angiography was performed 1 week after vascular grafting and before termination at 4 weeks after grafting. The excised carotid artery segment was used for histological assessment and RNA isolation.

2.4. Cannulation of tissue capsule

In each pig, one of two grafts was cannulated with a 16G dialysis needle. After removal of the needle, the cannulation site was digitally compressed for at least 3 min or until hemostasis was reached. Time to hemostasis was recorded.

2.5. Tissue capsule analysis

Tissue capsules before and after vascular grafting were processed and paraffin-embedded. Serial 5 μm cross-sections of the middle of the tissue capsules before and after grafting were made for morphometric and (immuno)histochemical analysis. Moreover, RNA was isolated from tissue capsules before and after vascular implantation and from a non-operated carotid artery. RNA was profiled (AROS, Danmark) using a porcine microarray (Affymetrix, Germany). A heatmap of all 3 groups was generated. Subsequently, gene profiles of tissue capsules before grafting were compared to tissue capsules after grafting. Genes with ≥8-fold difference and $p \leq 0.0001$ were selected. In addition, genes associated with ECM

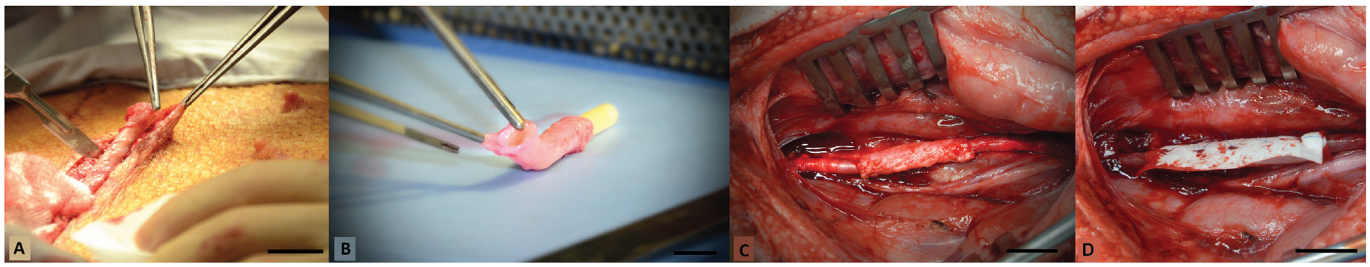

Fig. 1. Macroscopic pictures of the tissue capsule. A. Polymer rod *in situ* in the subcutaneous space is completely encapsulated by a fibrocellular tissue capsule. B. After harvesting of the tissue capsule, the tissue capsule is smoothly extruded from the polymer rod. C. Tissue capsule as carotid artery interposition. D. External sheet is applied around the tissue capsule and anastomosis. Scale bar represents 2 cm.

remodeling, endothelial cells and contractile VSMCs were selected and evaluated in our dataset. For mechanical evaluation, derived burst pressure was measured on 0.5 cm long tissue capsule segments before and after grafting. Suture retention strength (SRS) was measured on 1 cm segments of tissue capsules before grafting using 6-0 sutures and compared to similar specimens of porcine jugular veins and carotid arteries. Thirdly, compliance was measured on a 5 cm segment of tissue capsules before grafting. All analyses are described more detailed in the Supplementary Materials & methods section.

2.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data from the histological measurements and burst pressure before versus after grafting were analyzed with a paired t-test using SPSS. The other non-paired mechanical test results where tissue capsules were compared with native vessels were analyzed using a One-way ANOVA with Dunett post-hoc test with the tissue capsule values as reference. For luminal measurements where all groups were compared with each other, a One-way ANOVA test with a Tukey post-hoc test was performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Implant material

The smoothness of the unmodified rods was confirmed with SEM (Suppl. Fig. 1A). The roughness of the unmodified rods (Rq) was 68.68 ± 20.26 nm whereas this was 294.80 ± 25.58 nm for the chloroform etched rods. Chloroform etching resulted in all cases in homogenously spread porous structures of 0.5–2.5 μm in diameter (Suppl. Fig. 1B). SEM-images of the PCL sheet confirmed the specified fiber dimensions and porosity (Suppl. Fig. 1C). Gamma-irradiation did not affect the surface topography of the rods and sheets (data not shown). The Young's modulus of the PCL sheet after gamma-radiation was 9.0 ± 0.2 MPa.

3.2. Surgical procedure

Four weeks after subcutaneous implantation, a thick walled tissue capsule was formed around the rods (Fig. 1A). Tissue capsules could easily be harvested and subsequently smoothly extruded from the rod (Fig. 1B). All carotid artery interpositions went successful (Fig. 1C–D). One-week patency after vascular surgery was 100% (8/8) and 4-week patency was 87.5% (7/8). The initial tissue capsule diameter matched with the carotid artery diameter (Fig. 2C). One and 4 weeks after vascular surgery, some peri-anastomotic stenosis was observed on angiography (Fig. 2A–B) and confirmed by histology (Fig. 2D–E) and luminal measurements of the anastomosis (Fig. 2C). Subsequent to the anastomotic luminal narrowing, the luminal diameter of the tissue capsule increased throughout the graft reaching a maximum at the middle of the graft (Fig. 2C,F). The luminal diameter of the grafts increased with maintenance of adequate all wall thickness, thus suggesting outward remodeling in response to arterial blood flow and pressure.

3.3. Tissue capsule formation

Before vascular grafting, tissue capsules were mainly composed of ECM (Fig. 3B–E) and fibroblasts (Fig. 4E). Although wall thickness varied within each tissue capsule, no thin spots were observed. The ECM was composed of collagen I and III (Fig. 3B–C) and glycosaminoglycans (GAGs) (Fig. 3D). In contrast to the abundance of collagen, elastin was scarce (Fig. 3E). There was no calcification (Fig. 3F).

Tissue capsules were well-vascularized as illustrated by the von Willebrand factor staining (Suppl. Fig. 2B), but as expected lacked an endothelial luminal lining (Fig. 5A). Although some cells in the lumen were lectin+, they lacked the typical endothelial pattern as observed in native arteries (Fig. 5D). The first cell layers in the luminal area that approximated the synthetic rod were largely occupied by leukocytes (Fig. 4B), partly macrophages negative for M2-macrophage marker CD163 (Fig. 4C–D; Fig. 6). Hardly any foreign-body giant cells were observed. Apart from the luminal area, the majority of the tissue capsule was composed of vimentin+, α-SMA- and cytokeratin-fibroblasts (Fig. 4E–F; Fig. 6). In addition, some myofibroblasts (Fig. 4F; Fig. 6) were present, and very little cells expressed VSMCs markers; these cells were mainly part of the tissue capsule's microvasculature (Fig. 4G; Fig. 6).

3.4. Tissue capsule remodeling after vascular grafting

After grafting, tissue capsules largely remodeled into thick walled, homogeneous grafts. Paralleled by the increase in luminal diameter (Fig. 2C), the wall area and thickness increased as indicated by the collagen measurements (Fig. 6). The ECM remained largely composed of densely packed and circumferentially aligned collagen I and III (Fig. 3H–I) and GAGs (Fig. 3J). Elastin was scarcely present, only locally around the sheet at the “adventitial” site of the tissue capsule (Fig. 3K).

In contrast to the cellular composition before grafting (Fig. 4), tissue capsules after grafting were mainly composed of α-SMA+ and desmin+ VSMC-like cells (Fig. 6; Fig. 7E–G). Histomorphometry confirmed a 27-fold increase in desmin+ area and an almost 10-fold increase in percentage desmin-positivity of the total wall (Fig. 6). These VSMC-like cells were largely circumferentially aligned and in a non-proliferative state (Fig. 7H). The remaining cells in the tissue capsule were α-SMA+, desmin-myofibroblasts

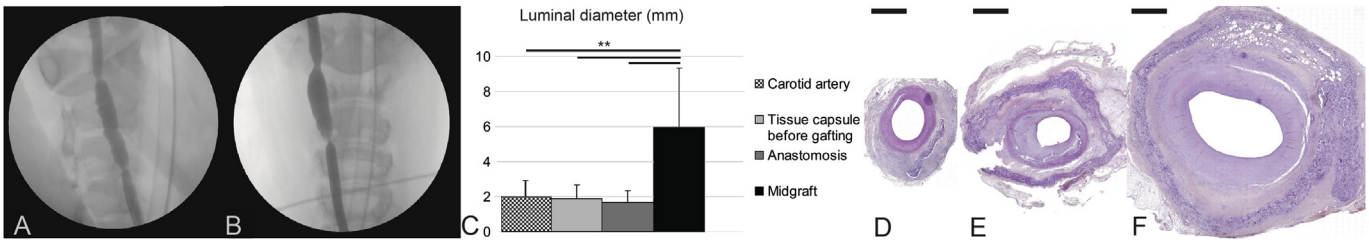

Fig. 2. Angiography of the carotid interposition after 1 (A) and 4 (B) weeks, showing a relative stenosis at the anastomotic area and some dilation of the tissue capsule. C. Quantification of the luminal diameter in mm indicates that the initial diameter of the tissue capsule fits well with the carotid artery. Four weeks after grafting the luminal diameter is declined at the anastomotic area but expands towards the middle of the graft. D–F: Haematoxylin phloxine saffron (HPS) stained cross-sections of D. carotid artery, E. anastomotic part of the tissue capsule and F. midgraft section of the tissue capsule. The stenosis in E is mainly composed of matrix and (myo)fibroblasts. Scale bar represents 2000 μm.

(Fig. 6; Fig. 7F–G). The leukocytes observed at the lumen before grafting (Fig. 4B) were barely present 4 weeks after grafting (Fig. 6; Fig. 7B). Instead, the lumen was largely covered with a confluent single endothelial layer demonstrated by a lectin stain exhibiting similar staining patterns as endothelial cells in a native artery (Fig. 5B–D).

The vascularization of the tissue capsule before grafting was interrupted during its harvesting. This could potentially affect the oxygenation of the tissue after grafting. However, 4 weeks after vascular grafting an extensive capillary network was present, especially in the outer layer of the capsule and in the external sheet (Suppl. Fig. 2C). Considering the above, these are likely in part newly formed capillaries. HIF1α and TUNEL staining confirmed no large areas of hypoxia or necrosis present (Suppl. Fig. 3). Throughout the tissue capsules, only little TUNEL+ spots were observed (Suppl. Fig. 3) which co-localized with the HIF1α staining. In addition, few Ki67+ proliferating cells were present in the tissue capsule 4 weeks after grafting (Fig. 7H).

3.5. mRNA profiles of tissue capsules

In total, 25,740 genes were profiled using RNA from the tissue capsules before and after grafting and from a non-operated carotid artery. Genetic profiles of the tissue capsule before grafting differed substantially from a porcine carotid artery as mRNA levels of 3749 genes were higher and mRNA levels of 3236 genes were lower expressed in the tissue capsules before grafting. In contrast, 4 weeks after grafting, these differences in genetic profile were considerably reduced. Compared to a carotid artery, mRNA expression of 1479 genes was higher and mRNA expression of 1548 genes was lower in the tissue capsules after grafting (Suppl. Fig. 4).

When tissue capsules after interposition were compared to before grafting, 662 genes were significantly differentially expressed ($P < 0.05$ with >2-fold difference, Suppl. Fig. 4B). Genes that both displayed ≥8-fold difference and $p \leq 0.0001$ were selected (Suppl. Fig. 4B, black box) and summarized in Suppl. Fig. 4C. In addition, genes associated with ECM remodeling, endothelial cells and VSMCs were selected. Differential expression of these genes is summarized in Suppl. Table 1–3. Whereas endothelial cell genes were both up and down-regulated (Suppl. Table 1), possibly reflecting both the luminal endothelialization as well as the turn-over of capillaries in the "adventitial" side, interestingly all VSMC genes were uniformly upregulated (Suppl. Table 2). Downregulation of tissue inhibitors of matrix metalloproteinases (TIMPs) combined with upregulation of some matrix metalloproteinases (MMPs) may suggest ECM remodeling (Suppl. Table 3).

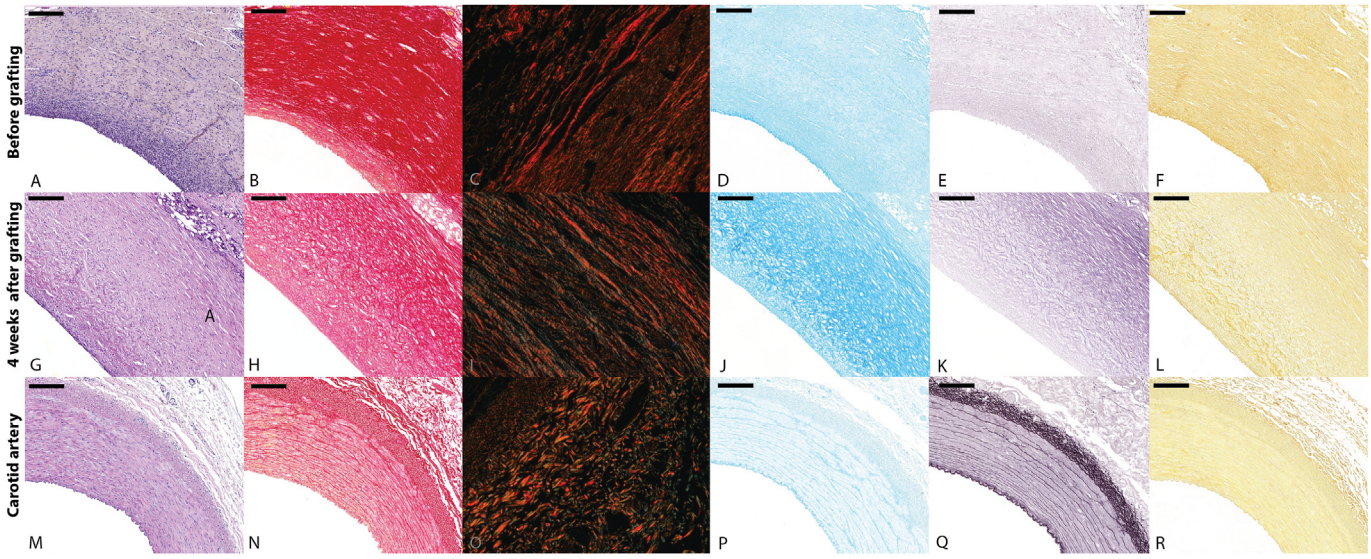

Fig. 3. Cross-sections of the matrix composition of the tissue capsule before grafting (upper row, A–F), tissue capsule four weeks after vascular grafting (middle row, G–L) and a native carotid artery (lower row, M–R). The lower row (M–R) can be used to compare the tissue capsule to a native blood vessel. From left to right: Haematoxylin phloxine saffron (HPS) staining, brightfield image of picrosirius red staining for collagen, polarized light image of picrosirius red staining, alcian blue staining for GAGs, Weighert's elastin staining for elastin and alizarin red staining for calcification. The matrix of the tissue capsules is largely composed of collagen type I and III (B–C, H–I, N–O) and GAGs (D, J, P). In contrast to a native vessel (Q), little elastin is present (E, K). Similar to a native vessel (R), no calcifications in the tissue capsules before (F) or after grafting (L) were observed. Scale bar represents 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

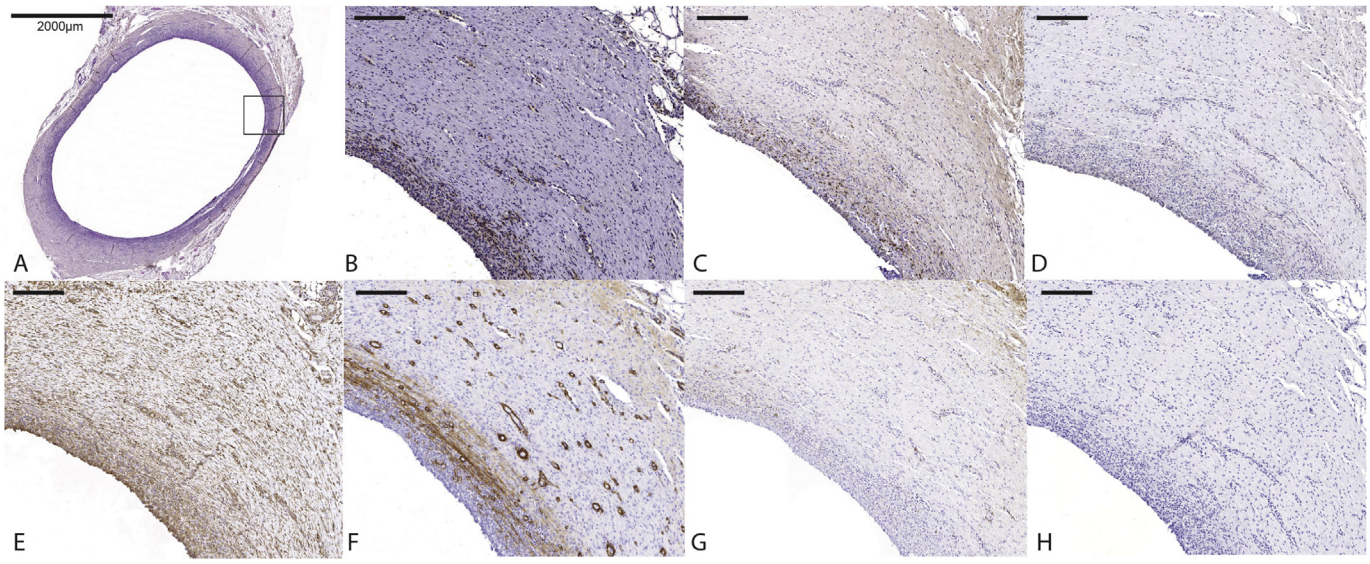

Fig. 4. Cross-sections of the cellular composition of the tissue capsule before vascular grafting. A. Haematoxylin phloxine saffron (HPS) overview, B. CD45, C. anti-macrophage, D. CD163, E. vimentin, F. α-SMA, G. desmin and H. Ki67. Tissue capsules were mainly composed of fibroblasts. The luminal area was covered with leukocytes, in part macrophages negative for M2 marker CD163. Scale bar represents 200 µm.

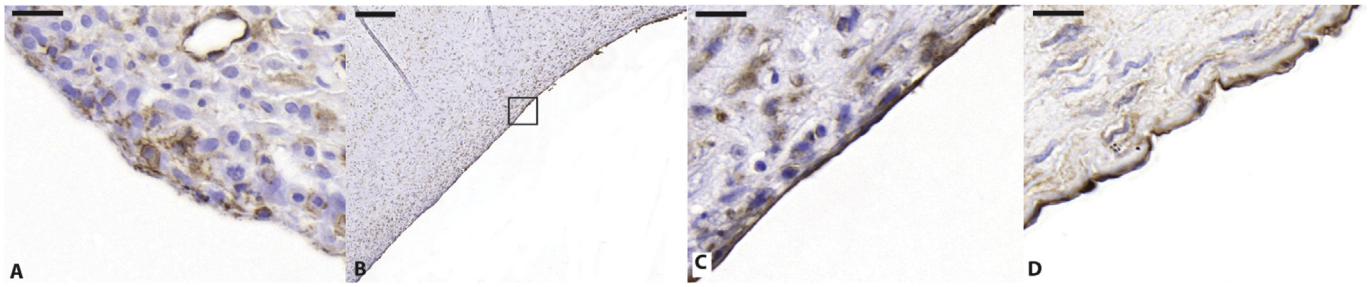

Fig. 5. Lectin staining of A. tissue capsule before grafting, B–C. tissue capsule after grafting, D. carotid artery. Although some cells in the tissue capsule before grafting (A) stain positively, this is not a typical endothelial staining pattern as observed in a native artery (D) and are more likely leukocytes that can also be lectin positive. In contrast, four weeks after vascular grafting, a confluent lectin positive line (B-c) typical for endothelial cells (D) was observed, suggesting endothelialization of the tissue capsule. Scale bar A, C–D represents 20 µm; scale bar B represents 200 µm.

3.6. Mechanical properties

The SRS, burst pressure and compliance of the tissue capsules before grafting as well as burst pressure 4 weeks after vascular grafting are summarized in Table 1. While the SRS was comparable to native vessels, the burst pressure was lower than in native veins and arteries. However, all tissue capsules had burst pressures well above proposed required values for TEBVs [25] and by far exceeding physiological values. Importantly, burst pressure remained stable 4 weeks after vascular grafting (Table 1). In contrast to the SRS and burst pressure, compliances values were low.

3.7. Cannulation of tissue engineered blood vessel

Tissue engineered carotid interpositions were cannulated *in vivo* with a dialysis needle. Time to hemostasis after digital compression

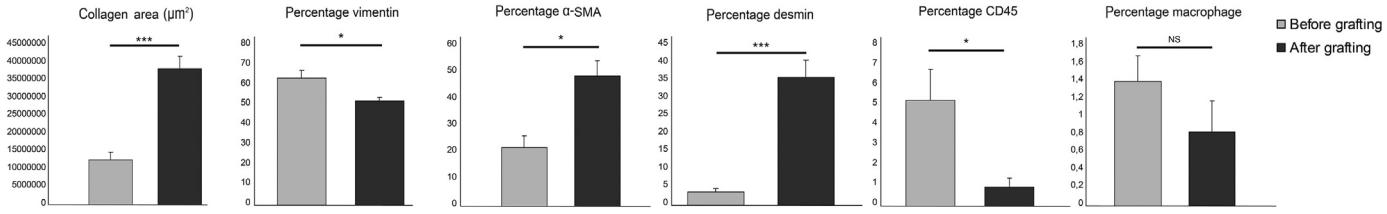

Fig. 6. Quantification of the tissue composition before and after vascular grafting. The total positive area in µm² and percentage DAB positivity in the tissue capsule before and after vascular grafting of fibroblasts (using a vimentin staining), myofibroblasts (using an α-SMA staining), VSMCs (using a desmin staining), leukocytes (using a CD45 staining), and macrophages (using a macrophage staining) as well as the total amount of collagen (using a picrosirius red staining) were quantified on brightfield images using ImageJ. Before vascular grafting, tissue capsules were mainly composed of fibroblasts as indicated by the vimentin quantification. Some leukocytes were present as indicated by the percentage of CD45+ cells; these were partly macrophages as indicated by the percentage of macrophages. After vascular grafting, tissue wall thickened as indicated by the increase in collagen content. In addition, the CD45+ leukocytes largely disappeared and more α-SMA and desmin positive cells were present compared with before grafting. NS: non significant, $^{*}p < 0.05$, $^{***}p < 0.001$.

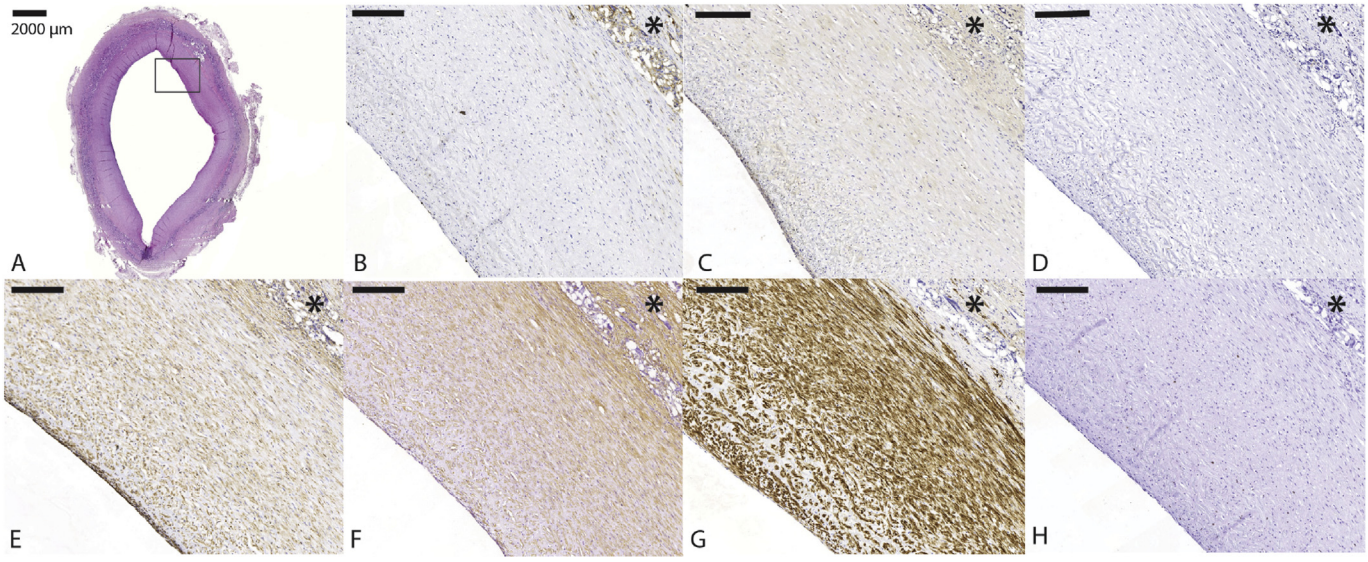

Fig. 7. Cross-sections of the cellular composition of the tissue capsule 4 weeks after vascular grafting. A. Haematoxylin phloxine saffron (HPS) overview, B. CD45, C. anti-macrophage, D. CD163, E. vimentin, F. α-SMA, G. desmin and H. Ki67. Tissue capsules largely remodeled during vascular grafting. Tissue was mainly composed of non-proliferative α-SMA+ and desmin+ VSMCs. The lack of proliferation suggests a steady state has been reached. The leukocytes present before vascular grafting largely disappeared. * indicates the location of the external sheet. The sheet is resolved due to laboratory processing. Scale bar of A. represents 2000 μm. All other scale bars represent 200 μm.

was in all cases ≤3 min, comparable to native vessels.

4. Discussion

In the present study, we developed an autologous, completely biological vascular conduit in only four weeks using the subcutaneous space as *in vivo* bioreactor, with sufficient mechanical strength to allow safe vascular implantation. During vascular grafting, this vascular conduit remodeled towards a vascular phenotype.

4.1. Remodeling of tissue capsule towards a vascular phenotype

Before vascular grafting, tissue capsules were mainly composed of ECM and fibroblasts. Fibroblasts are plastic cells that if stimulated can excrete and react to growth factors, chemokines and cytokines, differentiate, can acquire a contractile apparatus and play a key role in matrix synthesis and remodeling [26,27]. Importantly, flow and cyclic stretch are potent stimuli for ECM synthesis and remodeling [19,28,29], and even (myo)fibroblast to VSMC differentiation [21]. Indeed, 4 weeks after grafting, the capsule wall thickened and cell number and amount of ECM increased. This arterialization process is also observed in venous grafts exposed to arterial hemodynamic conditions [20,22,29]. In addition, after vascular grafting collagen fibers in the tissue capsule were more densely packed and circumferentially aligned, thus increasingly mimicking the medial layer of a native blood vessel. Probably, the exposure to arterial hemodynamics promoted further circumferential alignment of cells and collagen [30].

Remarkably, fibroblasts in the tissue capsule acquired contractile VSMC characteristics, as indicated by α-SMA and desmin expression, their non-proliferative state and the uniform upregulation of contractile VSMC-genes [31]. These VSMC-like cells were observed throughout the entire 4 cm length of the graft. Although migration of cells from the adjacent carotid artery cannot be fully excluded, the presence of these cells at substantial distance from the anastomosis rather suggests differentiation of fibroblasts into VSMC-like cells. Indeed, cyclic stretch of fibroblastic cells present in granulation tissue upregulates the volume fraction of myofilaments substantially *in vitro* and *in vivo* [21]. Underlining the above, total gene profiles suggested that tissue capsules 4 weeks after vascular grafting gained more similarity with a carotid artery in gene profiles as compared to tissue capsules before vascular grafting.

4.2. Extinction of the inflammatory response after removal of implant material

The luminal area of tissue capsules at baseline was covered with leukocytes. These leukocytes were attracted by the presence of the synthetic rod as part of the FBR [32]. Implantation of synthetic material evokes an inflammatory response dominated by neutrophils and macrophages [32,33]. This gradually shifts towards a more fibrotic response characterized by (myo)fibroblasts and ECM deposition [32,34]. The inflammatory lining of the tissue capsule was in part composed of M1 macrophages, a key effector cell in the foreign body response [32]. Remarkably, these leukocytes largely disappeared after vascular grafting. This suggests that removal of the foreign body largely extinguishes the associated inflammatory response. This observation is in line with

Table 1
Overview of mechanical test results of tissue capsule before and after grafting as compared with a native vein and artery.

	Compliance (%/100 mm Hg)	SRS (N)	Burst pressure (mm Hg)
Carotid artery	–	6,08 ± 1,51	10.010,92 ± 1090,09
Jugular vein	–	6,01 ± 1,99	10.686,44 ± 1.992,08
Tissue capsule before grafting	7,46 ± 2,61	5,81 ± 1,08	3.947,26 ± 576,66
Tissue capsule after grafting	–	–	5.211,04 ± 358,32

previous studies showing resolution of the FBR upon complete degradation of synthetic implants [35] or successful encapsulation of a foreign body [32].

4.3. Endothelialization

In contrast to native vessels, the generated tissue capsules did not have a luminal endothelial lining at time of vascular implantation. The tissue capsules' lumen is rich of collagen and Tissue Factor, thus intrinsically thrombogenic. Nonetheless, no acute thrombosis was observed. The occlusion of the non-patent graft in our study was attributed to peri-anastomotic intimal hyperplasia rather than primary thrombosis. Although preferable, the absolute necessity of an endothelial lining in TEBVs prior to vascular implantation in especially larger diameter and high flow situations remains a matter of debate. For larger diameter, high flow applications such as hemodialysis vascular access and above the knee bypass grafts, it has been proposed to use TEBVs without endothelial lining [13]. Conventional ePTFE grafts do not have an endothelial lining either, yet show little acute thrombosis. Indeed, if present, thrombosis is mainly secondary to stenotic intimal hyperplastic lesions [24]. Possibly, if the flow is high enough, in the presence of anti-platelet therapy, platelets will not adhere substantially to the surface. Although most vascular tissue engineering approaches aim to endothelialize their vascular constructs, some reports of TEBVs with very little or no initial endothelial coverage showed excellent long-term patency in small [35] and large [13] animal models. Currently, large clinical trials with non-endothelialized blood vessels are being performed with little thrombosis in the first months of follow-up [14] (clinicaltrials.gov, NCT01872208 and NCT01744418).

Four weeks after grafting, an endothelial lining was observed largely throughout the vascular graft. Unfortunately, there are no porcine antibodies for endothelial markers that exclusively stain the endothelium on large blood vessels. Indeed, a vWF antibody only stained the vaso vasorum in porcine native arteries. In our experience, the commonly used CD31 marker does not cross-react with porcine endothelium when using immunohistochemistry. Therefore, lectin was used as alternative marker [36]. However, α-SMA positive cells and leukocytes can also express lectin [37,38]. Although sufficient α-SMA positive cells were present in the graft, only a single luminal layer stained lectin+, in a similar pattern as endothelial cells of a native artery. In addition, the single luminal cell layer that stained lectin positive, was negative for other markers such as α-SMA, CD45 and keratin, thus ruling out many other potential cell types. Although not irrefutable, in our opinion the above strongly suggests the presence of an endothelial lining 4 weeks after vascular grafting. In contrast to histology, RNA profiling results showed a mixed pattern of up and downregulation of endothelial genes. This may likely reflect the disruption of capillaries in the tissue capsule after harvesting from the subcutaneous space, the newly formed capillaries as well as the luminal lining of endothelial cells.

4.4. Mechanical properties of TEBV

Tissue capsules showed adequate mechanical strength to allow safe implantation. SRS was comparable to values of native arteries and veins, and well above required values [25]. Burst pressure values were lower than native vessels, but still well above the required 1600 mm Hg [25], and remained stable after vascular grafting. The amount and especially the circumferential alignment of collagen likely contributed to the mechanical strength [39]. In contrast, compliance was relatively low due to the little amount of elastin present in the tissue capsule. Indeed, induction of elastogenesis in adult cells is a major challenge in vascular tissue engineering [40]. Interestingly, flow and cyclic stretch are potent stimuli for elastogenesis [18,41] for elastogenic cells such as (myo)fibroblasts and VSMCs [42] present in the tissue capsule. Longer-term follow-up experiments should reveal if in time elastogenesis and consequently compliance are increased.

4.5. Intimal hyperplasia

Angiographies at 1 and 4 weeks after vascular grafting showed some luminal narrowing at the anastomotic site. Although our engineered grafts lack a well-defined intimal layer, histology confirmed a local accumulation of matrix, randomly aligned (myo) fibroblasts and VSMCs, all hallmarks for intimal hyperplasia [43]. However, this did not result in decreased patency. Indeed, we showed a patency of 87.5% after 4 weeks (7/8). Our end-to-end anastomotic configuration of the interposition is prone for luminal inversion of the rims creating a slight surgical stenosis. Unfortunately, no angiographies were performed directly after surgery to evaluate the surgical procedure. However, these lesions were present at the angiography one week after vascular grafting which suggest a significant contribution of surgically induced tissue injury. In addition, compliance mismatch of the graft with the carotid artery may contribute to intimal hyperplastic lesions [44]. As end-to-end configurations are less commonly performed in vascular surgery, future studies focusing on patency instead of tissue remodeling should use a more clinically relevant vascular model, such as an end-to-side bypass configuration or arteriovenous graft.

4.6. Other vascular tissue engineering approaches

Since the first TEBV created by Weinberg and Bell, the vascular tissue engineering field has rapidly expanded. Currently, a wide variety of vascular tissue engineering methods are being developed. Their common aim is to create a completely biological blood vessel that is rapidly available and cost-effective. Some groups have already reached clinical implantation. L'Heureux et al. were the first to generate a clinically applied biological TEBV, without the use of a synthetic scaffold. Concentric layers of cell sheets were generated from isolated fibroblasts to create a tubular structure and endothelial cells were seeded in the luminal side. Using this tissue engineering by assembly (TESA) method, TEBVs were created with sufficient mechanical properties to allow human implantation as arteriovenous graft [45]. Despite promising primary patency rates of 60% in 6 months [46], the TEBVs were encumbered with high production costs and long production time, thus limiting wide spread clinical use. Therefore, recently these TEBVs were devitalized and implanted as acellular arteriovenous graft [14]. In addition, the Human Acellular Vascular Graft as developed by Niklason et al. has recently reached clinical implementation. In this scaffold-based method, allogenic VSMCs were isolated and seeded on tubular, degradable polyglycolic acid scaffolds *in vitro* and cultured in a pulsatile bioreactor system. The scaffold was gradually replaced by ECM *in vitro* whereupon grafts where decellularized to remove all allogeneic cells. After successful studies in baboons and dogs [13], these TEBVs were recently evaluated in a clinical trial as hemodialysis access conduit [47]. Primary patency of these TEBV was 60% at 6-months, which is comparable to prosthetic arteriovenous grafts.

In contrast to the methods above, our *in situ* tissue engineering method rapidly generates cellular, biological, autologous TEBVs. In addition, laborious and expensive *in vitro* steps are circumvented.

4.7. In situ vascular tissue engineering

The concept of *in vivo* vascular tissue engineered has previously been studied. Sparks pioneered this method in 1969, using a so-called Sparks mandril [48]. This multi-layered implant device was typically implanted intramuscularly for several months and generated autologous tissue capsules with a Dacron mesh incorporated in the graft wall [48,49]. Although initial results as vascular graft in canine and human studies were encouraging [49,50], these grafts largely failed due to thrombosis and aneurysm formation [51–53]. Likely, these dismal results can be attributed to the random selection of biomaterials, the continuous presence of synthetic material and the lengthy implantation period of the implant device comprising several months, yielding tissue capsules with low cellularity. Indeed, the FBR is dynamic, starting with an inflammatory phase that in weeks shifts to a fibrotic response and after months, a relatively acellular collagenous tissue capsule remains [32]. It was concluded not to use this method anymore until more was known about the FBR [54]. Many of the questions regarding the mechanisms of the FBR, limiting the usage of this method, are presently answered, enabling optimization of this ingenious method. Recently we developed and tested a set of different physical, chemical and biological surface modifications on their ability to actively steer the tissue capsule formation showing distinct differences in tissue composition, arrangement and cell distribution per implant material [17]. The best functioning surface was used in the present study. Thus, in contrast to Sparks' studies, here we used preselected material specifically designed to evoke a controlled inflammatory response thus enabling tailored tissue formation. In addition, implant period was shortened, as implant period is crucial to the cellularity and composition of the FBR and the subcutaneous space was used as implant site rather than the muscle.

A similar *in vivo* approach was described using the peritoneal cavity [55]. Importantly, not all rods implanted in the peritoneal cavity were encapsulated by a tissue capsule [55,56], making it necessary to implant multiple rods. In addition, using the peritoneal cavity as bioreactor is much more invasive with risk of adhesions, limiting clinical application. Instead, the subcutis is easily accessible.

4.8. Study limitations

Our study has some potential limitations. Importantly, this is a short-term follow-up study especially valuable to describe the remodeling process after vascular grafting. Although there are promising short term results, long term follow-up studies are required to evaluate patency and complications typically associated with longer term follow-up, such as aneurysm formation.

Second, our blood vessels were evaluated in young, healthy pigs instead of cardiovascular compromised individuals, using an end-to-end interposition model. This model is frequently used to evaluate vascular grafts. Yet, in the clinical setting, end-to-end interpositions are less commonly used. In future long-term studies, the TEBV should be tested in a clinically more relevant setting. In addition, these pigs are known to have a high re-endothelialization capacity. Although the latter constrains direct extrapolation to the human situation, it does imply that endothelial cells are able to attach to the luminal surface of the tissue capsule.

Lastly, the question arises if the use of the external sheet is necessary in view of the high burst pressure. Indeed, the burst pressure of the tissue capsule before vascular grafting was sufficiently high. However, burst pressure measurements are short term measurement that do not account for tissue fatigue in the longer term after vascular implantation. Therefore, we started using an external sheet that might be omitted if the tissue capsule demonstrated to be fatigue proof after vascular grafting. Four weeks after vascular grafting, the burst pressure of the tissue capsule remained stable and was even non-significantly increased, likely due the increase of circumferentially aligned collagen. Thus, the TEBV seems to withstand the high arterial pressure. The application of an external sheet may limit the usefulness of our approach. Therefore, future experiments should be performed without the external sheet.

5. Conclusion

In conclusion, using the subcutaneous space as *in vivo* bioreactor, autologous tissue engineered blood vessels were rapidly developed with sufficient mechanical strength to allow safe implantation in the arterial circulation. Within 4 weeks, the TEBV further remodeled and “arterialized” into an adequate vascular conduit. Future experiments should reveal the long-term patency of these TEBVs.

Disclosures

All the authors declared no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.023.

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