Decellularization as a method to generate a new generation of vascular grafts

In vitro results and preclinical testing

Robin Simsa

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy at the University of Gothenburg
Sweden

UNIVERSITY OF GOTHENBURG

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Robin.simsa@gu.se

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The question is so good that I don't

want to spoil it with my answer

Robert Koch

Deadicated to the European Union, that

funded this research and enables me to

live a life in peace and prosperity

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ABSTRACT

Decellularization of blood vessels is a technique to remove cells from the extracellular matrix (ECM), which can be used as a vascular graft for peripheral or coronary blood vessel bypass surgery. This thesis focuses on the optimization of decellularization strategies for blood vessels such as porcine *vena cava*, to determine the optimal decellularization protocol (Paper I) and the ideal method of applying liquids during the decellularization process (Paper II). Our optimized strategy for blood vessel decellularization, which removes all cells from the ECM but leaves the mechanical properties and ultrastructure of the ECM intact, employs the detergents TritonX-100 and Tri-n-butyl phosphate in combination with the enzyme DNase, applied either by agitation or perfusion at low velocities. To test the utility of the decellularized vascular grafts, a preclinical animal study was performed by transplanting *vena cava* grafts in a pig animal model (Paper III). This study utilized decellularized blood vessels that were reconditioned with whole peripheral blood before transplantation. The results showed that blood vessels remained patent, resisted mechanical pressure and did not lead to a major immunogenic response. Taken together, this thesis describes a promising technique to generate novel vascular grafts based on decellularization on reconditioning of the ECM.

Keywords: Decellularized vascular graft, Tissue engineering, Reconditioning, Preclinical animal models, Reendothelialization

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SAMMANFATTNING PÅ SVENSKA

Blodkärl, som vener eller artärer, är organ i kroppen vilka kan betraktas som rör, eftersom deras huvudsakliga uppgift är att transportera blod från punkt A till punkt B. Blodet transporteras till alla delar av kroppen för att leverera syre och näringsämnen till cellerna och tillbaka till lungorna för att plocka upp nytt syre och ta bort biprodukter från cellernas metabolism. Hjärtat är pumpen som ger ett kontinuerligt flöde av blod genom hela kroppen. I medicinsk litteratur beskrivs ofta blodkärl och hjärta som det kardiovaskulära systemet. Sjukdomar i hjärt-kärlsystemet står för den största delen av naturliga dödsfall i industrialiserade länder.

Orsakerna till många av dessa sjukdomar är en kombination av miljö-, genetiska och livsstilsfaktorer. Minskat blodflöde som observeras vid kärlsjukdomar orsakas ofta av arterosklerotiska plack eller förtjockning av kärlväggen, vilket minskar kärlets innerdiameter. Detta kan leda till att vävnader inte tillförs den mängd blod som behövs, vilket i sin tur leder till brist av syre och näringsämnen. De flesta av dessa sjukdomar är kroniska, men patienter har sällan symtom under sjukdomens progression, vilket kan leda till akuta besvär.

Det finns många strategier för att återupprätta ett fysiologiskt blodflöde, det kan vara allt från medicinering till insättning av s.k stents, implantat som spänner ut det förträngda kärlet, eller kirurgi. Stentbehandling fungerar bra för mindre komplexa sjukdomar, men när sjukdomen är mer avancerad rekommenderas kirurgiskt avlägsnande av det sjuka kärlet och ersättning med ett vaskulärt transplantat. Vaskulära transplantat kan till exempel vara friska blodkärl som tas från ett område i patienten där borttagning inte orsakar dramatiska konsekvenser. Detta ses fortfarande som den ideala metoden för vaskulär transplantation, eftersom blodkärlet endast innehåller celler och proteiner från patienten, vilket innebär att det inte kommer att orsaka en immunreaktion och kärlet kan integreras väl i patienten. Problemet med denna metod är att den här typen av sjukdom oftast drabbar äldre personer där inga lämpliga blodkärl av god kvalitet finns att tillgå. En annan vaskulär transplantationsstrategi är då att använda syntetiska kärl. Denna behandlingsstrategi har emellertid nackdelen att den bara fungerar bra för blodkärl med stor diameter. I dessa typer av transplantat är återcellulariseringen med endotelceller mycket långsam eller obefintlig, vilket kan leda till fler komplikationer i framtiden. Med tanke på dessa begränsningar är utveckling av nya vaskulära transplantat ett viktigt mål för att förbättra behandlingen av patienter som lider av hjärt-kärlsjukdom.

I denna studie har vi skapat nya vaskulära transplantat baserade på decellularisering. Decellularisering är en metod där celler tvättas bort från den omgivande vävnaden, den extracellulära matrisen (ECM). ECM är en blandning av strukturella och funktionella proteiner vilka ger form och mekanisk stabilitet till alla vävnader i kroppen. Vid decellularisering används olika typer av kemiska tvättlösningar för att både förstöra cellerna och bindningarna mellan celler och ECM. Medan celler är specifika för varje individ, och immunsystemet kan upptäcka och angripa celler från en annan givare, orsakar ECM-proteiner inte ett så drastiskt immunsvar. Vår hypotes är att decellulariserade kärl accepteras av kroppen, både vad gäller immunogenicitet och mekanisk stabilitet. Hypotesen testades först genom att undersöka decellulariseringsprocessen och utveckla ett protokoll som tar bort alla celler men som inte signifikant skadar ECM. Detta transplantat användes sedan för implantation i gris för att utvärdera om detta vaskulära transplantat accepteras i djuret.

Vi har jämfört många olika protokoll med avseende på deras effektivitet att ta bort celler från ECM i blodkärl, utan att påverka kärlets mekaniska egenskaper och proteininnehåll. Vi fann att tvättmedlet "Triton X100" i kombination med DNase var det protokoll som fungerade bäst. Sedan använde vi detta optimerade protokoll för att utvärdera hur olika decellulariseingsmetoder påverkar den mekaniska stabiliteten av kärlets ECM. Vi gjorde detta genom att tillföra tvättmedel och enzym till kärlet på olika sätt; genom perfusion, skakning eller statisk inkubation (ingen rörelse). Där fann vi att skak eller perfusion med låg hastighet fungerar bäst för att ta bort celler samt att hålla de mekaniska egenskaperna intakta. När vi hittat en idealisk decellulariseringsmetod användes dessa decellulariserade kärl i en djurstudie. De decellulariserade kärlen förbehandlades under en vecka med blod från grisarna i studien. Blodkärlen (vena cava) sattes in i sex grisar och fick sitta i upp till 5 veckor. Vi fann att alla blodkärl förblev öppna den testade tidsperioden. Vi såg också att blodkärlet återuppbyggdes med celler från grisen, vilket är ett bra tecken på läkning av kärltransplantatet.

Sammanfattningsvis har vi upprättat ett protokoll för decellularisering av blodkärl med stor diameter och visat på att de erhållna vaskulära transplantaten kan användas säkert i en djurmodell. Detta resultat är en viktig förutsättning innan man testar detta nya vaskulära transplantat i kliniker på människor.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Simsa R**, Padma AM, Heher P, et al. Systematic in vitro comparison of decellularization protocols for blood vessels. PLoS ONE. 2018;13(12):e0209269.

II. **Simsa R**, Vila XM, Salzer E, et al. Effect of fluid dynamics on decellularization efficacy and mechanical properties of blood vessels. PLoS ONE. 2019;14(8):e0220743.

III. Håkansson J, **Simsa R**, Bogestål J et al. Personalized tissue-engineered veins as vascular graft transplants: a proof of concept study in pig. In Manuscript.

CONTENT

ABBREVIATIONS

CEC	Circulating endothelial cells
CVD	Cardiovascular disease
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
DAPI	4′,6-Diamidin-2-phenylindol
DC	Decellularization / Decellularized
DNase	Deoxyribonuclease
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ECs	Endothelial cells
FGF	Fibroblast growth factor
LDL	Low density lipoproteins
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
H&E	Hematoxylin-Eosin
HUVECs	Human umbilical vein endothelial cells
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
iPSC	Induced pluripotent stem cells

MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
PDGF	Platelet derived growth factor
PDGF	Platelet derived growth factor
PTFE	Polytetrafluoroethylene
RC	Reconditioned
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SMC	Smooth muscle cells
TEM	Transmission electron microscopy
TEVG	Tissue engineered vascular graft
t-PA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 GENERAL OVERVIEW

The heart and blood vessels are often collectively described as the *cardiovascular system*. The cardiovascular system transports blood throughout the body to supply cells in all tissues with oxygen and nutrients and remove carbon dioxide and other metabolites. The blood is also an important transport system for immune cells, proteins, hormones, signaling molecules etc. Hence, a functional cardiovascular system is critical for proper homeostasis.

Cardiovascular disease is responsible for a majority of natural deaths in industrialized countries [1–3], and the major part of cardiovascular disease is due to atherosclerosis. Atherosclerosis is caused by an inflammatory reaction to blood lipids that are retained within the vessel wall. Over time, the inflammatory response leads to the formation of atherosclerotic plaques that may significantly reduce blood flow and cause reduced blood supply to downstream tissues. The plaques may also rupture and cause acute thrombosis. Although the formation of atherosclerotic plaques starts early in life (20-30 years), patients often do not experience symptoms, such as angina pectoris, stroke and myocardial infarction, until decades later.

Many strategies exist to re-establish blood flow following clinical symptoms of atherosclerosis. The most common treatments are medication, stent implantation, and vascular surgery. Stent implantation following angioplasty has improved patency rate in recent decades, showing only 10% restenosis after 10 years post-surgery [4,5]. However, certain complex diseases or several affected vessels require surgical methods where the diseased blood vessel is replaced with a new vessel, a so-called *vascular graft*. Vascular grafts can, for example, be another blood vessel from the patient, such as the saphenous vein or the left internal mammary artery. The patient's own vessels often integrate well at the target site without adverse immune responses. However, this procedure is often performed in elderly patients, whose vessels might not be suitable for replacement due to comorbidities or previous bypass operations. Also, this procedure increases surgery time, cost and healing time of the patient.

Other types of vascular grafts are synthetic grafts, produced from different polymers. However, synthetic grafts are not suitable for the replacement of small diameter vessels (<6 mm), as occlusion due to thrombosis often occurs.

A way to avoid thrombosis is to seed the luminal surface of synthetic grafts with endothelial cells from the patient, but this is complicated and takes several weeks before the graft is ready for implantation. Given these limitations, developing new types of vascular grafts is an important goal to improve the treatment of patients suffering from atherosclerotic cardiovascular disease.

In this thesis, we created new vascular grafts based on the technology of decellularization. Decellularization is a method that removes cells from the extracellular matrix (ECM) of a tissue. The ECM is a mixture of structural and functional proteins that give form and mechanical stability to all tissues in the body. Chemical detergents are often applied for decellularization of the tissue, to weaken the bond between cells and the ECM. Commonly, these detergents lead to a disintegration of molecules in the membrane of cells, which therefore lose their integrity. Removal of cells makes the tissue much less immunogenic when transplanted since it is the cells that express the transplant antigens (HLA antigens). In contrast, ECM proteins do not cause a drastic immune response. To observe the suitability of decellularized vascular grafts for peripheral vascular bypass, we first investigated the decellularization process and developed a protocol that efficiently removes cells while keeping the ECM intact. Then, we tested if transplantation of decellularized vascular grafts is accepted without major complications in a porcine vein graft model.

1.2 BLOOD VESSELS

Blood vessels of the human body form a closed delivery system, which begins and ends with the heart [6]. Different types of blood vessels are separated by size and function and include arteries, arterioles, capillaries, venules and veins (Fig 1) [7]. Arteries and veins are the largest vessels and are important for the transport of blood from and to the heart. Arteries are vessels that carry blood away from the heart, and veins are vessels that carry blood back to the heart. In the systemic circulation, arteries carry oxygenated blood and veins deoxygenated blood, while the opposite is true in the pulmonary circulation. This thesis mainly describes observations and studies of larger-sized vessels, e.g. the *inferior vena cava*, which is the vein that brings all blood below the diaphragm back to the heart.

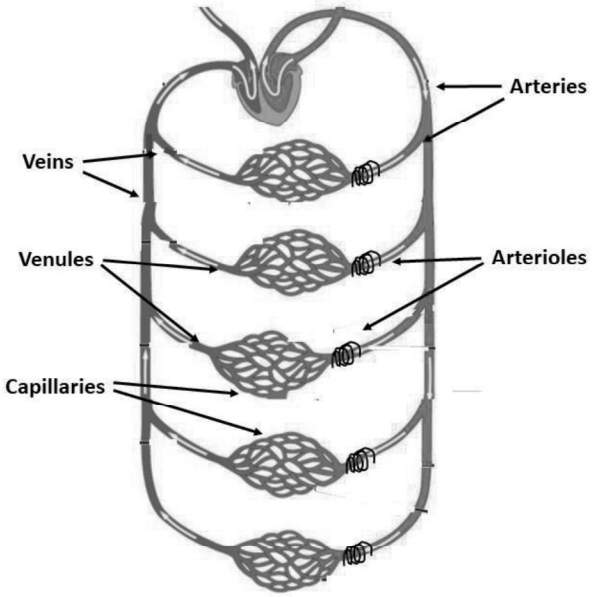

Figure 1: Structure of blood vessels in the human body. Source: wikimedia.org [8].

Morphologically, arteries and veins have a structure of 3 layers (*tunica*); the tunica intima, tunica media, and tunica adventitia (Fig 2). The **tunica intima** is the layer closest to the bloodstream and consists of a single layer of endothelial cells (endothelium) that serves as an interface between the blood and the vessel wall. In human arteries, the intima also often contains smooth muscle cells and ECM [9]. The **tunica media** consists mainly of smooth muscle cells and structural proteins such as elastic fibers, which are important for the mechanical properties of arteries and veins. The **tunica adventitia** consists mainly of collagen and fibroblasts that enforce and stabilize the blood vessel [6]. It is connected to nerves, capillaries and lymph vessels and acts as a reservoir of progenitor/stem cells which can act to repair vascular damage [10–13]. A morphological difference between veins and arteries is that arteries usually have a smaller luminal diameter and a thicker tunica media compared to veins [7]. Furthermore, veins especially in the lower extremities contain valves, which prevent the backflow of blood during transport back to the heart [14].

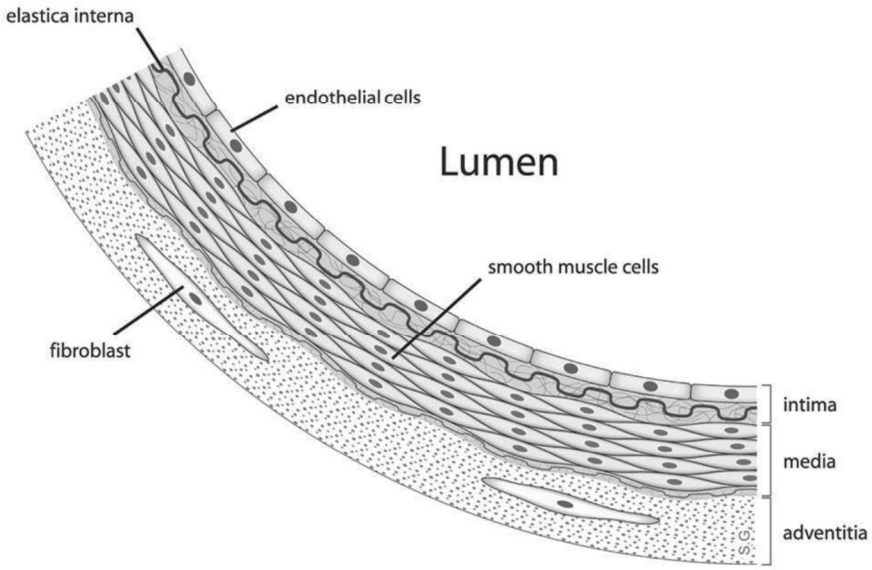

Fig 2: Graphical representation of the three layers of blood vessels. Source: wikimedia.org [15].

1.3 CELLS OF BLOOD VESSELS

Different cell types populate the blood vessels, which have a diverse distribution among the 3 layers of blood vessels. The most common vascular cells are endothelial cells (ECs) and smooth muscle cells (SMCs), that are described below. Other cells found in blood vessels include fibroblasts, pericytes and a variety of progenitor/stem cells [12].

1.3.1 ENDOTHELIAL CELLS (ECs)

Endothelial cells (ECs) are forming a homogenous lining on the lumen of the blood vessel, directly facing the blood. A continuous EC layer is important for the regulation of hemostasis, in multiple ways. First, ECs shield the blood from the underlying ECM proteins such as collagen, which can induce thrombotic events upon contact with blood [16]. Second, ECs secrete prostacyclins, nitric oxide and tissue plasminogen activator, which regulate thrombosis and fibrinolysis [17]. Another important function of ECs is the regulation of inflammatory reactions within the vessel wall, which is done by membrane proteins such as cell adhesion molecule 1, intercellular adhesion molecule 1, P-selectin or E-selectin, which bind circulating leukocytes and guide them to the vessel wall [18].

Given these important functions, damage or absence of the EC layer on the luminal surface can promote thrombosis, coagulation and formation of intima hyperplasia [19]. Therefore, treatment options for vascular diseases often try to reestablish a healthy EC layer.

1.3.2 SMOOTH MUSCLE CELLS

Smooth muscle cells (SMCs) are primarily found in the tunica media of blood vessels and are mainly responsible for mechanical functions, such as vasodilation and vasoconstriction of the vascular wall [20]. This is performed by contractile elements within the cells, composed of the proteins actin and myosin [21]. SMCs furthermore produce and secrete extracellular matrix (ECM) proteins, which give structure and support to the tissue. Arteries, in general, have a larger number of smooth muscle cells and higher elastin content compared to veins [16]

1.4 EXTRACELLULAR MATRIX

The structure which surrounds the vascular cells is the extracellular matrix (ECM). The ECM consists of many structural and functional proteins, minerals (e.g. calcium) and polysaccharides. ECM proteins are produced and secreted mainly SMCs and fibroblasts. The ECM should not be thought of as a permanent and unchangeable structure, as it can be remodeled dynamically by enzymes secreted by the cells (e.g. matrix metalloproteases) to adopt to tissue growth or react to diseases [22]. The exact protein composition of the ECM is organ-, and tissue-dependent and affects the mechanical properties and display of functional proteins. The most common proteins in the ECM of blood vessels include collagen, elastin, fibronectin, proteoglycans and laminins [23].

Collagen is the most abundant protein in the ECM, and up to now, 28 different forms have been described [24]. Collagen type I, III and V are predominantly present in the tunica media and adventitia of the blood vessel, while Collagen type IV is mainly present in the tunica intima directly underneath the EC layer. This has important implications for the initiation of thrombosis upon injury, as collagen is a strong trigger for thrombosis, however, type IV Collagen forms more basic structures, being less potent in initiating thrombosis [25]. Deeper injuries that expose the collagen types of the tunica media and adventitia therefor lead to a stronger thrombosis initiation, while milder injuries that only expose the collagen type of the tunica intima lead to a weaker thrombosis initiation. **Elastin** is a protein of major importance for the biomechanical properties of the ECM, due to the formation of insoluble elastic fibers which give tensile strength to the vessel and show recoil potential after deformation

[26]. **Fibronectin** and **Laminin** are two glycoproteins that are primarily found in the tunica intima and have been associated with cell attachment, as well as tissue growth and wound healing [27,28]. All of these proteins facilitate binding of cells to the ECM, by displaying different integrin receptor binding sites to which cells can attach [29]. Other functional proteins include **proteoglycans** and their **glycosaminoglycan** (GAG) sidechains, which act as binders of growth factors, cytokines and signaling molecules. Growth factors bound to GAGs include FGF, VEGF, HGF, IGF or PDGF.

While cells are highly immunogenic due to differences in surface antigens (e.g. HLA proteins) between individuals, the ECM has been reported as being less immunogenic [30]. This makes the ECM an interesting biomaterial for transplantation purposes, but only if it is ensured that cellular antigens are properly removed [31,32]. Previous studies have reported a T-cell driven immunogenic reaction of the ECM [33] and monocyte recruitment [34] when cellular components such as the HLA proteins or alpha/gal epitopes are not properly removed. Therefore, careful evaluation of antigen presence must be ensured before testing ECM in clinical trials [35].

1.5 CARDIOVASCULAR DISEASES (CVD)

Cardiovascular disease (CVD) is one of the leading causes of death and disability in the world. In 2014, 17.3 million people died worldwide due to CVD, and that number is expected to increase to 23.6 million in 2030 [36,37]. The term CVD includes a wide range of diseases, affecting either the heart or the vasculature. Vascular diseases are furthermore separated into *coronary heart disease*, which affects the arteries of the heart, and *peripheral vascular disease*, which affects blood vessels in other parts of the body. Vascular diseases often lead to a disturbance of blood flow, for example, due to an atherosclerotic plaque or venous valve insufficiency. This can lead to a restricted blood supply to tissues (ischemia) or disturbed return of blood to the heart. Many of these diseases may progress asymptomatic for many years [1]. Risk factors to develop CVD include genetics [38], age [39], smoking [40], obesity [41], high blood pressure [42], high cholesterol levels [43] or general diet [44]. Different treatment strategies to re-establish normal blood flow in vascular diseases are currently being applied.

1.6 CURRENT TREATMENT STRATEGIES FOR CVD

For prevention and stabilizing of vascular diseases, medications such as statins or antihypertensive drugs are being applied. For more progressed diseases with

significant luminal narrowing, vascular interventions such as angioplasty with stent implantation are performed to ensure blood flow to downstream tissues. However, thrombosis or intima hyperplasia leading to restenosis is observed in approximately 10% of the cases following the procedure [4,5]. To prevent initial thrombosis, anti-thrombotic or anti-coagulation drugs, such as aspirin, heparin, platelet glycoprotein receptor blockers or Ticlopidine are often applied [45]. For cases where stenting is not a suitable option, e.g. at injury sites near vessel branches or if a large area of the vessel is affected, bypass surgery is the recommended treatment [46]. For vascular bypass surgery, the diseased vessel is replaced with a vascular graft [47,48]. In the USA alone, 1.4 million arterial bypass operations are performed annually [36].

The main choice for vascular grafts today is autologous grafts, which utilize non-diseased blood vessels from the patient that can be removed without severe complications, such as the left internal mammary artery, the radial artery or the saphenous vein [49,50]. While arteries usually integrate better when used as an arterial bypass, harvest of veins commonly shows less severe complications and is less invasive [51,52]. The advantage of using an autologous graft is that it contains cells from the patient, and therefore does not cause an immune reaction [53]. However, autologous blood vessels can be of insufficient quality, or not available in cases where multiple vessels need to be replaced [54]. Donor site morbidity and longer surgery times are additional disadvantages [55]. Furthermore, patency rates for vein grafts are suboptimal, as 50% of the grafts fail after 10 years when used as coronary artery bypass grafts [56]. Other potential grafts are cryopreserved allografts, e.g. blood vessels harvested from a different donor and stored frozen until the time of implantation [57]. This method, however, shows complications with immune responses leading to graft rejection [58]. Other vascular grafts that can be used are synthetic grafts, such as grafts produced from polyethylene terephthalate (PET, marketed under the name “Dacron”) or polytetrafluoroethylene (PTFE) [59]. These grafts consist of polymers, which work well as large diameter graft replacements (>6 mm) and are conveniently available off-the-shelf. However, failure of graft in case of small diameter vessel replacement (<6 mm) is often observed due to thrombosis, and poor recellularization with endothelial cells *in vivo* decreases long-term efficacy [60]. Also, infection of synthetic vascular grafts is often observed [61]. Due to these unbeneficial attributes of both natural and synthetic vascular grafts, there is a clinical interest to develop new biomaterials with improved properties to be used as vascular grafts.

1.7 TISSUE ENGINEERED VASCULAR GRAFTS (TEVGs)

Due to the mentioned limitations of currently used vascular grafts, there have been numerous attempts to find suitable alternative grafts using tissue engineering. **Tissue engineered vascular grafts (TEVGs)** are based on biological materials and scaffolds [55,62]. One of the first descriptions of a TEVG was already published in 1986 by Weinberg and Bell, who used a collagen-based scaffold approach, which however was lacking mechanical stability [62]. Design requirements to generate TEVGs should consider the following points:

TEVGs should mimic the **mechanical properties** of the respective native blood vessel as close as possible [63]. This is important to ensure withstanding of the arterial blood pressure without bursting or failure at anastomosis sites. Moreover, the TEVG should have elastic properties to ensure compliance with the pulse waves [64] and to match the elasticity of the native blood vessel at the surgery site. When the viscoelastic behavior of the graft does not match the native blood vessel at the site of surgery, *compliance mismatches* can lead to disturbed blood flow and subsequent delayed healing or injury to the EC layer. Finally, the mechanical properties of the vascular graft also influence cell attachment and proliferation [65], which is crucial for re-endothelialization.

The geometry of the graft should resemble the size and inner diameter of the native diseased blood vessel that needs to be replaced, to avoid compliance mismatches that can lead to disturbed blood flow [66].

The graft should **not be immunogenic**, **thrombogenic** or **cytotoxic**. Immunogenicity can lead to (chronic) graft inflammation and ultimately graft failure. Thrombogenicity can lead to early graft failure due to thrombosis upon blood contact, and cytotoxicity can lead to cell death at the surrounding tissue and inability of cells to repopulate the graft.

Clinical and regulatory requirements should be taken into account for the safe and reliable production of TEVGs. Producing TEVGs should be operator-independent and easily reproducible, to ensure high quality and independence of batch-to-batch variations. Also, reagents and processes should be utilized which are approved for medical usage. Ideally, TEVG could be stored for some time and does not need to be used directly after production, to allow flexible adaptation to surgery scheduling. Also, the TEVG should allow suturing and adjustment of length by the surgeon prior to or during the operation. Additionally, quality control of each individual TEVG must be ensured with analytical methods that do not damage the graft [67].

Biodegradability of the graft is seen as important to allow the repopulation and remodeling of the graft by cells from the patient *in vivo,* to reestablish a functional cell population and avoid a foreign body reaction [68]. This is especially important for pediatric patients, whose bodies are still growing and who may require follow-up surgeries if the vascular graft does not follow this growth [69].

Multiple strategies to develop TEVGs have been tested to date [54]. Methods can be described as "Bottom-up" or "Top-down" approaches. "Bottom-up" utilizes cells or proteins to create a TEVG *de novo*. Strategies that apply this approach commonly utilize biodegradable polymers [70], silk [71], fibrin [72], collagen [73], chitosan [74] or stacked cell sheets [75,76]. Interesting approaches also include the generation of ECM by human fibroblasts, either obtained by the patient (autogenous) or from a fibroblast cell line (autologous), which can then be utilized following decellularization procedures. This process is currently in stage III clinical trials [77–79]. Other efforts to generate TEVGs also include 3D printing of blood vessels with a variety of hydrogels [80].

"Top-down" methods utilize an already existing 3D architecture as an initial starting point. Examples include cells seeded on biodegradable scaffolds that replace the scaffold with ECM over time [81,82] or decellularization of native cadaveric blood vessels from human or animal donors [83]. While many potential methods to generate TEVGs have been described, common clinical complications of vascular grafts must be considered for choosing an ideal alternative.

1.8 COMMON COMPLICATIONS OF VASCULAR GRAFTS

1.8.1 BLOOD COAGULATION AND THROMBOSIS

Blood coagulation is a result of platelet activation, which accumulate at a site of vessel injury and initiate a clotting cascade which ultimately leads to the deposition and maturation of a fibrin mesh on the injury site. It is an acute event, and occurrence in vascular grafts happens most drastically shortly after surgery.

The initial hemostasis is termed **Primary Hemostasis** and includes vasoconstriction and formation of a platelet plug. The platelet plug is commonly formed by exposure of collagen to the blood stream. Also, compliance mismatches between the native blood vessel and the synthetic graft

can disturb the blood flow and lead to platelet activation [84,85]. Platelets can bind directly to collagen via Glycoprotein Ia/IIa, and this bond is furthermore strengthened by molecules secreted from ECs, such as van Willebrand Factor (vWF). Binding of platelets to collagen initiates a signaling cascade, which includes release of platelet factors, such as thromboxane A2 (TXA2) and adenosine diphosphate (ADP). These factors activate Glycoprotein IIa/IIIb on the surface of platelets, which mediates binding of single platelets with each other.

Secondary Hemostasis is described as the process leading from activated platelets to the formation of a fibrin mesh at the site of injury. This process can be initiated either by intrinsic factors or extrinsic factors. The intrinsic pathway is slower and involves the exposure of platelets to collagen and the membrane of activated platelets. The extrinsic pathway is a rapid response to injury and initiated by exposure of tissue factor on SMCs and fibroblasts. The final enzyme of the coagulation cascade is thrombin. Thrombin cleaves soluble fibrinogen into the polymeric fibrin, which is a sticky mesh that stabilizes the platelet plug. During wound healing, the fibrin clot is degraded in a process called fibrinolysis. Fibrinolysis is a process mediated by ECs, which secrete tissue plasminogen activator (t-PA), which activates the fibrin-degrading molecule plasmin.

In vivo, blood coagulation in synthetic grafts and TEVGs is most commonly observed in smaller sized vessel with a diameter of less than 6 mm, due to lower blood flow velocity, resulting in contact activation at the blood-graft anastomosis sites [84]. Given the activation of platelets upon contact with collagen, decellularized or collagen-based grafts might exhibit a risk for initial thrombosis and coagulation. However, collagen types in the luminal ECM facing the blood flow are type IV, VIII or XVIII, which are less thrombogenic than other collagen types present in deeper layers of the tissue [16], even though immune responses and inflammation may still be observed [35,86,87]. Due to the potential thrombogenicity of collagen as well as for long-term viability, recellularization or reconditioning of a vascular graft is often seen as advantageous.

1.8.2 GRAFT INFECTION

Vascular graft infection describes the contamination of the graft with microorganisms. Contamination may occur due to intraoperative contamination or by extension of a nearby infection [88]. Occurrence depends on the anatomic location of the graft and varies between 1-5 % [89]. Mortality of graft infection is high, and can reach up to 30% and may require limb

amputation for survivors of peripheral vascular graft infections [90]. Microorganisms mainly responsible for graft infection include members of the *Staphylococcus* family, such as *Staphylococcus aureus*, *Coagulase-negative staphylococci* or *Staphylococcus epidermidis* [89].

1.8.3 INTIMAL HYPERPLASIA

Intima hyperplasia is defined as an increase in the number of cells in the tunica intima and media of a blood vessel. Increased cell number and deposition of ECM molecules leads to the occurrence of "intima thickening", which disturbs the blood flow by reducing the inner diameter size of the vessel. It is commonly observed in vascular grafts from 1 to 12 months or more following surgery [91]. Cells contributing mainly to intima hyperplasia are SMCs, migrating from the tunica media due to damage of the tunica intima, for example during surgery by cutting of the anastomosis [92]. A healthy endothelium inhibits the formation of intimal hyperplasia by the release of growth inhibiting factors, such as nitric oxide and prostacyclin [93]. Hence, at vascular injury this endothelial protection is lost and underlying SMCs start to migrate and proliferate in the intima [94]. Formation of intimal hyperplasia is associated with the severity of EC loss, as larger areas with loss of ECs leads to greater degree of intima hyperplasia than smaller areas [95]. Additional causes of intima hyperplasia include platelet activation, which act as a chemoattractant for SMCs to migrate from the tunica media to the intima, and inflammation, due to increased secretion of inflammatory cytokines and PDGF [94]. Intima hyperplasia is one of the major complications observed after surgical interventions, such as stenting or bypass procedures [96]. In TEVGs, intima hyperplasia can furthermore develop as a result of compliance mismatches [97].

1.8.4 ATHEROSCLEROSIS

Atherosclerosis is a disease of arteries, which manifests itself as a plaque formation, leading to narrowing of the vessel when plaque formation appears on the tunica intima. The atherosclerotic plaque is developing due to the accumulation of fatty and fibrous substances, and initiation is caused by low-density lipoprotein (LDL) particles.

Atherosclerosis is a chronic process that may develop in native arteries during decades without patients noticing symptoms. However, atherosclerosis is also a common mode of failure for vascular grafts and may be observed after time periods of 12+ months following surgery [98]. As atherosclerosis in vascular graft is often observed at accelerated time scales compared to native atherosclerosis, it is often referred to as *accelerated atherosclerosis* [99].

Accelerated atherosclerosis has been observed both in synthetic and autologous vascular grafts [100,101]. In autologous graphs, it is mainly observed when utilizing vein grafts such as the saphenous vein [98,102]. Causes for accelerated atherosclerosis include occurrence of intima hyperplasia [99] or compliance mismatches [103].

1.9 DECELLULARIZATION

As the ECM is the native environment of cells, both in terms of structure and protein composition, it is perceived as an ideal scaffold for many medical applications, such as vascular grafting [55], organ transplantation [104], hydrogel generation [105] or culturing meat from animal cells [106,107]. A promising approach to generate ECM grafts is decellularization (DC) of native blood vessels, which has been investigated for many decades already and has been described in multiple previous studies [108–115]. DC is the process of removing cells, cellular material and cellular antigens from the ECM [116,117]. Different DC methods exist (described below), however, all of them seek to find a balance between efficient removal of cells and antigens while preserving the integrity of the ECM. Unwanted effects of DC on the ECM may include denaturation or removal proteins, damage to the ultrastructure and surface structure or alteration of mechanical properties such as stiffness or tensile strength. While no standardized definition exists regarding whether a tissue can be regarded decellularized or not, less than 50 ng DNA per mg dry tissue and absence of nuclei in histological staining is often cited as a criterion [118]. It is likely that this definition may be refined and more specialized for different tissues as the research field advances.

1.9.1 DECELLULARIZATION METHODS

Different decellularization methods have been described, such as high hydrostatic pressure [119], supercritical carbon dioxide [120] or freeze-thaw cycles [121], however, the most common method of blood vessel DC is by using chemical and/or enzymatical methods [122,123]. Deciding on a method depends on the respective tissue and whether the structure of the tissue should remain intact or if structural integrity is not a priority (e.g. for hydrogel generation).

Common chemicals include ionic, nonionic or zwitterionic detergents (See Table 1). Ionic detergents are considered denaturing, while nonionic detergents and zwitterionic detergents are non-denaturing [124]. All these detergents consist of a hydrophobic “tail” and a hydrophilic “head” and are water-soluble. By binding to nonpolar proteins with their hydrophobic tail, while presenting

the hydrophilic head outwards towards the H_2O molecules, protein solubilization can be achieved [124]. Ionic detergents have an anionic or cationic headgroup (Fig 3). Due to their charged headgroup, ionic detergents can bind to both hydrophilic proteins and hydrophobic proteins (e.g. membrane proteins) [125]. By disrupting non-covalent protein bonds, such as electrostatic or van-der-Waals forces, ionic detergents lead to protein denaturation [126]. Non-ionic detergents have an uncharged rigid headgroup and do not penetrate hydrophilic proteins, which thus retain their native form. They can disrupt lipid-lipid and lipid-protein interactions by forming lipid-detergent aggregates [127]. Zwitterionic detergents have headgroups with both anionic and cationic charges, with a net sum of zero. They share characteristics of both ionic and nonionic detergents, as they have a neutral charge, but can in some cases break protein-protein bonds [128]. A summary of the most common detergents is given in table 1.

Fig 3. Sample image of the molecular structure of SDS, an ionic detergent. Source: Wikimedia [129].

While DC only with detergents has been described in the past, DC only with enzymes (e.g. trypsin) is rather rare and leads either to insufficient DC or damage to the ECM [130]. More commonly, DNA-degrading enzymes are applied following chemical treatment to remove remaining DNA content which easily sticks to the ECM. DNase is an endonuclease that cleaves DNA nonspecifically by cutting phosphodiester bonds [131]. Benzonase is another potential enzyme to remove DNA and RNA by cleaving nucleotides mid-sequence [131].

Table 1: Summary of common detergents utilized for decellularization.

Reagent	Category	Mode of action	Advantage (+) Disadvantage (-)	Refs
Triton-X 100	Non-ionic Detergents	Breaks protein-lipid and lipid-lipid bonds	+ Mild detergent, does not break protein-protein bonds - Might require more incubation time, DC efficacy can be lower	[132,133]
Sodium dodecyl sulfate (SDS)	Ionic detergent	Breaks protein-lipid, lipid-lipid and non-covalent protein-protein bonds	+ strong, effective DC agent - Aggressive detergent, can damage ECM proteins and surface structure	[111]
Sodium Deoxy cholate (SDC)	Ionic detergent	Similar to SDS	+ Similar effects as SDS, but less aggressive - Can also damage ECM proteins and ultrastructure	[134]
EDTA	Chelating Agent	Binds calcium, which is needed for protein bonding -> no re-bonding after lysis	+ Good to assist DC after detergent application - Not efficient alone as a DC reagent	[135]
CHAPS	Zwitterionic detergent	Solubilizing of membrane proteins (but no denaturation)	+ Mediocre detergent, balance between efficient cell removal and ECM protein integrity - Mainly applied for thin tissues as less effect is seen on thicker tissue	[136]

1.10 RECELLULARIZATION / RECONDITIONING

Recellularization or reconditioning (RC) of vascular grafts intends to recreate an endothelial cell layer on the luminal surface. RC is seen as a necessary step to prevent thrombosis and other undesirable effects upon surface contact

between blood and the graft, especially in smaller diameter grafts (less than 6 mm) [137].

Reestablishing the endothelial layer can take place naturally *in vivo* after transplantation by transanastomotic outgrowth [138,139]. While animal models often show complete recellularization of synthetic grafts weeks or months after surgery, this is not observed in humans, even after decades post-surgery [140]. In part, this has been contributed to animal studies utilizing grafts of small length and young animals with higher regenerative capabilities than typically elderly human patients receiving vascular grafts [140].

As natural healing of the endothelial cell layer is limited in humans, multiple strategies for *in vitro* endothelial cell seeding on the graft prior to transplantation have been tested [138,139]. However, it has often been observed that seeded cells do not survive long on the graft after transplantation, but are rather removed from the graft due to pulsatile blood flow [141,142]. This shows the need to further optimize vascular graft recellularization methods, both in terms of cell source and cell seeding.

1.10.1 CELL SOURCES

Endothelial cells can be obtained as primary cells, progenitor cells, stem cells or induced pluripotent stem cells (iPSCs). Primary and progenitor cells can be obtained by taking tissue biopsies or collecting circulating endothelial cells from the blood [143]. A disadvantage of utilizing primary cells is the limited population doubling number due to cell senescence and variation of cell populations between individuals [144]. Stem cells can be obtained from tissue biopsies; however, the biopsy is more invasive and stem cells require a robust differentiation protocol for endothelial cell generation. iPSCs can be generated from easily obtainable cells, such as fibroblasts, and can be kept in culture for prolonged periods [145,146]. However, this method is resource-, and time-intensive and displays some technical difficulties due to limited clinical experience and regulatory hurdles [147]. Given these limitations, more research into ideal starting cell sources and for clinical application is needed [55].

1.10.2 CELL SEEDING TECHNIQUES

Seeding ECs on the vascular grafts should be performed reproducible and lead to a homogeneous EC layer over the whole surface area of the graft. Multiple methods for cell seeding on vascular grafts have been described in the past, which vary in their level of complexity [148]. The simplest seeding method is by statically seeding cells onto the luminal surface of the graft. A limitation

however is that it is hard to achieve a homogenous cell population over the full luminal surface, and the method is highly operator dependent. Furthermore, the absence of physiological flow can have a negative impact on cell attachment and development [149]. A method to overcome some of these limitations is to coat the graft with proteins aiding attachment before static seeding, such as fibronectin, collagen or laminin [148]. This is predominantly performed in synthetic grafts. Dynamic cell seeding is a more advanced method and includes rotation, perfusion and vacuum-based systems, or combinations of these systems [148]. These systems lead to a more homogenous cell population, however seeding efficacy can be low, longer seeding times can be required and higher rotation speed may harm cell viability [55]. On the other hand, *in vivo* studies utilizing rotation-perfusion bioreactors in the past have also reported positive recellularization results [150]. Due to conflicting results, many of these systems need to be optimized to allow clinical implementation.

1.10.3 RECONDITIONING WITH BLOOD

As current methods to recellularize vascular grafts show limitations in terms of cell source and cell seeding, new or improved recellularization methods are of interest. One promising new method that has been described in the past is the reconditioning or recellularization of DC blood vessels with whole peripheral blood. This method has been tested in clinical application in several pediatric patients and has shown positive initial results [132,151,152]. The method is based on slow perfusion of whole peripheral blood from the patient through the DC vascular graft for one week. Whole blood contains plasma proteins, platelets, red blood cells and mononuclear cells, and the initial rationale behind this method was that peripheral blood contains circulating ECs (CEC) and endothelial progenitor cells which have the potential to recellularize the vascular graft surface [153,154]. While initial studies suggested recellularization of the vascular grafts both with ECs and SMCs in large numbers [132], these claims have probably been incorrect due to misinterpretation of immunostaining results, as another study on this topic concluded [155]. Indeed, the amount of ECs in the blood is rather low, as most studies report a range of 10-100 ECs per mL of whole blood [156–161], which is an insufficient cell number to fully recellularize a vascular graft with a luminal surface area of several cm^2.

While initial claims about “recellularization” might be incorrect, “reconditioning” with whole peripheral blood could have positive effects on vascular graft before transplantation. This is due to the attachment of proteins and other molecules from the blood on the vascular graft surface, which could

improve the incorporation of the vessel at the target site [162]. Perfusion of donated organs with whole blood or blood-derived solutions prior to conventional transplantation is an established method called *normothermic perfusion*, that has been shown beneficial for preserving the organ physiology and functionality previously [163–168]. While normothermic perfusion is commonly applied on donated organs to ensure cell viability, decellularized organs might also show beneficial effects of this procedure. One hypothesis is that priming of the ECM surface with whole peripheral blood prior to exposure to the bloodstream *in vivo* could reduce the risk of thrombotic events. Furthermore, reconditioned vascular grafts could assist the recellularization of the DC graft by endothelial outgrowth from the anastomosis sites [169].

1.11 ANIMAL MODELS

Different animal species are commonly used for preclinical testing of vascular grafts, including rodents, rabbits, dogs, pigs, sheep and non-human primates [170–172]. All these animals have certain advantages and disadvantages as a model for human immune response and blood clotting mechanism. Non-human primates such as baboons are often considered as an ideal model for vascular grafting, due to similarity to human cardiovascular physiology, immunological and blood coagulation mechanisms. However, large ethical concerns and costs limit their use for preclinical studies with vascular grafts. Sheep and goats are often used as models for carotid artery grafts, due to easy accessibility of the long neck, and have been described as possessing a coagulation system more similar to humans than pigs [173]. Pigs are other common models, having similarity to the human cardiovascular system, but quick growth often poses difficulties in terms of animal handling. Minipigs are sometimes used as an alternative as their growth is limited. Dogs and rabbits were more commonly used for vascular graft studies in the 1990s, following recommendations by the Cardiovascular Society of North America [174], but decreased in recent years due to animal welfare concerns. Rodents are very cheap models but can only be used for very small diameter graft studies. Given these limitations, animal models should be chosen with care with respect to the goals of the study.

2 AIM

The general aim of this thesis is to improve knowledge in the field of tissue engineering and regenerative medicine, mainly for application in the cardiovascular field for the development of new vascular grafts.

Specific aims of the different projects were:

a) To compare different protocols utilizing detergents and enzymes for large diameter blood vessel (pig vena cava) decellularization in terms of efficacy, mechanical properties, antigen removal and recellularization potential. (Paper I)

b) To observe the effect of different fluid dynamics on blood vessels during decellularization, such as treatment in static, agitational or perfusion conditions with detergents (Paper II)

c) To test vascular vein grafts, generated by decellularization of native porcine vena cava and subsequent reconditioning with whole peripheral blood, in a preclinical pig animal trial for a total time of up to 5 weeks. (Paper III)

3 METHODOLOGY

A summary of the study design, the aim, the research question, the tissue preparation and the analysis methods can be seen in Figure 4.

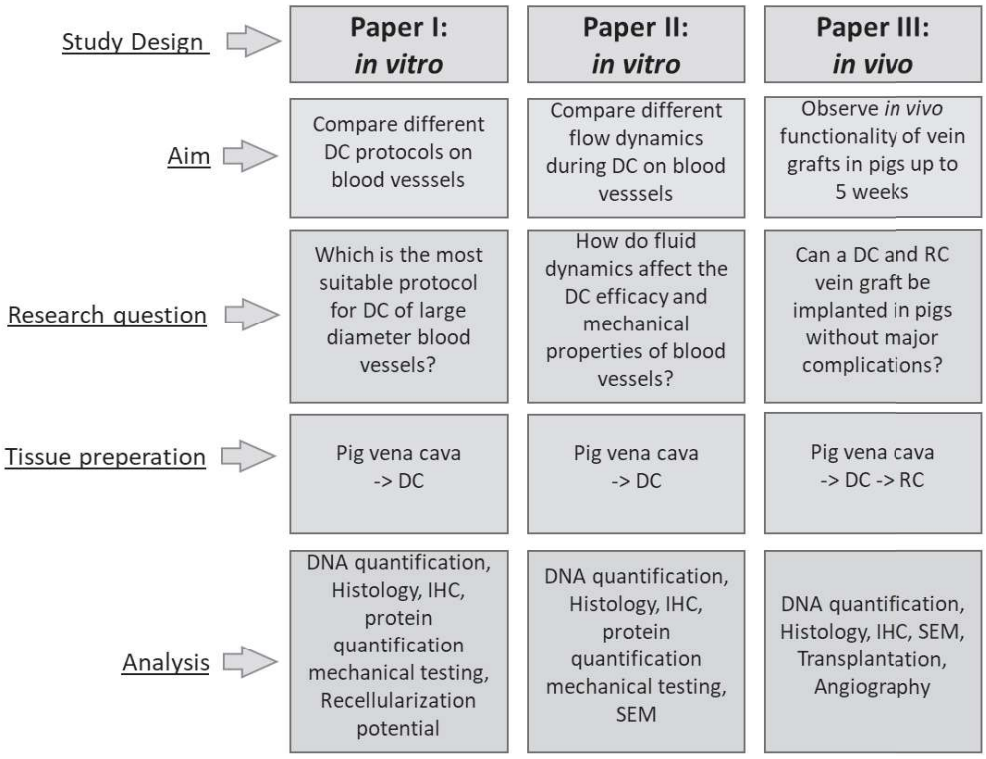

Fig 4: Schematic overview of different studies performed

3.1 ETHICAL CONSIDERATION

Ethical considerations were taken for multiple aspects of these studies. Vena cava utilized in Paper I and II were obtained directly from the slaughterhouse from pigs slaughtered for the production of meat for consumption. As pigs were not slaughtered specifically for this study and vena cava was a by-product of the slaughter, no ethical permit was required. Given that vena cava is not

usually utilized as a food product, but is rather discarded, we did not take considerations into limiting our sample numbers. Cells used in this study were obtained from commercial suppliers, not requiring ethical permission. For paper III, we obtained ethical permission for the transplantation of vascular grafts into pigs (#863-16) by the regional ethics board in Gothenburg. Animals were treated with care at a professional animal handling facility that gave the pigs access to outside areas and unnecessary suffering of animals was avoided at all steps.

3.2 DECELLULARIZATION

Blood vessels were obtained from a local slaughterhouse (Paper I and II) or the animal facility of Gothenburg University, EBM (Paper III). A suitable decellularization method was determined by comparison of the effect of various detergents/enzymes (Paper I) as well as different fluid application modes (Paper II) onto the native vessels.

3.2.1 PAPER I

For testing different DC methods, four different previously published DC protocols for blood vessels were chosen, due to being highly cited and applying a variety of different detergents (SDS, SDC, CHAPS, TritonX). Additionally, one novel protocol utilizing TritonX was developed, which consisted of longer incubation periods with detergents and DNase. As a strong effect of increased DNase incubation was observed, we tested the same protocol without DNase. All protocols were tested on pig vena cava in a custom build perfusion bioreactor (Fig 5). The bioreactor consists of a peristaltic pump which circulates liquids through the vena cava in a closed-loop system. The vena cava is connected with the bioreactor tubing via Luer connectors.

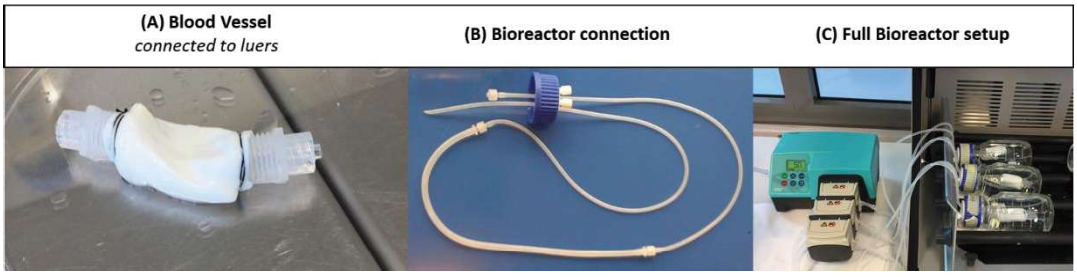

Fig 5: Decellularization bioreactor setup. (A) Vena cava ligated to female Luer connectors. (B) Bioreactor connection, which allows the connection of vena cava to

male Luer connector. The lid can then be screwed together with vena cava on a 250 mL glass flask. (C) Full bioreactor setup, showing 250 mL flasks with connected vena cava inside an incubation shaker. Tubings connect the blood vessel with a peristaltic pump to allow perfusion of detergents.

3.2.2 PAPER II

After mechanical alterations were observed with increased perfusion times, we wanted to observe if different perfusion speeds or other fluid application modes alter the mechanical properties of *vena cava* during DC. 5 different fluid application modes were tested with the DC protocol optimized in Paper I (Fig 6):

- Static DC – *Vena cava* was immersed in detergents/enzymes without movement.
- Agitation DC – *Vena cava* was incubated on an incubation shaker.
- Perfusion DC – *Vena cava* was connected to a perfusion bioreactor as described above, and perfused at a perfusion rate on 25, 100 or 400 mL/min.

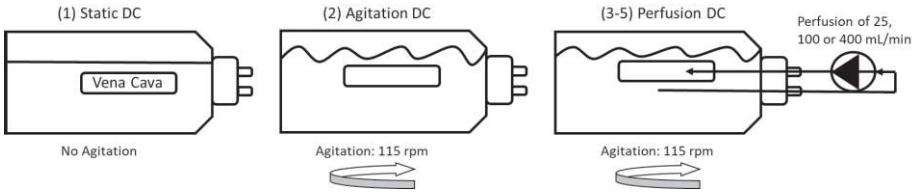

Fig 6: Schematic overview of the different DC setups tested in paper II.

3.3 MECHANICAL TESTING

Mechanical properties such as stiffness, maximum tensile strength and theoretical burst pressure were analyzed in a ringlet elongation test with a Zwick Roell testing machine (Fig 7) [175]. Firstly, a simple device was constructed to enable cutting of a DC blood vessel into equally sized ringlets with a width of 5.5 mm. Then, the length and thickness of the ringlet were measured for further calculation with the supplied software. Ringlet was put between 2 U-shaped metal holders and clamped into the testing machine. Then, the test was started by stretching the ringlet with a constant strain rate. The resulting stress-strain curve was reported, and mechanical properties calculated by the software.

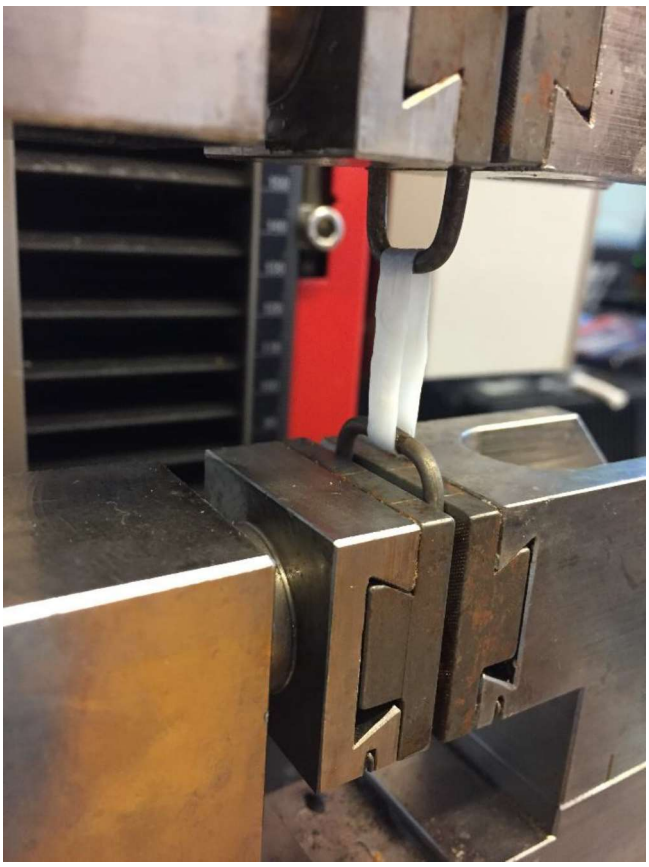

Fig 7: Mechanical testing setup. Vein ringlets of equally sized width were clamped into the testing machine, stretched and resulting stress-strain curve reported until vein ringlet failure.

3.4 IMMUNOSTAINING

Immunostaining is a staining method to detect specific proteins in a sample. While methods such as mass spectrometry, ELISA or western blot can give quantitative information about the protein content, immunostaining can give information about the location and distribution of proteins within a sample. First, a sample is fixed e.g. in paraformaldehyde to stop cell metabolism and prevent degradation of proteins or biological alterations within the sample over time. Then, the sample can be stained directly or embedded for paraffin-, or cryosectioning. If embedded, antigens should be retrieved by heating in a buffer or application of TritonX. Next, the sample is blocked with fetal bovine serum or milk to prevent unspecific binding of the antibodies to non-target proteins. A primary monoclonal antibody which is reactive with a specific protein is then incubated with the sample. The primary antibody is raised in a specific animal species and can be labeled by incubation with a secondary antibody which is reactive towards antigens from this species. The secondary antibody is usually conjugated with a fluorochrome, allowing visualization with a fluorescent microscope.

In our study, we used primary antibodies against alpha-smooth muscle actin (ASMA), collagen type I/III, CD31, CD45, laminin, fibronectin, vitronectin and elastin.

3.5 HISTOLOGY

Histology is a technique that is commonly used to examine biological structures and morphology with a microscope. Histological stains are not very specific to species or single proteins but can be used as a simple general assessment of the structure. Commonly, sections of tissues are stained with a solution that allows contrast between specific areas and highlighting of tissue features. Routine histology stains include hematoxylin-eosin (H&E), which stains nuclei (hematoxylin) in blue and collagen or muscle fibers (eosin) in red. Histological stainings are not as specific as immunostainings regarding single proteins or individual species.

3.6 SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is a method that visualizes the surface structure of a sample by scanning with an electron beam. While SEM only shows surface properties, methods such as transmission electron microscopy (TEM) would be more suitable to also observe deeper layers of the tissue. As

a preparation, the samples need to be dehydrated first with alcohol, which is later removed by critical point drying or the use of drying reagents. Samples are then coated with a non-conducting material, usually gold or platinum, to inhibit the charging of the sample and improving signal detection. Sample can then be observed in a vacuum condition in a suitable SEM.

3.7 RECELLULARIZATION IN VITRO (PAPER I)

The potential of DC blood vessels to be recellularized with endothelial cells was tested *in vitro*. Pieces of DC ECM were seeded with human umbilical vein endothelial cells (HUVECs) on the luminal surface to observe attachment and proliferation of these cells. For this, ECM pieces were soaked in growth media and incubated at 37°C for 1h to adapt to cell culture conditions. Then, HUVECs cultured until 80% confluency were added in a droplet on the ECM surface to allow attachment of the cells. After 90 min, more media was added to prevent drying out of the sample. After incubation for 7 days, cell viability was observed with calcein AM staining and quantified with MTS assay. The MTS assay is based on the reduction of the added tetrazolium compound to a colored formazan dye product, which is carried out only in metabolically active mammalian cells by dehydrogenase enzymes.

3.8 RECONDITIONING WITH WHOLE PERIPHERAL BLOOD (PAPER III)

Reconditioning was performed by taking blood from an animal donor (pig), mixing it with STEEN solution and a protein mixture and perfusing it through the vessel for up to 7 days in an upright position at room temperature. The vessel was at all times emerged in the blood-protein mixture. A bioreactor that was previously designed and presented specifically for this purpose was utilized (Fig 8).

3.9 ANIMAL MODEL AND SURGERY

Reconditioned vena cava were surgically transplanted into six pigs of the Yorkshire, Hampshire and Swedish Pigham breed. Additionally, two pigs were operated in a sham operation, e.g. by cutting the vena cava and resuturing it, as a control for the effects of the surgical procedure. The pigs were given antithrombotic medication (Acetylsiacylic Acid) from 6 days prior to the study, and then throughout the whole study until the pigs were killed. Surgery was performed by experienced human surgeons.

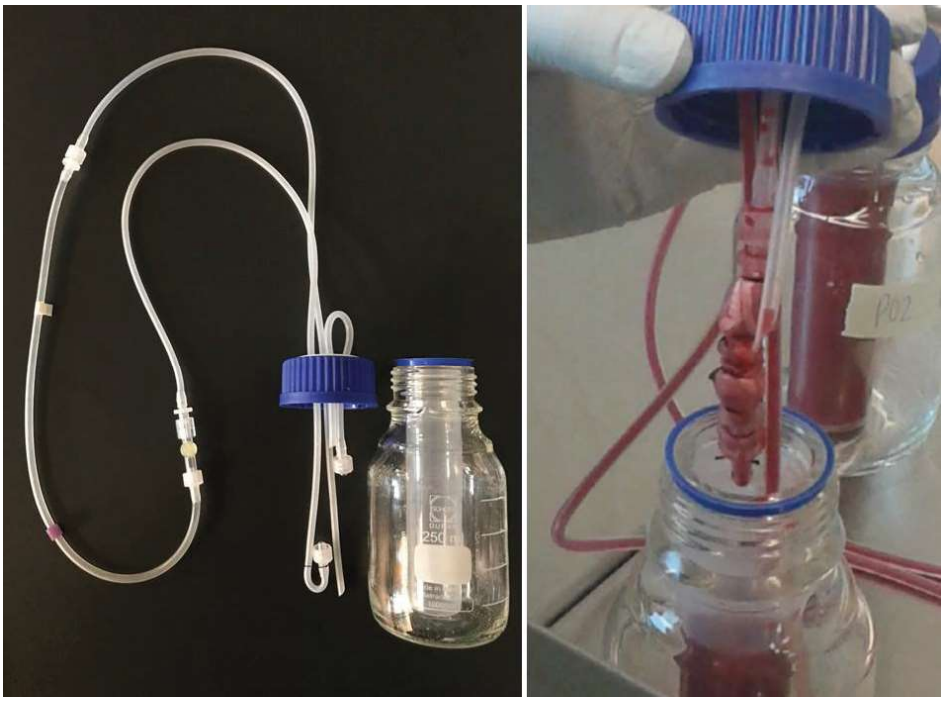

Figure 8: Recellularization bioreactor. 60 mL flat-bottom tube is fixed within a 250 mL Duran flask. Then, the blood-protein mixture is added and DC vascular graft connected to the Luers. Connection to a peristaltic pump allows closed-loop perfusion of the vascular graft with the blood-protein mixture.

3.10 PATENTS

The company VERIGRAFT holds a patent on the technology to recondition a decellularized blood vessel with whole peripheral blood from a specific donor to generate personalized blood vessels (USA patent office number: US9433706B2; European patent office number: EP2782995B1). No patents on decellularization processes are being held.

4 RESULTS

4.1 SYSTEMATIC IN VITRO COMPARISON OF DECELLULARIZATION PROTOCOLS FOR BLOOD VESSELS. (PAPER I)

In this study, we compared different previously published blood vessel DC protocols as well as a novel DC protocol. As published protocols typically have been tested on different blood vessels in terms of size, animal species or DC process setup, it is important to perform DC with different protocols in a standardized way in an equal process setup, to allow proper comparison of the effect of each protocol on blood vessels.

Comparison of DC with protocols using the detergents SDS [111], SDC [134], CHAPS [136] or TritonX [176] in combination with DNA removing enzymes were applied on porcine *vena cava* in a perfusion bioreactor setup. Also, a novel protocol utilizing TritonX was applied, which reduced total process time and resource input. DNA quantification after DC showed that all protocols successfully removed more than 99% of the nuclear content. These results were confirmed by histology staining, which did not show cell remnants on cross-sections of decellularized vena cava. Interestingly, when no DNase was applied, nuclear content was significantly higher, showing the importance of enzymatic treatment during DC.

Importantly, antigens were efficiently removed in all groups, as observed by ASMA staining. Proteins such as laminin and collagen were retained after DC, showing the preservation of ECM integrity. Mechanical properties of the DC vessels were tested in a ringlet elongation test, which showed an increased stiffness in groups with prolonged process times, showing a potential correlation between stiffness and perfusion time. Cytotoxicity was tested by adding small pieces of ECM to HUVECs grown on a 96-well-plate, which showed that HUVECs growth was not inhibited by the ECM in any group.

While all DC protocols showed similar results in terms of DC efficacy, antigen removal and protein retention, bigger differences were observed in terms of recellularization potential. DC ECM obtained by different protocols were seeded with HUVECs *in vitro* and incubated for one week. Then, cell viability was observed visually and measured quantitatively. The protocols utilizing TritonX led to a much higher recellularization with HUVECs compared to the other protocols. Also, the novel protocol utilizing TritonX showed an even

higher increase in recellularization potential than the previously published TritonX protocol. In summary, these results suggest that TritonX is a preferable detergent for blood vessel DC for recellularization *in vitro* or *in vivo*.

4.2 EFFECT OF FLUID DYNAMICS ON DECELLULARIZATION EFFICACY AND MECHANICAL PROPERTIES OF BLOOD VESSELS (PAPER II)

In Paper I, we observed that DC protocols with longer perfusion times led to increased mechanical stiffness of the grafts. Due to these results, we further investigated the correlation between blood vessel perfusion with detergents and subsequent mechanical stiffness of the ECM.

The effect of fluid dynamics on porcine vena cava was tested with the previously optimized protocol based on TritonX and DNase (Paper I). Detergents were either applied in a static, agitation or perfusion setting. Perfused groups were additionally tested at different perfusion velocities of 25, 100 or 400 mL/min. All groups were treated equally in terms of detergents and process times. We found that DC efficacy was equally sufficient in all the groups, as shown by DNA quantification and histology. Immunohistochemistry and protein quantification furthermore revealed that all protocols could retain the ECM protein Fibronectin, Vitronectin, Elastin and Collagen type I/III similar to the native blood vessel. Soluble collagen and GAGs, however, showed to be removed in all protocols.

Mechanical properties were retained in terms of stiffness and maximum tensile strength, however vascular wall thickness measurement revealed that the wall thickness decreased with increased perfusion velocities. These results indicate that while structural proteins responsible for the mechanical properties of the blood vessel are retained (elastin, collagen), other proteins might be washed out at higher perfusion velocities. The ultrastructure of the luminal ECM surface was observed with SEM, which showed that cellular remnants were present at the statically incubated group, while the 400 mL/min perfused group showed local tearing of the surface.

Taken together, these results suggested that DC either with agitation or low-speed perfusion (25 or 100 mL/min) is preferable compared to static or high-speed perfusion (400 mL/min) DC.

4.3 TRANSPLANTATION OF DE-, AND RECELLULARIZED VENA CAVA IN A PIG ANIMAL MODEL (PAPER III)

After optimizing the decellularization strategies for porcine *vena cava*, the next goal was to test the application of these grafts in a preclinical pig study. For this, native *vena cava* were treated with two steps:

1) *Vena cava* was decellularized with a previously optimized method, by utilizing TritonX and DNase in a low perfusion setup.
2) Decellularized *vena cava* was reconditioned with whole peripheral blood from individual pig donors. For this, blood was obtained one week prior to transplantation and perfused through the decellularized vessel for one week. Then, vessels were transplanted as vena cava replacement grafts into six pigs.

During the study period of five weeks, angiography confirmed that grafts remained patent. Observation of the grafts after explanation furthermore confirmed the absence of thrombosis or blood clotting. Also, no signs of intima hyperplasia were observed macroscopically. DNA quantification showed repopulation of the vessel with cells from the pig, and DAPI staining confirmed recellularization already from day 3 onwards. Additionally, the presence of a homogenous lining of endothelial cells on the luminal surface was confirmed by CD31 staining, which was fully covering the lumen after 5 weeks. Leucocytes were present at sites near the anastomosis, as confirmed by CD45 immunostaining, but less in the center of the graft, which is a sign that no general immune rejection occurred.

Taken together, this study showed first preclinical data that suggests that the utilized vascular grafts are safe to use in an animal model for up to five weeks and might be considered for clinical trials as a potential vascular graft.

5 DISCUSSION

The ECM has been investigated for many decades for its potential application in regenerative medicine and tissue engineering, both in preclinical animal studies and in clinical human studies [104,123,177–180]. ECM scaffolds are obtained by decellularization, which has been demonstrated for many different tissues and even organs, and a large number of protocols exist for blood vessels decellularization [108,109,111–113,181,182]. Clinical trials utilizing decellularized vascular grafts have mainly focused on hemodialysis access grafts, and only a few clinical trials have focused on vascular bypass grafts [179,183,184]. While recent developments show promising results for decellularized vascular grafts for peripheral artery bypass [185], also more mixed results have been reported previously [183,186], showing the need to further optimize the production of decellularized vascular grafts.

As the number of protocols and studies on decellularization increases, there is also a growing concern about the lack of standardized definitions regarding "successful decellularization", e.g. what data needs to be presented to define a decellularized tissue as suitable for clinical trials [187,188]. Optimizing the decellularization processes is critically important, as transplanting suboptimal decellularized tissues can have tragic consequences. This has been shown in a study with decellularized porcine heart valves, which were incompletely decellularized when transplanted into four pediatric patients, leading to the death of 3 of the patients due to a severe foreign body reaction [189].

To address these concerns, we first performed a systematic evaluation of different protocols and process parameters for blood vessel decellularization (Paper I and Paper II). Then, we tested the optimized decellularized vascular grafts in a pre-clinical animal trial (Paper III). All these tests were performed on porcine *vena cava*, which is considered a relevant model for human vessels due to similar geometry and histological structure [110].

The systematic comparison of different DC protocols based on the detergents SDS, SDC, CHAPS or TritonX interestingly showed very similar outcomes in terms of removal of DNA content, protein retention, and biodegradability. Mechanical properties such as stiffness showed to be slightly influenced by the total process time. However, the biggest variations were observed in the recellularization potential *in vitro* with HUVECs, as only TritonX-based protocols showed successful recellularization. TritonX based decellularization protocols have already shown successful recellularization potential in previous studies [190–192]. It has been shown that blood vessels decellularized with

TritonX retain growth factors such as FGF and VEGF, which might play a role in the increased recellularization potential [176]. Previous studies also showed low recellularization potential of tissues decellularized with ionic detergents [128,193,194], which might reflect on the potential of ionic detergents to denature proteins and disrupt protein-protein interactions [126]. Interestingly, the positive recellularization potential of blood vessels decellularized with CHAPS has been shown in the past [109,195], however, these results might reflect a different process setup and starting material, confirming the difficulty to compare individual decellularization studies.

While mechanical properties of decellularized blood vessels in all tested protocols were similar, there appeared to be a tendency towards increased stiffness when longer incubation periods were applied. The mechanical properties of blood vessels are of crucial importance for the integration of the vascular graft at the transplantation site and for withstanding physiological blood pressure. Additionally, stiffness and elasticity influence cell spreading, differentiation and attachment to the graft *in vivo* and *in vitro* [65,196]. For example, increased stiffness has been shown to lower the recellularization potential of the ECM with endothelial cells [197]. Synthetic grafts such as PTFE are usually stiffer than native blood vessels and do not show recellularization with endothelial cells even after decades. Increased stiffness can also cause compliance mismatches, which may result in pathological flow disturbances and formation of stenotic lesions [198]. Bottom-up generated vascular grafts (derived from cells or proteins) often report lower mechanical stability of obtained grafts compared with bottom-down generated vascular grafts (e.g. DC grafts) [54,62], however exceptions have been reported [199]. Given the importance of the mechanical properties of vascular grafts, we further investigated the influence of fluid application on the mechanical properties of decellularized blood vessels (Paper II).

To our knowledge, only a few studies have investigated the fluid application mode on DC efficacy to date [200–202]. Interestingly, a study investigating decellularization of lungs showed that no significant difference in the mechanical properties was found when comparing two perfusion decellularization modes [200]. Also in our study, we did not observe a change of mechanical properties when blood vessels were decellularized statically, under agitation or under perfusion. However, vessel wall thickness was significantly decreased in all groups, showing the highest decrease in the perfused groups. This indicates that structural proteins that influence the mechanical properties remain intact after DC, however other proteins that do not or only minorly affect mechanical properties are being washed out [24,26,203]. Given that whole organ DC often relies on perfusion DC [118],

these results can give valuable insights for the optimization of perfusion speeds, while less complex tissues can also be treated by agitation.

Together, the results of paper I and paper II show an optimized strategy for decellularizing large diameter blood vessels, specifically porcine *vena cava*.

After the decellularization method was optimized for *vena cava*, we tested it in a pig animal model, to observe the occurrence of adverse effects when tested as a vein-to-vein model (Paper III). Prior to transplantation, a novel reconditioning method was tested, consisting of perfusion of the decellularized vessel with autologous whole peripheral blood for one week. Reconditioning of vascular grafts with whole peripheral blood and a protein mixture has been already applied in the clinic on three pediatric patients for compassionate use [132,204]. These studies suggested a complete recellularization of the graft *in vitro* with endothelial cells from the blood after one week of incubation. However, another recent study on this topic suggested that recellularization does not take place, and that initial claims were a misinterpretation of immunostaining results [155]. Given these conflicting results, we aimed to further investigate the method of reconditioning with whole peripheral blood.

Indeed, our results did not show a complete recellularization of the vascular grafts with cells after one week of incubation *in vitro*. On the other hand, rapid recellularization of the transplanted blood vessel was observed *in vivo* after five weeks, as the luminal surface showed complete coverage with CD31 positive cells, which is a marker for endothelial cells. Also, the graft showed to be patent, non-immunogenic and mechanically stable under physiological blood pressure. Care must be taken when extrapolating these animal results to humans, as animals commonly show graft recellularization at a much higher rate, which is not usually observed in humans even after decades [137,205]. While the reconditioned grafts were patent in the animal trials, no comparison with only decellularized grafts (no reconditioning) was performed. It is possible that the reconditioning has no major effect on the graft outcome, which would eliminate the need to perform the blood reconditioning procedure and thus simplify the graft preparation process. This needs to be tested in future studies.

In summary, our results show the potential of decellularized blood vessels to be used as vascular grafts, potentially as vein-to-vein grafts for patients suffering from chronical venous insufficiency. While many protocols for blood vessel decellularization exist, the studies performed in paper I and II help to compare the advantages and deepen the understanding of previously published protocols. Animal studies, such as paper III, are important for testing immune

reactions, mechanical properties, and grafts remodeling *in vivo* before initiating clinical trials.

Decellularized tissues are holding the promise of being ideal biomaterials for recellularization with relevant cells to regain function and to alleviate the need for organ donors. Despite the long history of research on DC biomaterials, few clinical products have been approved so far [54]. As DC processes are continued to be better understood and optimized, ours and other results suggest that the main challenge for clinical application lies in the re-functionalization of DC structures [178]. Preparing DC materials with the cell-, and protein environment as observed *in vivo* will remain a challenge that needs to be tackled in future studies.

6 CONCLUSION

In conclusion, we were able to optimize DC protocols for large diameter blood vessels, both in terms of detergents/enzymes, as well as in terms of application of these fluids to the vessels during the DC process. Furthermore, we showed that decellularized and reconditioned vascular grafts can successfully be transplanted into pig animal models. The most important findings of each paper are listed below.

Paper I:

A combination of the non-ionic detergent TritonX with the enzyme DNase leads to the best decellularization and *in vitro* recellularization results on porcine *vena cava*.

Paper II:

Decellularization of porcine *vena cava* using detergents and enzymes is most efficient when liquids are applied with agitation or low-velocity perfusion (25 or 100 mL/min).

Paper III:

Perfusion of porcine *vena cava* with whole peripheral blood does not lead to recellularization *in vitro*. However, grafts can be transplanted into pigs without major complications such as thrombosis, infection or intima hyperplasia for up to 5 weeks. Also, complete recellularization with endothelial cells *in vivo* can be observed.

7 FUTURE PERSPECTIVES

Decellularized blood vessels are already applied clinically as hemodialysis grafts, however, there is only limited use as peripheral vascular replacement grafts. Our results from the animal trials are promising, as rapid recellularization with ECs was observed *in vivo*. However, previous studies suggest that recellularization results observed in animals are not observed to the same extent in humans. While decellularization is an attractive method to recreate the native cell environment, batch-to-batch variations, variabilities and operator dependency in the process design are a disadvantage for standardization efforts [206]. Already small alterations in the decellularization protocol might lead to a different outcome and reduce reproducibility. Also, donor starting material both for human or animal donors can be highly variable in terms of animal origin, race and breeding conditions; age; gender or diet. Especially age of animal could greatly affect the ECM composition, as the ECM is dynamically remodeled and shows a different protein composition at different life stages [207,208]. Studying the ECM as the native environment of tissues and organs can however increase our basic understanding of the native environment of cells, as well as help to define proteins and other factors which are crucial for proper cell function and might therefore lead to an improved design and manufacturing of synthetic or natural biomaterials in the future.

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