# STABILISED DECELLULARISED VASCULAR GRAFTS IN AN OVINE CAROTID MODEL

A dissertation submitted in fulfilment of the requirements for the degree:

**Master of Medicine (Surgery)**

Department of Surgery

Faculty of Health Sciences

University of Cape Town



by

**Dr Keno Mentor**

*BBusSc (UCT), MBChB (UCT), FCS (SA)*

MNTKEN001

Supervisors:

Assoc. Prof. Deon Bezuidenhout

Dr Tim Pennel

# DECLARATION

I, *Keno Mentor*, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: ..... Signed by candidate

Date: ……03 February 2018…….

# ACKNOWLEDGEMENTS

Our collaborators from Clemson University in South Carolina, USA - Prof Dan Simionescu & his research team

This project would not have been possible without the assistance of multiple stakeholders based at the UCT Cardiovascular Research Unit theatre, namely:

Dr Chima Ofoegbu

Theatre staff:
- Sr Rose Boltman
- Raymond Michaels

Laboratory
- Helen Ilsley
- Anel Oosthuyzen
- Joel Du Toit

Animal Unit:
- Dr Bert Mohr
- Rodney Lucas
- Sr Inge Botes

# ABSTRACT


Background

There is an urgent clinical need for an alternative vascular graft, especially for smaller artery applications such as in below-knee and coronary artery bypass. Currently available synthetic grafts have unacceptably low patency rates, while autologous saphenous vein grafts are not feasible in one third of patients.

Decellularised vascular grafts have been investigated as alternative conduits, but this chemical treatment results in degradation of the extracellular matrix. Chemical stabilization of elastin with penta-galloyl glucose (PGG) combined with collagen stabilisation during covalent heparinisation was previously investigated by our group in a small animal model and shown to be effective and safe. The current study describes their evaluation in a large animal (ovine) model.

Methods

Porcine mammary arteries were harvested, decellularised according to an established protocol involving rinsing with sodium hydroxide, alcohol (ETOH), treatment with DNAse/RNAse enzymes, immersion in PGG and subsequently surface modified with covalently bound heparin. Samples of the grafts were also tested for radial and suture retention strength.

The prepared grafts were implanted as interposition grafts into the carotid arteries of 6 sheep, using industry standard 6mm expanded polytetrafluoroethylene (ePTFE) on the contralateral side of each animal as control. *In-situ* patency was determined by ultrasound and angiography at two months, following which the grafts were explanted for macro- and microscopic analysis.

Results

*In-vitro evaluation:* Grafts showed significant levels of bound heparin (14.56 mg/g vs 0.69mg/g in untreated tissue) and demonstrated similar mechanical properties to those of human carotid arteries.

*Survival:* Five out of six sheep survived the full 2-month implant period, while the remaining animal developed sepsis shortly after implantation and was euthanized on day 4.

*Patency*: None of the decellularised grafts were patent at explant, as assessed by ultrasound, angiography and macroscopic examination. Two of the five control (ePTFE) grafts were patent.

*Microscopic analysis*: An inflammatory cell infiltrate with vascularised granulation tissue was found encasing the decellularised xenografts with little or no sign of endothelial cell infiltration. Signs of early occlusion, likely due to technical factors, was noted at the sites of anastomosis.

## Conclusion

Although demonstrating similar mechanical properties to human carotid arteries, and promising results in the small animal model, the stabilised decellularised vascular grafts failed to achieve endothelialisation or patency in this sheep carotid model. Significant calibre mismatch between the test graft and the native artery is thought to be the primary factor in the failure of these grafts, highlighting the potential difficulty in acquiring grafts of an appropriate size from animal sources.

# TABLE OF CONTENTS

## CHAPTER 1 - LITERATURE REVIEW



## CHAPTER 2 - MANUSCRIPT



## APPENDIX

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide | EDC |
| Cardiovascular disease | CVD |
| Coronary artery disease | CAD |
| Deionized water | DI |
| Endothelial cells | Ecs |
| Endothelial progenitor cells | EPCs |
| Ethanol | EtOH |
| Ethylenediaminetetraacetic acid | EDTA |
| Expanded polytetrafluoroethylene | ePTFE |
| Extracellular matrix | ECM |
| Global Burden of Diseases | GBD |
| Hematoxylin & Eosin | H&E |
| Matrix metalloproteinases | MMP |
| *N*-Hydroxysuccinimide | NHS |
| Nitrous acid degraded | NAD |
| Nitrous acid solution | HNO2 |
| Penta-galloyl glucose | PGG |
| Phospahte buffered saline | PBS |
| Poly-epsilon-caprolactone | PCL |
| Polyglycolic acid | PGA |
| Polylactic acid | PLA |
| Sodium azide | $NaN_3$ |
| Sodium cyanoborohydride | $NaCNBH_3$ |
| Sodium hydroxide | NAOH |
| Ultrasound | US |
| Wall shear stress | WSS |

# LIST OF FIGURES & TABLES

# CHAPTER 1

## *Literature Review*

# 1. INTRODUCTION

The morbidity and mortality associated with the various manifestations of cardiovascular disease (CVD) viz. cerebrovascular, coronary artery and peripheral arterial disease, and the complications that result from their surgical management cause profound suffering for many patients and is a major burden on health care systems worldwide. Peripheral arterial disease (PAD) has an overall prevalence of 3-10% and is present in 20% of people over 70 years of age.[1] Loss of limbs to amputations is a frequent occurrence in these patients, resulting in profound lifestyle-limiting disability.

## 1.1. Epidemiology of cardiovascular disease (CVD)

Epidemiological data for sub-Saharan Africa is not as clearly defined due to a lack of reliable health records. As more African communities adopt a westernized lifestyle, the prevalence of CVD risk factors continues to increase. A review of the available health statistics in sub-Saharan Africa showed that, although the incidence of CAD is under-reported, the rising prevalence of CVD risk factors including hypertension, obesity, physical inactivity and tobacco use, predicts an exponential increase in the incidence of CAD. The true impact of CVD is also overshadowed by the large burden of infectious and trauma-related deaths.[2] The Global Burden of Diseases (GBD) study showed that, although the relative prevalence of these diseases is smaller than in Europe and America, the absolute rates have increased dramatically between 1990 and 2010. Furthermore, the prevalence of PAD was greater among adults younger than 55 years old. This contrasts with the pattern in the first world and is further cause for concern as the disease affects adults in their productive years of life.[3]

## 1.2. Current surgical management of PAD

The basis of surgical management of PAD is a bypass procedure with either autologous vein or synthetic vascular grafts. Although advancements in tissue engineering technologies have provided alternative conduits for revascularization procedures, autologous vein remains the superior conduit. In a meta-analysis of randomized trials comparing autologous vein grafts to polytetrafluoroethylene (PTFE) in above-knee bypasses, 80% of venous bypasses remained patent at 2 years compared to 69% of PTFE bypasses. At five years, the patency rates dropped to 74% and 39% respectively, demonstrating the superior durability of vein grafts over synthetic grafts.[4] Similarly, whereas sepsis of vein grafts is rare, infection rates of synthetic grafts occur in up to 5% of patients.[5]

In below-knee bypass, the patency rate of synthetic grafts differs even more significantly from autologous vein. In their multicentre randomized trial comparing autologous vein to ePTFE for below-knee bypasses, Veith et al reported a patency rate of ePTFE at four years of 12% compared to 49% in autologous vein bypasses for the same implant period .[6]

### 1.3. Strategies to improve patency of synthetic grafts

In an attempt to improve the patency of synthetic grafts, various modifications to the graft material and surgical technique have been developed. These include vein cuffing (where a short segment of autologous vein is interposed between the graft and native vessel), heparin bonding and the creation of arterio-venous fistulae. Heparin bonding appears to increase the 1-year patency rate,[7] but this benefit is lost beyond 2 years.[8] Similarly, vein-cuffing improves primary patency rates but this does not reduce the risk of limb loss within 3 years.[9] More recent data shows that these synthetic graft modifications have conferred little benefit. In their review of the last 20 years of study into below-knee bypasses, Ziegler et al (2012) reported an average primary patency rate of 24% for ePTFE incorporating these modifications, compared to 69% of autologous vein grafts.[10]

Although the saphenous vein is most often used in these clinical situations, it is unsuitable in up to one third of patients due to small size, varicosities or anatomic variation.[11] Despite extensive study into tissue engineering and surgical techniques, the patency rate of synthetic grafts in below-knee bypass is still unacceptably low. There is thus still an urgent need for a suitable alternative vascular conduit, especially for smaller artery applications such as in below-knee bypass. Drawing on work done in vascular dynamics, haematological physiology and tissue engineering, the factors which determine graft success have come to be increasingly understood. Strategies for mitigating or modifying these various factors has formed the basis for continued study in this field.

# 2. FACTORS WHICH DETERMINE GRAFT PATENCY

Synthetic vascular grafts perturb vascular physiology in two primary ways, often leading to graft occlusion. First, as it lacks an endothelial lining, the synthetic material of the luminal surface of the graft is exposed to the proteins and platelets that comprise the coagulation system, resulting in clot formation. Second, differing mechanical responses between native arteries and implanted grafts results in intimal hyperplasia, especially at sites of anastomosis.

## 2.1. Endothelium and thrombogenicity

The endothelium, which acts as the interface between blood and the underlying tissues, is a complex and highly specialized cellular barrier. By responding to a myriad of hormones and cytokines and controlling the movement of platelets and inflammatory cells, it plays a central role in the regulation of thrombosis, inflammation, perfusion and healing.[12] Recognising the importance of the endothelium in maintaining graft patency, much of the research into vascular grafts has examined various techniques for achieving luminal endothelialisation. There are three known mechanisms of spontaneous *in-vivo* graft endothelialisation:

- transanastomotic migration of endothelial cells (ECs) from the native vessel[13]
- adherence of circulating endothelial progenitor cells (EPCs)[13]
- transmural endothelialisation from capillary ingrowth through the graft wall[13–15]

While in humans, transanastomotic endothelialisation has been shown to be limited to approximately 1-2cm of growth,[16] many animal studies have demonstrated complete endothelialisation of a variety of graft materials, including ePTFE and Dacron (Polyethylene terephthalate). These apparent successes have, however, not translated to similar results in the clinical setting, highlighting the wide species variation in vascular physiology and the consequent inherent limitation of animal studies.

## 2.2. Intimal hyperplasia and anastomotic stenosis

Intimal hyperplasia occurs as a result of the migration of smooth muscle cells and increased production of cellular matrix at anastomotic sites. The mechanisms that stimulate this cellular migration are incompletely understood but involve an imbalance between inhibitory and stimulatory paracrine signals between endothelial and smooth muscle cells, and as a result of surgical injury.[17]

It is known that the endothelium responds to physical forces within the lumen of blood vessels. Via various chemical mediators (e.g. nitric oxide), the diameter and compliance of arterial walls vary

dynamically in response to luminal pressure. A low mean wall shear stress (WSS) has been shown to be the primary mechanical parameter affecting endothelial cell physiology and predicts the development of atherosclerosis. Synthetic vascular grafts such as ePTFE and Dacron graft are more rigid and disrupt this normal physiology. Size and compliance mismatch between the graft and native artery results in abnormal flow patterns, which leads to decreased WSS[17] and abnormal stress patterns at the anastomotic site. Remodelling occurs in an attempt to compensate for this mechanical mismatch, leading to progressive occlusion of the graft.[18] The importance of these effects in determining the eventual success of vascular grafts has been reported in numerous studies and reviews.[17–21]

# 3. DEVELOPING THE IDEAL VASCULAR GRAFT

Understanding the mechanisms which underlie graft failure, further work in the development of alternative vascular conduits has sought to mitigate these factors by simulating physiological conditions. In addition to strategies to decrease luminal thrombogenicity and intimal hyperplasia, factors which determine safety and clinical feasibility also need to be considered.[22,23] A model for the ideal vascular graft would thus be characterised by:

- an endothelial-lined luminal surface or the capacity to facilitate *in vivo* endothelialisation
- mechanical properties similar to that of native arteries
- safe and non-toxic host interactions
- favourable surgical handling properties
- a feasible and scalable manufacturing process

## 3.1. Tissue engineering strategies to produce the ideal vascular graft

Combining techniques from engineering, cell biology and materials science, tissue engineering strategies to produce the ideal vascular graft have evolved steadily over the last 50 years. Two essential elements are common to most approaches: a scaffold, which provides the substrate for cellular infiltration, and cell seeding or infiltration of the scaffold.

The scaffolds, which are utilised as a substrate for cellular infiltration, are either composed of synthetic materials or are derived from animal sources. Currently investigated synthetic conduits are composed of biodegradable polymers similar to those used for suture materials, such as polylactic acid (PLA), polyglycolic acid (PGA), or poly-epsilon-caprolactone (PCL).[23] Animal-derived materials are harvested from a variety of tissues such as blood vessels, skin, liver, intestinal mucosa, and can be harvested from both human or non-human sources.[24]

Endothelialisation of these scaffolds is then achieved either *in-vitro* by cell-seeding in a bio-reactor prior to implantation, or *in-vivo* with infiltration of various cell populations (endothelial, fibroblasts and inflammatory cells) occurring after implantation within the graft recipient. Each approach has its relative advantages and disadvantages, with research groups dedicated to the development of a particular approach working toward ameliorating the disadvantages. For example, conduits composed of synthetic biodegradable materials have the potential to facilitate cell infiltration and remodelling and thus adapt to the needs of the host, analogous to the angiogenesis that occurs in juveniles. However, as the chemical and mechanical characteristics of these materials often differ

significantly from native arteries, the maintenance of patency and adequate mechanical strength during the remodelling process is challenging.[23]

Compared to *in-vivo* models, *in-vitro* methods of achieving endothelialisation allow more sophisticated preparation techniques such as flow and pressure pre-conditioning, which increase the probability of successful endothelial formation. Additionally, the graft has an intact endothelium at the time of implantation, which could prevent early graft thrombosis and occlusion. In their 15-year series of 310 patients implanted with *in-vitro* endothelialised ePTFE grafts of 6-7mm, Deutsch et al showed a patency rate of 69% at 5 years.[25] However, these advantages come at a significant cost in terms of both time and resources, decreasing the clinical feasibility of these strategies.[26] For example, in this same series the mean delay to implantation was 27 days.

Thus, despite the successes achieved with more complex *in-vitro* approaches, the trend in the study of tissue engineering of vascular grafts has shifted toward strategies which seek to facilitate *in-vivo* endothelialisation.[22] It is thought that the combination of decellularised vascular scaffolds with *in-vivo* endothelialisation has the potential to harness the relative advantages while minimising the disadvantages of the various tissue engineering approaches. The extracellular matrix (ECM) of decellularised vascular grafts possesses the three-dimensional architecture and cell-signalling proteins which could facilitate the migration, adhesion and proliferation of the various cell populations of each layer of an artery, including the endothelium. Additionally, it is primarily the connective tissue framework comprised of elastin and collagen fibres present in the ECM which determines the mechanical properties of arteries.[23]

### 3.2. Producing a decellularised graft

Decellularised vascular scaffolds are produced by removing cellular compounds such as DNA, lipids and glycosylated products, which are antigenic and incite an inflammatory response and would result in rapid graft rejection. The decellularisation process can, however, also remove or degrade desirable ECM compounds, which can alter the mechanical properties of the graft. The goal is thus to remove the immunogenic compounds while maintaining the mechanical and chemical integrity of the ECM.[24,27]

Decellularisation protocols used for vascular grafts originated in the advancements achieved in tissue engineering of other tissues such as heart valves and have continued to evolve, with various biochemical techniques developed and their utility validated. Most protocols involve a combination

of washing with detergent and/or alkaline treatments and specific enzymes directed at cellular compounds (e.g. DNase, RNase, lipase and proteases).[27] Using a combination of DNase and sodium deoxycholate (detergent) on porcine carotid arteries, Pellagata et al demonstrated that all cellular material was removed while maintaining the mechanical integrity of the graft. There was no statistical difference in the major parameters of mechanical testing including Young's modulus, compliance, ultimate stress, burst pressure and suture retention strength. There was a significant difference in ultimate strain, which the authors attributed to the absence of smooth muscle cell interaction with ECM fibers.[18] In contrast, Williams et al, applied a different decellularisation protocol and found major ultra-structural alterations in collagen and elastin fibres, which caused significant differences in the mechanical properties of decellularised vessels compared to native arteries.[28] The contrast of these results highlights the importance of utilising a decellularisation protocol which preserves collagen and elastin in order to maintain the mechanical properties of naturally derived vascular grafts.

### 3.3. Elastin stabilisation – preventing degradation and calcification

Elastin is an important ECM protein which imparts the capacity to distend and recoil in response to luminal pressure. It is present to varying degrees in all layers of blood vessels depending on its location in the vascular tree. For example, the tunica media of the aorta and pulmonary arteries are comprised of multiple layers of elastin which allows greater distensibility to receive blood directly from the heart and enables the elastic recoil which maintains blood flow during the diastolic phase of the cardiac cycle. More peripheral arteries such as the femoral artery are predominantly muscular and have fewer elastin fibres, which are concentrated in two distinct layers - the internal and external elastic lamellae.[29]

In contrast to other proteins present in the ECM, which are derived from multiple complex genes, the precursors of elastin are encoded on the single ELN gene. Active elastin production occurs *in-utero* and during the first few years of life, after which ELN gene expression rapidly decreases.[30] The inability to replenish elastin necessitates a stability uncharacteristic of other ECM proteins. The turnover rate of elastin under normal conditions is thus very slow, with a mean half-life of 74 years.[31] The clinical implication of this reduced turnover is that elastin damage and degradation tends to accumulate, causing persistent arterial disease even after the inciting pathological process has resolved.[32]

Despite much study into the mechanisms of elastin degradation, the molecular basis of arterial stiffening and aneurysm formation is incompletely understood. The pathophysiology involves a complex interaction of numerous factors including endothelial damage, inflammatory responses,

matrix metalloproteinase (MMP) activation and calcification of elastin fibres.[33] Elastin is inherently resistant to proteolysis but is susceptible to degradation by a limited number of proteinases in the MMP family.[34] MMP-mediated degradation, in turn, facilitates calcification of elastin fibres via unknown mechanisms and is thought to be the initiating event in the sequence culminating in arterial calcification. Animal models have demonstrated that chemical stabilisation of elastin against MMP degradation effectively prevents calcification of elastin-rich tissues.[35]

The importance of chemical stabilisation of elastin was first investigated in naturally derived cardiac valves[35,36] and later, similar techniques applied to decellularised arteries. Simionescu et al first described the use of penta-galloyl-glucose (PGG), a stabilising polyphenolic tannin, to reduce the degradation of elastin. Using a combination of alkaline wash (Sodium hydroxide, NaOH) and PGG treatment of porcine carotid arteries, this group investigated various aspects of PGG stabilisation.[37] *In vitro* testing of the grafts demonstrated strong resistance to elastase conferred by PGG.[38] *In-vivo* tests, by subdermal implantation of segments of the grafts in rats, demonstrated the infiltration of fibroblasts and smooth muscle cells and showed signs of early remodelling. Finally, mechanical testing showed no statistical difference in overall strength and compliance compared to fresh arteries.[37] PGG-stabilised grafts were stiffer than fresh arteries at low strains but the elastic moduli approached that of fresh arteries at higher strains.

### 3.4. Heparinisation and stabilisation of collagen

Collagen is the major determinant of tensile strength in arteries and other structures such as tendons and bone. In contrast to elastin, collagen metabolism is ongoing throughout life, and responds to several biomechanical and chemical cues. It is produced by multiple cell populations present in the ECM including fibroblasts and macrophages, but is also susceptible to breakdown by a wide range of proteases.[39]

The bonding of heparin to decellularised vascular grafts has been found to have the added effect of stabilising collagen. Passive absorption by soaking decellularised grafts in heparin solutions is not effective as the collagen within these grafts lack heparin binding (amine) sites. To overcome this limitation, methods of covalent linkage of heparin by pre-treatment with amine groups were developed.[40,41] Based on this earlier work, Pennel et al developed and validated such a protocol for heparin bonding which involves first treating the decellularised grafts with Jeffamine® (a diamine) followed by heparin coupling. This method achieves significantly increased heparin content of the grafts compared to those which were reacted with activated heparin alone.[42,43] Also, as the

carbodiimide activated amination of the tissue results in crosslinking of the collagen, increased denaturation temperatures and a 3-4 fold resistance to collagenase in grafts was demonstrated.[42]

### 3.5. Combined elastin and collagen stabilisation

Having validated elastin and heparin stabilisation separately, our group then evaluated the biological feasibility of combining these methods in a single protocol. Elastin stabilisation with PGG and collagen stabilisation with diamine cross-linking and covalent heparinisation was applied to decellularised porcine renal arteries. These stabilised decellularised grafts were implanted as interpositional grafts in rat abdominal aortas to compare patency rates and the degree of elastin and collagen degradation to untreated control grafts. Patency rates were high in both untreated and stabilised grafts but the rate of aneurysm formation and degree of elastin degradation was significantly decreased in the chemically stabilised grafts.[42]

# 4. NEED FOR LARGE ANIMAL EXPERIMENTATION

The rat aorta is a useful *in-situ* model for demonstrating biocompatibility and to assess the degree and nature of cell infiltration (including endothelialisation) and degradation of the graft material. However, it is not a robust test of graft patency as various vascular conduits have frequently demonstrated excessively high rates of patency in rats.[13] For example, van Der Lei et al demonstrated 100% patency for 1.5mm ePTFE and polyurethane grafts implanted in the rat aorta[44] as did Pennel et al in testing PGG-stabilised decellularised vascular grafts.[42] In contrast, when small calibre synthetic grafts were investigated for their use in coronary artery bypass graft (CABG) in humans, the results were consistently poor, with patency rates as low as 14% after 3 years.[45] Large animal models are thus required that are more representative of human vascular physiology and flow dynamics to provide a more clinically relevant test of vascular grafts.

## 4.1. Rationale for selection of ovine carotid model

Several factors are considered in selecting an appropriate animal model for testing vascular grafts. Both the animal species and anatomical site of the implant are considered to most accurately simulate human physiology. Secondary considerations include cost, ease of handling and response to anaesthesia.[13] As the ultimate clinical goal is the implantation of animal-based vascular grafts into humans, demonstrating cross-species viability is fundamental to the study of decellularised grafts.

Non-human primates simulate human physiology most accurately, but ethical concerns have precluded their use internationally. Canine models were regarded as the next best model as, like humans, they lacked the ability to completely endothelialise synthetic grafts.[46,47] However, more recent animal rights considerations also limited the use of canine models in animal experiments.[13] Based on all of these factors, the ovine model is most feasible and is currently utilised in most large animal model testing of vascular conduits.

## 4.2. Comparative haematology

Compared to that of the dog or pig, the coagulation system of sheep bears more similarity to human physiology.[48] The rate of platelet activation by thrombin and platelet-activating factor is similar to humans but there is an increased number (3-8 x $10^5$/mL vs 2-5 x $10^5$ /mL) and adhesiveness of platelets in sheep. This, and decreased fibrinolytic activity, mean there is a somewhat greater tendency toward hypercoagulability in sheep.[49] There are also differences of the other constituents of blood including

increased red blood cell fragility and a predominant lymphocytic component of white blood cells, compared to a relative neutrophilia in humans.[50]

The changes in coagulation physiology in response to anaesthesia and heparin administration have been shown to be similar in humans and sheep. Tillman et al examined platelet function and coagulation parameters of sheep at various times during a 5-hour vascular procedure and demonstrated little change caused by anaesthesia and surgery, and a response to heparin comparable to that in humans.[51]

In contrast, the effectiveness of anti-platelet agents such as aspirin and clopidogrel is reduced in sheep. Higher doses of clopidogrel are required to achieve levels of anti-platelet activity in sheep which are comparable to humans,[52,53] while aspirin has minimal anti-platelet activity.[53]

### 4.3. Comparative anatomy

The ovine carotid artery is the most commonly used model for the assessment of vascular grafts intended for peripheral artery applications (i.e. 4-6mm diameter applications). The ovine common carotid artery has sufficient length and is surgically accessible and implantation in the neck is well tolerated with minimal postoperative morbidity.[13]

Anatomically, there are extensive anastomotic connections between the left and right side, and the anterior (carotid artery) and posterior circulation (basilar artery). Unilateral occlusion results in dynamic responses in the remaining vascular beds, shunting blood to compensate for the loss of flow.[54] Simultaneous bilateral occlusion may cause reversible cerebral ischaemia but sequential loss over time, as could occur post-operatively after bilateral graft implantation, is tolerated with little morbidity.[55]

# 5. REVIEW OF SHEEP CAROTID MODEL STUDIES

A review of studies which have utilised the ovine carotid model to evaluate vascular grafts again highlight the importance of an intact endothelial layer in maintaining patency. In their study comparing decellularised porcine arteries, which were seeded with ovine endothelial progenitor cells (EPC) versus the same porcine graft without seeding, Kaushal et al demonstrated 100% patency in the endothelialised group vs 0% in the un-seeded group.[56] An intact endothelium also appears to confer the ability to heal from injury. Tillman et al subjected EPC-seeded decellularised porcine grafts to multiple puncture over the course of several months. The grafts remained patent for a mean of 4.4 (± 0.9) months, which is comparable to venous grafts under similar conditions.[57] Like other studies which have employed this method to achieve luminal endothelialisation, these promising results came at a significant cost. In the Kaushal study for example, three weeks of culture time was required to produce sufficient EPCs. Also, as previous work in EPCs have demonstrated that 80% of these cells are lost when exposed to shear stress *in vivo*, the seeded grafts were subjected to 4 days of pressure pre-conditioning in a bioreactor prior to implantation.[56]

In contrast, studies relying on *in-vivo* endothelialisation have thus far yielded disappointing results. In their study of 6mm diameter vascular grafts composed of hydrated small intestinal mucosa, Pavcnik et al, demonstrated a 30% patency rate at 3 months.[58] Microscopic examination of the specimens from this and other studies show migration of fibroblasts and other inflammatory cell populations but limited endothelialisation of the luminal surface. To facilitate the transmural migration of endothelial cells, physical modifications to alter the porosity of the grafts have been employed. Bergmeister et al used laser perforations of decellularised grafts to increase porosity. Compared to non-perforated grafts, endothelialisation was slightly improved at 6 weeks but there was no difference in the extent of endothelialisation at 3 months.[59] Additionally, the perforated grafts developed a thicker neointima, which resulted in a lower patency rate in the perforated group.

These attempts to achieve *in-vivo* endothelialisation in an ovine carotid model have not yet achieved results superior to PTFE grafts of comparable size which are implanted under similar conditions. After 6 months, 17% - 50% of 5-6mm ePTFE grafts range remain patent.[60–62] This rate is also similar to the patency rate of PTFE in below-knee bypass in humans, making the use of PTFE in the ovine carotid model a suitable control in testing grafts against the current clinical standard.

# 6. CONCLUSION

Despite 50 years of ongoing research, there remains a need for a suitable alternative conduit for small and medium sized bypass applications. The successes achieved with *in-vitro* endothelialisation with EPC culture and seeding reaffirms the pivotal role an intact endothelium plays in maintaining patency. However, with the time required to produce these grafts, their wide-scale application is prohibitively resource intensive. In attempting to produce an 'off-the-shelf' vascular graft, the strategy has shifted toward achieving *in-vivo* endothelialisation by harnessing the ultra-structure and chemistry of biological scaffolds to facilitate migration of essential cell groups, while maintaining their mechanical characteristics. The complex interaction between these different cell populations, the extracellular milieu they are exposed to, and various host factors such as flow dynamics and the immune response, are the main determinants of the long-term patency of these vascular conduits. Elucidating the intricate nature of these interactions and attempting to modify these processes is the subject of ongoing study in this field.

# 7. AIMS AND OBJECTIVES

The aim of this study is to assess the clinical feasibility of decellularised vascular grafts, which have been produced by utilising protocols based on earlier work, using an established large animal model. The objectives of the study include:

- determination of the rate of patency of the vascular grafts in a large animal model compared to ePTFE grafts as control
- assessment of the extent of host cellular infiltration of the graft material, including the degree of endothelialisation of the luminal surface of the graft
- analysis of the mechanical characteristics of the graft material and comparison to human physiological conditions

# 8. REFERENCES


1. Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Eur J Vasc Endovasc Surg*. 2007;33 Suppl 1(1):S1-75. doi:10.1016/j.ejvs.2006.09.024
2. Onen CL. Epidemiology of ischaemic heart disease in sub-Saharan Africa. *Cardiovasc J Afr*. 2013;24(2):34-42. doi:10.5830/CVJA-2012-071
3. Moran A, Forouzanfar M, Sampson U, et al. The Epidemiology of Cardiovascular Diseases in Sub-Saharan Africa: The Global Burden of Diseases, Injuries and Risk Factors 2010 Study. *Prog Cardiovasc Dis*. 2013;56(3):234-239. doi:10.1016/j.micinf.2011.07.011.Innate
4. Klinkert P, Post PN, Breslau PJ, et al. Saphenous vein versus PTFE for above-knee femoropopliteal bypass. A review of the literature. *Eur J Vasc Endovasc Surg*. 2004;27(4):357-362. doi:10.1016/j.ejvs.2003.12.027
5. Darouiche RO. Treatment of infections associated with surgical implants. *N Engl J Med*. 2004;350(14):1422-1429. doi:10.1056/NEJMra035415
6. Veith FJ, Gupta SK, Ascer E, et al. Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal arterial reconstructions. *J Vasc Surg*. 1986;3(1):104-114.
7. Neville RF, Capone A, Amdur R, et al. A comparison of tibial artery bypass performed with heparin-bonded expanded polytetrafluoroethylene and great saphenous vein to treat critical limb ischemia. *J Vasc Surg*. 2012;56(4):1008-1014. doi:10.1016/j.jvs.2012.03.020
8. Lösel-Sadée H, Alefelder C. Heparin-bonded expanded polytetrafluoroethylene graft for infragenicular bypass: five-year results. *J Cardiovasc Surg (Torino)*. 2009;50(3):339-343.
9. Khalil A a M, Griffiths G, Boyd A. Interposition vein cuff for infragenicular prosthetic bypass graft. *Cochrane Database Syst Rev*. 2012;12(9). doi:10.1002/14651858.CD007921
10. Ziegler K, Muto A, Eghbalieh S, et al. Basic Data Related to Operative Infrainguinal Revascularization Procedures: A Twenty Year Update. *Ann Vasc Surg*. 2012;25(3):413-422. doi:10.1016/j.avsg.2010.10.010.Basic
11. Veith FJ, Moss CM, Sprayregen S, et al. Preoperative saphenous venography in arterial reconstructive surgery of the lower extremity. *Surgery*. 1979;85(3):253-256.
12. van Hinsbergh VWM. Endothelium--role in regulation of coagulation and inflammation. *Semin Immunopathol*. 2012;34(1):93-106. doi:10.1007/s00281-011-0285-5
13. Byrom MJ, Bannon PG, White GH, et al. Animal models for the assessment of novel vascular

conduits. *J Vasc Surg*. 2010;52(1):176-195. doi:10.1016/j.jvs.2009.10.080

14. Davis L, Dower T, Zilla P. The lack of healing in conventional vascular grafts. In: Zilla P, Greisler H, eds. *Tissue Engineering of Vascular Prosthetic Grafts*. Landes Company: Austin, TX, USA; 1999:3-44.
15. Greisler H. Characteristics and healing of vascular grafts. In: Callow A, Ernst C, eds. *Vascular Surgery: Theory and Practice*. Appleton & Lange: Stamford (Conn), New York; 1995:1181-1212.
16. Zilla P, Bezuidenhout D, Human P. Prosthetic vascular grafts: wrong models, wrong questions and no healing. *Biomaterials*. 2007;28(34):5009-5027. doi:10.1016/j.biomaterials.2007.07.017
17. Salacinski HJ, Goldner S, Giudiceandrea A, et al. The mechanical behavior of vascular grafts: a review. *J Biomater Appl*. 2001;15(3):241-278. doi:10.1106/NA5T-J57A-JTDD-FD04
18. Pellegata AF, Asnaghi MA, Stefani I, et al. Detergent-enzymatic decellularization of swine blood vessels: insight on mechanical properties for vascular tissue engineering. *Biomed Res Int*. 2013;2013:918753. doi:10.1155/2013/918753
19. Haruguchi H, Teraoka S. Intimal hyperplasia and hemodynamic factors in arterial bypass and arteriovenous grafts: A review. *J Artif Organs*. 2003;6(4):227-235. doi:10.1007/s10047-003-0232-x
20. Sarkar S, Salacinski HJ, Hamilton G, et al. The mechanical properties of infrainguinal vascular bypass grafts: their role in influencing patency. *Eur J Vasc Endovasc Surg*. 2006;31(6):627-636. doi:10.1016/j.ejvs.2006.01.006
21. Prichard HL, Manson RJ, DiBernardo L, et al. An early study on the mechanisms that allow tissue-engineered vascular grafts to resist intimal hyperplasia. *J Cardiovasc Transl Res*. 2011;4(5):674-682. doi:10.1007/s12265-011-9306-y
22. Li S, Sengupta D, Chien S. Vascular tissue engineering: from in vitro to in situ. *Wiley Interdiscip Rev Syst Biol Med*. 2014;6(1):61-76. doi:10.1002/wsbm.1246
23. Bergmeister H, Strobl M, Grasl C, et al. Tissue engineering of vascular grafts. *Eur Surg*. 2013;45(4):187-193. doi:10.1007/s10353-013-0224-x
24. Catto V, Farè S, Freddi G, et al. Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration. *ISRN Vasc Med*. 2014;2014:1-27. doi:10.1155/2014/923030
25. Deutsch M, Meinhart J, Zilla P, et al. Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. *J Vasc Surg*. 2009;49(2):352-362. doi:10.1016/j.jvs.2008.08.101

26. Chong DST, Lindsey B, Dalby MJ, et al. Luminal surface engineering, "micro and nanopatterning": potential for self endothelialising vascular grafts? *Eur J Vasc Endovasc Surg*. 2014;47(5):566-576. doi:10.1016/j.ejvs.2014.02.007
27. Piterina A V, Cloonan AJ, Meaney CL, et al. ECM-based materials in cardiovascular applications: Inherent healing potential and augmentation of native regenerative processes. *Int J Mol Sci*. 2009;10(10):4375-4417. doi:10.3390/ijms10104375
28. Williams C, Liao J, Joyce E, et al. Altered structural and mechanical properties in decellularized rabbit carotid arteries. *Acta Biomater*. 2009;5(4):993-1005. doi:10.1016/j.actbio.2008.11.028.Altered
29. Paxton S, Peckham M, Adele K. The Leeds Histology Guide. http://www.histology.leeds.ac.uk/circulatory/arteries.php. Published 2003. Accessed May 11, 2017.
30. Bashir MM, Indik Z, Yeh H, et al. Characterization of the complete human elastin gene. Delineation of unusual features in the 5'-flanking region. *J Biol Chem*. 1989;264(15):8887-8891.
31. Shapiro SD, Endicott SK, Province MA, et al. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. *J Clin Invest*. 1991;87(5):1828-1834. doi:10.1172/JCI115204
32. Robert L, Hornebeck W. *Elastin and Elastases, Volume 2*. CRC Press; 1989.
33. Cecelja M, Chowienczyk P. Molecular Mechanisms of Arterial Stiffening. *Pulse (Basel, Switzerland)*. 2016;4(1):43-48. doi:10.1159/000446399
34. Basalyga DM, Simionescu DT, Xiong W, et al. Elastin degradation and calcification in an abdominal aorta injury model: role of matrix metalloproteinases. *Circulation*. 2004;110(22):3480-3487. doi:10.1161/01.CIR.0000148367.08413.E9
35. Bailey M, Xiao H, Ogle M, et al. Aluminum chloride pretreatment of elastin inhibits elastolysis by matrix metalloproteinases and leads to inhibition of elastin-oriented calcification. *Am J Pathol*. 2001;159(6):1981-1986. doi:10.1016/S0002-9440(10)63048-9
36. Isenburg JC, Simionescu DT, Vyavahare NR. Elastin stabilization in cardiovascular implants: improved resistance to enzymatic degradation by treatment with tannic acid. *Biomaterials*. 2004;25(16):3293-3302. doi:10.1016/j.biomaterials.2003.10.001
37. Chuang T-H, Stabler C, Simionescu A, et al. Polyphenol-Stabilized Tubular Elastin Scaffolds for Tissue Engineered Vascular Grafts. *Tissue Eng Part A*. 2009;15(10):2837-2851. doi:10.1089/ten.TEA.2008.0394
38. Isenburg JC, Simionescu DT, Starcher BC, et al. Elastin stabilization for treatment of

abdominal aortic aneurysms. *Circulation*. 2007;115(13):1729-1737. doi:10.1161/CIRCULATIONAHA.106.672873

39. Minor RR. Collagen metabolism: a comparison of diseases of collagen and diseases affecting collagen. *Am J Pathol*. 1980;98(1):225-280.
40. Liao D, Wang X, Lin PH, et al. Covalent linkage of heparin provides a stable anti-coagulation surface of decellularized porcine arteries. *J Cell Mol Med*. 2009;13(8 B):2736-2743. doi:10.1111/j.1582-4934.2008.00589.x
41. Raghunath K, Biswas G, Panduranga Rao K, et al. Some characteristics of collagen-heparin complex. *J Biomed Mater Res*. 1983;17(4):613-621. doi:10.1002/jbm.820170406
42. Pennel T, Fercana G, Bezuidenhout D, et al. The performance of cross-linked acellular arterial scaffolds as vascular grafts; pre-clinical testing in direct and isolation loop circulatory models. *Biomaterials*. 2014;35(24):6311-6322. doi:10.1016/j.biomaterials.2014.04.062
43. Bezuidenhout D, Davies N, Black M, et al. Covalent surface heparinization potentiates porous polyurethane scaffold vascularization. *J Biomater Appl*. 2010;24(5):401-418. doi:10.1177/0885328208097565
44. van der Lei B, Robinson PH, Bartels HL, et al. Microarterial grafting into the carotid artery of the rabbit: some considerations concerning species-dependent thrombogenicity. *Br J Plast Surg*. 1989;42(1):59-64.
45. Desai M, Seifalian AM, Hamilton G. Role of prosthetic conduits in coronary artery bypass grafting. *Eur J Cardiothorac Surg*. 2011;40(2):394-398. doi:10.1016/j.ejcts.2010.11.050
46. Abbott WM, Callow A, Moore W, et al. Evaluation and performance standards for arterial prostheses. *J Vasc Surg*. 1993;17(4):746-756.
47. Swartz DD, Andreadis ST. Animal models for vascular tissue-engineering. *Curr Opin Biotechnol*. 2013;24(5):916-925. doi:10.1016/j.copbio.2013.05.005
48. Konya A, Wright K, Gounis M, et al. Animal models for atherosclerosis, restenosis, and endovascular aneurysm repair. In: Conn P, ed. *Sourcebook of Models for Biomedical Research*. NJ: Humana Press; 2008:369-384.
49. Blunt MH. Cellular Elements of Ovine Blood. In: Blunt MH, ed. *The Blood of Sheep: Composition and Function*. Berlin, Heidelberg: Springer Berlin Heidelberg; 1975:29-44. doi:10.1007/978-3-642-66115-0_2
50. Didisheim P. Comparative hematology in the human, calf, sheep and goat. Relevance to implantable blood pump evaluation. *ASAIO J*. 1985;8(3):123-127.
51. Tillman P, Carson SN, Talken L. Platelet function and coagulation parameters in sheep during experimental vascular surgery. *Lab Anim Sci*. 1981;31(3):263-267.

52. Connell JM, Khalapyan T, Al-Mondhiry H a, et al. Anticoagulation of juvenile sheep and goats with heparin, warfarin, and clopidogrel. *ASAIO J*. 2007;53(2):229-237. doi:10.1097/MAT.0b013e31802e192b
53. Weigand A, Boos AM, Ringwald J, et al. New aspects on efficient anticoagulation and antiplatelet strategies in sheep. *BMC Vet Res*. 2013;9:192. doi:10.1186/1746-6148-9-192
54. Baldwin BA, Bell FR. The effect on blood pressure in the sheep and calf of clamping some of the arteries contributing to the cephalic circulation. *J Physiol*. 1963;167(3):463-479.
55. May ND. Experimental studies of the collateral circulation in the head and neck of sheep (Ovis aries). *J Anat*. 1968;103(Pt 1):171-181.
56. Kaushal S, Amiel G, Guleserian K. Functional Small Diameter Neovessels using Endothelial Progenitor Cells Expanded Ex Vivo. *Nat Med*. 2001;7(9):1035-1040. doi:10.1038/nm0901-1035.Functional
57. Tillman BW, Yazdani SK, Neff LP, et al. Bioengineered vascular access maintains structural integrity in response to arteriovenous flow and repeated needle puncture. *J Vasc Surg*. 2012;56(3):783-793. doi:10.1016/j.jvs.2012.02.030
58. Pavcnik D, Obermiller J, Uchida BT, et al. Angiographic evaluation of carotid artery grafting with prefabricated small-diameter, small-intestinal submucosa grafts in sheep. *Cardiovasc Intervent Radiol*. 2009;32(1):106-113. doi:10.1007/s00270-008-9449-7
59. Bergmeister H, Boeck P, Kasimir M-T, et al. Effect of laser perforation on the remodeling of acellular matrix grafts. *J Biomed Mater Res B Appl Biomater*. 2005;74(1):495-503. doi:10.1002/jbm.b.30228
60. Sardelic F, Fletcher JP, Ao PY, et al. Comparison of fluoropolymer passivated Dacron and polytetrafluoroethylene grafts in a sheep model. Cardiovascular surgery (London, England). http://www.ncbi.nlm.nih.gov/pubmed/8049954. Published 1994. Accessed July 23, 2015.
61. Pedersen G, Laxdal E, Ellensen V, et al. Improved patency and reduced intimal hyperplasia in PTFE grafts with luminal immobilized heparin compared with standard PTFE grafts at six months in a sheep model. *J Cardiovasc Surg (Torino)*. 2010;51(3):443-448.
62. Baek I, Bai CZ, Hwang J, et al. Paclitaxel coating of the luminal surface of hemodialysis grafts with effective suppression of neointimal hyperplasia. *J Vasc Surg*. 2012;55(3):806-814. doi:10.1016/j.jvs.2011.09.012

# CHAPTER 2

*Manuscript*

# 1. INTRODUCTION

There is significant morbidity and mortality associated with cardiovascular disease, which exert a major burden on healthcare systems worldwide. The worldwide increase in the co-morbid illnesses of diabetes, hypertension and dyslipidaemia have resulted in an increase in the prevalence of coronary artery and peripheral vascular disease.[1,2] With more populations in the developing world adopting a western lifestyle and diet, there is also an expected increase in these illnesses in Africa.[3]

When medical and endovascular therapeutic modalities fail, surgical bypass of the diseased segment of artery is required. For larger arteries, synthetic vascular grafts such as polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (Dacron) offer viable curative options and have good long-term patency rates. However, for smaller vessel disease, such as in below-knee bypass, patency rates are unacceptably low.[4] An autologous vein graft, usually involving harvest of the saphenous vein, is required in these clinical situations. However, due to anatomic variation or disease, this is not available in one third of patients.[5]

The primary causative factors that result in graft occlusion are a lack of endothelium, and mechanical mismatch between the native artery and graft. The endothelium plays a central role in regulating thrombosis and thus much of the current study into alternative vascular grafts aims to achieve endothelialisation of the luminal surface of these grafts.[6] Calibre and compliance mismatch cause abnormalities of flow, which stimulate a smooth muscle cell mediated deposition of extracellular matrix at sites of anastomosis.[7] The progressive narrowing eventually results in thrombosis and occlusion.

Various tissue engineering approaches have been adopted to address these two problems. *In-vitro* endothelisation utilizing endothelial cell culture and complex bioreactor systems have demonstrated the best results,[8] but are resource intensive and require long lead times to produce, and are thus not suitable for large-scale production.[9] Consequently, the focus has shifted toward *in-situ* endothelialisation of animal-derived decellularised vascular grafts. The extracellular matrix (ECM) of these grafts have similar mechanical characteristics to native arteries[10] and possess the cell signalling proteins that could facilitate endothelial cell migration. In producing decellularised grafts, a balance is sought between effectively removing all antigenic material while maintaining the mechanical and chemical integrity of the ECM. There is significant variation in the mechanical properties between grafts produced with different decellularisation protocols,[11,12] highlighting the importance of refining the process used to produce these grafts.

Collagen and elastin, the ECM proteins which confer strength and distensibility to arteries, are prone to degradation by both the decellularisation process and by host proteases. Our group previously described the use of penta-galloyl glucose (PGG), a polyphenolic tannin, to reduce the degradation of elastin.[13,14] PGG treatment of decellularised porcine arteries demonstrated strong resistance to elastase and similar mechanical properties to fresh native arteries. Collagen stabilisation was achieved as an added effect of the heparinisation process. Using Jeffamine (a diamine) heparin was covalently bound to collagen and cross-links between collagen molecules were formed.[15] This method also achieves significantly higher heparin content compared to passive soaking in heparin solutions[15,16] and does not reduce its anticoagulant effects.[17]

The viability and efficacy of this decellularisation and stabilisation protocol was tested in a small animal model by utilising it to prepare harvested porcine renal arteries for implantation into rat aortas as isolation loop implants. Compared to untreated grafts, the stabilised grafts demonstrated higher patency and reduced elastin degeneration, effectively preventing aneurysmal dilatation.[15]

Having demonstrated the biological safety and viability of this graft preparation protocol, a large animal study was undertaken to more closely simulate human vascular flow dynamics and haematological physiology. The ovine carotid model best fulfils the requirements to test conduits intended for use in below-knee and coronary bypass (4-6mm). The diametric size of the sheep common carotid artery closely matches that of the test conduits and is of sufficient length.[18] Additionally, ePTFE within this size range implanted in sheep has been shown to have patency rates similar to that seen in humans.[19]

By harnessing their mechanical and chemical properties, it is hypothesised that the stabilised decellularised grafts will both facilitate endothelialisation and reduce intimal hyperplasia, thereby improving patency rates. By comparing the current clinical standard (ePTFE) with the tested graft within the same animal, this study aims to produce a robust, clinically relevant test of the stabilised decellularised grafts.

# 2. METHODS

## 2.1. Harvesting and decellularisation

Fresh porcine mammary arteries were sourced from Animal Technologies© (Inc.) and received on ice by our collaborators. The fresh arteries were cleared of excess tissue and checked for leaks from side branches, which were ligated with 3-0 braided polyethylene suture. The samples were treated overnight in 30 mM ethylenediaminetetraacetic acid (EDTA) and 0.02% sodium azide ($NaN_3$) on an orbital shaker at 3.5 rpm at 4°C.

The prepared specimens were decellularised by our collaborators in a bioprocessing protocol previously designed and validated by them.[20] Briefly, the arteries were mounted in a perfusion bioreactor, which facilitates treatment of the outer and luminal surfaces of the graft under pressure. Using this perfusion system, the arteries were first rinsed with double distilled water ($ddH_2O$). The detergent sodium dodecyl sulfate (SDS) was used as the primary cell lysing agent. The grafts were incubated for 12 days in 1% SDS solution. Following rinsing of the SDS with $ddH_2O$, the grafts were treated with 70% ethanol (EtOH) for 3 hours and sodium hydroxide (NaOH) for 2 hours. Overnight treatment with phosphate buffered saline (PBS) was followed by 96 hours of enzymatic treatment with DNAse and RNAse (720munits/ml). Sterilisation of the grafts was achieved with peracetic acid.

## 2.2. Elastin stabilization with penta-galloyl glucose

Following decellularisation and sterilization, the grafts were soaked in 0.15% penta-galloyl glucose (PGG, N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium) in 50 mM dibasic sodium phosphate buffer with 20% isopropanol in saline (pH 5.5) for 24 hours. The grafts then underwent a 6-day rinse with $ddH_2O$ and PBS, and stored in sterile PBS + 1% antimicrobial solution (antibiotic and antifungal) for shipping to Cape Town, South Africa.

## 2.3. Heparinisation

The heparinisation process was conducted by our group and was achieved with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC) / *N*-Hydroxysuccinimide(NHS) activated Jeffamine® (D-230 polyoxypropylenediamine) coupling prior to heparinisation.

The decellularised grafts were first pre-soaked in 240mM Jeffamine solution for 30 minutes to infiltrate the tissue with excess Jeffamine. Samples were then immersed in a combination of Jeffamine/EDC/NHS solution (2.3g EDC and 0.56g NHS per 50mls 240mM Jeffamine solution) for two

and half hours. Following a PBS rinse, nitrous acid degraded heparin (Celsus Glycoscience, Inc) was combined in solution with the reducing agent sodium cyanoborohydride ($NaCNBH_3$, 2mg/ml) and used to treat the samples for 18 hours. The samples were then rinsed with PBS and normal saline, and stored individually in PBS-filled tubes.

2.4. Heparin content assessment

A heparinised graft and untreated graft (as control) were excluded from *in-vitro* testing and utilised to assess heparin content. Three specimens were sampled from the untreated graft, and three each from the mid-section and end of the heparinised graft (i.e. 6 heparinised samples). The specimens were subjected to a colorimetric procedure as previously described[16] from a modified technique developed by Riesenfeld and Roden.[21] Briefly, the samples were treated with deionized water (DI) and nitrous acid solution (0.025M HNO2, 0.9M HCl), which selectively cleaves N-sulfated glucosamine residues from heparin. These residues are reacted with 3-methyl-2- benzothiazoninone hydrazone (MBTH) solution, which is then complexed with FeCl3 (500 mL of an 0.031M solution, 50C, 20 min). The absorbance of the resultant solution is determined by UV spectroscopy (660nm, ShimadzuUV-1601PC) and the heparin content of the sample (mg/g of tissue) thus determined from standard curves. To assess the degree of loss of heparin content during storage, the assessment was repeated on a similar set of specimens sampled from grafts held in cold-storage ($4^o$ C) for 11 weeks.

2.5. Mechanical properties

Pre-heparinisation and post-heparinisation grafts were selected for mechanical testing using an Instron® 5544 load frame (Illinois Tool Works Inc.) strain extensometer. For circumferential tensile strength testing, 1cm samples were applied to the machine attachments and extended at 1mm/sec to failure. The rate of stretch and force required were recorded and used to derive stress-strain curves.

Suture retention strength was assessed by passing size 4/0 braided suture through the graft at 1mm from the graft edge and fixing the graft and the looped suture to the machine attachments and similarly extended to failure. The procedure was repeated at 3 separate points on the same test graft edge to assess for consistency of mechanical properties.

2.6. Implant procedure

Six adult female Merino sheep weighing 75-80kg were used for the experiments. The protocol of animal experiments and care was approved by the Animal Ethics Committee of the University of Cape Town (Ethics No: 013/023).

Pre-operative preparation included a 12-hour fasting period, antibiotic prophylaxis (Cefotaxime 1g IM) and analgesia (Buprenorphine 0.01mg/kg). Induction of anaesthesia was established with Medetomidine (25µg/kg IM) and Ketamine (1mg/kg), and maintained with Isoflurane (2- 3%) in oxygen and nitrous oxide.

Sterile technique was maintained throughout the procedure. The common carotid artery was exposed on both sides via bilateral 10-12cm incisions made in the mid-neck. Heparin (5000iu IV) was administered prior to arterial cross-clamping. A 5cm section of common carotid artery was excised and the grafts were implanted as carotid interposition grafts using 6/0 polypropylene sutures (Prolene®, Ethicon). ePTFE (6mm, GORE-TEX®, Gore) was implanted on the left side as a control and the decellularised grafts implanted on the right side.

Enoxaparin (Clexane® 80mg sub-cutaneous, Sanofi-Aventis) was administered daily for 1 week following implantation and Clopidogrel (Plavix® 75mg orally, Sanofi-Aventis) was given for the full implant period. After an average implant period of two months the grafts were assessed for patency using ultrasound and angiography. The grafts were then explanted under general anaesthesia and the animal euthanized.

2.7. Assessment of patency

Patency was assessed prior to explantation with duplex doppler ultrasound (US), on angiogram and post-explantation by macroscopic assessment. Samples from animals euthanised before the end-point were excluded from the analysis.

US assessment was performed by a registered ultrasonographer who specialises in human carotid artery assessment. Anastomotic sites were assessed for intimal hyperplasia using B-mode US and the presence and velocity of flow with Doppler-mode US.

Angiography was performed by the operating surgeon with the animal anaesthetised just prior to explant. The femoral artery (left or right) was accessed percutaneously under US guidance and the

angiogram catheter positioned using the seldinger (over-guidewire) technique at the root of the aorta under X-ray guidance. IV contrast (Iohexol 300mg/ml, Omnipaque™) was injected and X-Ray screening of the neck was taken to assess the presence of contrast in both common carotid arteries.

Direct macroscopic assessment of patency was done by palpation of the surgically exposed grafts. Following resection of the grafts, injection of saline through the grafts was attempted to assess patency.

### 2.8. Specimen preparation & histological analysis

The explanted grafts were fixed in formalin solution for 24 hours before being embedded in paraffin. Sections were cut longitudinally and dewaxed with 2,2,4-trimethylpentane. Haematoxylin (Merck, Damstadt, Germany) and eosin (BDH; WWR International, Poole, England) (H&E), Miller and Masson elastin trichrome stain Willebrand (Dako, AS, Glostrup, Denmark). Histology analysis was performed with a Nikon eclipse 90i microscope (Nikon, Tokyo, Japan) at 4x, 10x and 40x magnification.

### 2.9. Statistical analysis

Statistical tests were performed with STATA (StataCorp. 2013. Stata Statistical Software:
Release 13.0 College Station, TX: StataCorp LP). Results were expressed as mean ± standard deviation (SD). Significant differences between means were determined by Student's t- test (paired, two-tailed) with significance set at $p<0.05$.

# 3. RESULTS

## 3.1. Decellularised graft dimensions & handling

Acquiring animal derived grafts of suitable dimensions proved to be challenging. The harvested grafts were more than 50% larger than expected and the mean tapering ratio (ratio of diameter at proximal end to diameter at distal end) was 48% (Figure 1 & Table 1). This calibre mismatch between the test graft and the native sheep carotid artery resulted in difficulties in constructing the anastomosis, which necessitated altering the surgical technique by spatulating either the graft or the native artery. This both compromised the quality of the anastomosis and increased the operating time.

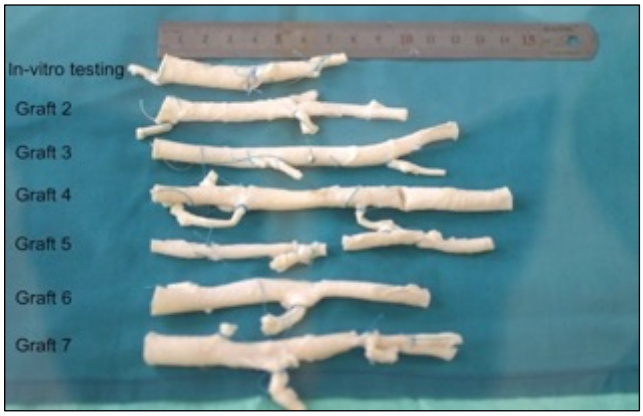


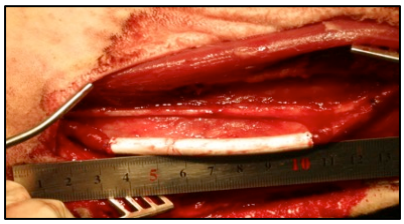

Figure 1: Decellularised grafts showing heterogeneity of dimensions

Table 1
Graft dimensions

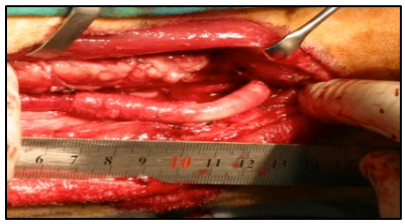
| *Graft* | *Length (mm)* | *Diameter(mm)* | | |
|---|---|---|---|---|
| | | Proximal | Mid | Distal |
| *1* | 63 | 11 | 8 | 10 |
| *2* | 98 | 11 | 8 | 5 |
| *3* | 123 | 8 | 8 | 6.5 |
| *4* | 139 | 9 | 7.5 | 6 |
| *5* | 124 | 5 | 5 | 5 |
| *6* | 100 | 10 | 9 | 6.5 |
| *7* | 112 | 11.5 | 8 | 6.5 |

The decellularised grafts had good surgical handling which was judged by the surgeon to be superior to that of the ePTFE grafts. Needle passage was smooth, and the graft edge held the suture material well. The graft demonstrated good shape memory, maintaining its cylindrical shape and after being compressed with digital pressure, returned to its original shape, albeit more slowly than ePTFE. While the graft length of ePTFE could be doubled with longitudinal traction, the decellularised graft had minimal capacity for expansion (Figure 2). The anastomotic line of the test graft formed a good seal with the native artery with minimal extravasation of blood once flow was re-established, comparable to that of the ePTFE graft.

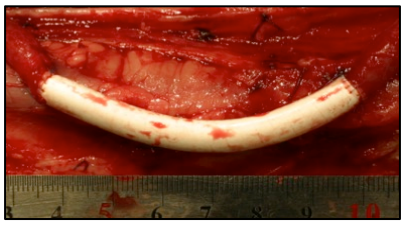
**Sheep 1 - Left**



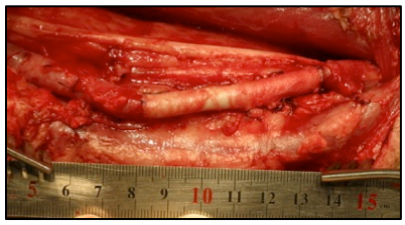
**Sheep 1 – Right**



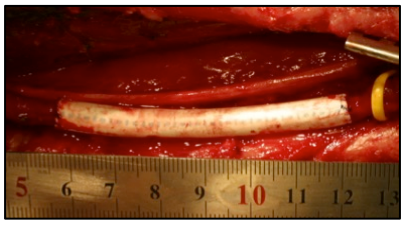
**Sheep 2 - Left**



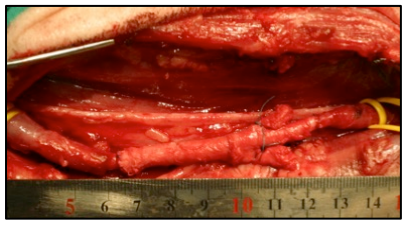
**Sheep 2 - Right**



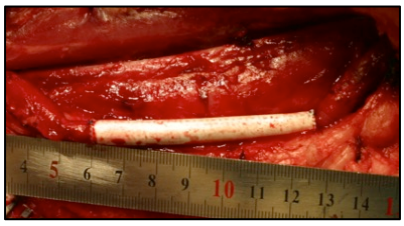
**Sheep 3 - Left**



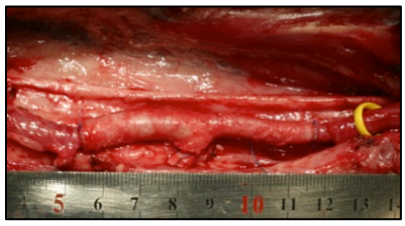
**Sheep 3 - Right**



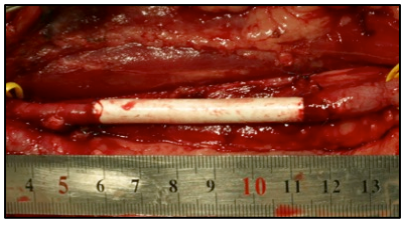
**Sheep 4 - Left**



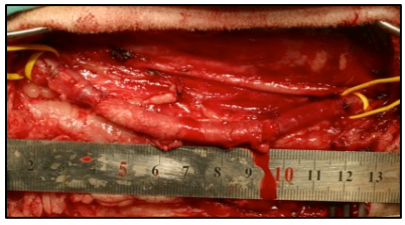
**Sheep 4 - Right**



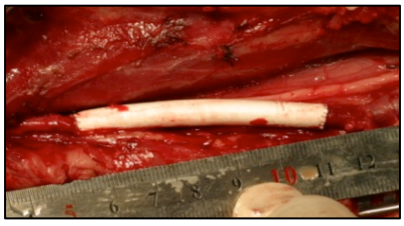
**Sheep 5 - Left**



**Sheep 5 - Right**



**Sheep 6 - Left**



**Sheep 6 - Right**

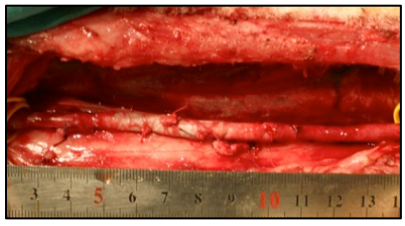


Figure 2: *In-situ* grafts at implantation. Left: ePTFE (6mm), Right: decellularised graft

3.2. Heparin content

The control grafts had negligible heparin content (mean 0.69 ±0.12mg/g of tissue).

The mean heparin content of the treated grafts (3 end- and 3 mid-section specimens) shortly after the heparinisation process was completed (0 weeks) was 14.56 ±4.01mg/g. After 11 weeks of cold storage of these grafts in PBS solution, the mean heparin content had not decreased significantly (14.56 ±4.01mg/g vs 13.41 ±3.88mg/g, NS).

There was a significant difference of heparin content between mid- and end-sections of the graft at both 0 weeks (11.03 ±0.33mg/g vs 18.08 ±1.72mg/g, p = 0.002) and after 11 weeks of cold storage (10.52 ±1.02mg/g vs 16.30 ±3.38mg/g, p = 0.047).

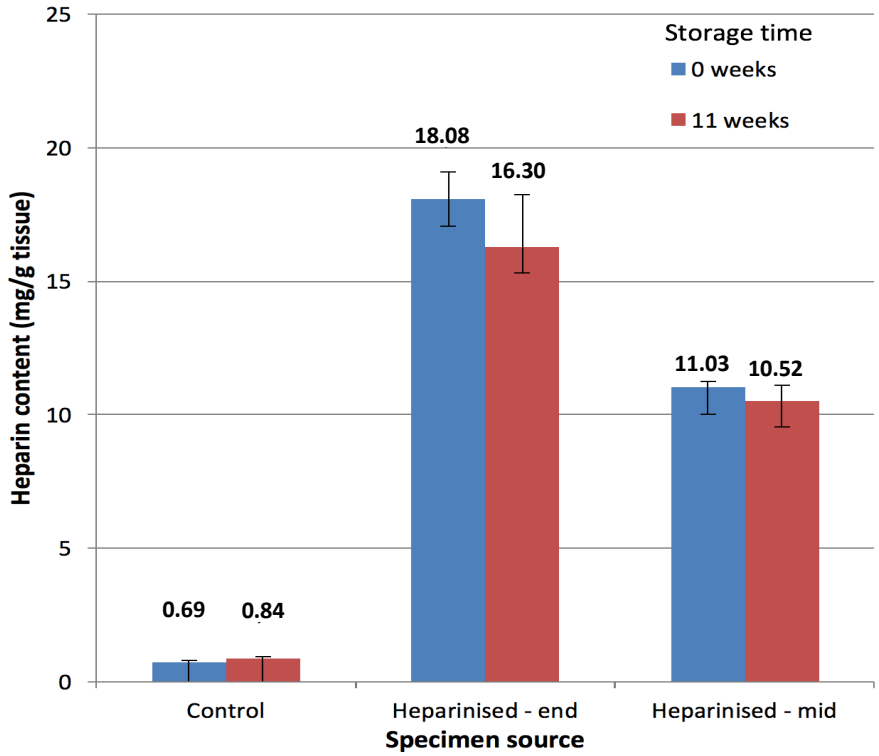



Figure 3: Heparin content after covalent cross-linking with Jeffamine demonstrating a significant difference in heparin concentration between the mid and end sections of the graft

### 3.3. Mechanical properties

The maximum circumferential load tolerated by the unheparinised graft sample before breaking was 43.79N compared to 47.54N for the heparin-treated sample. The heparinised sample was slightly larger than the untreated sample (surface area 24.54mm$^2$ vs 21.71mm$^2$) and when taking these dimensions into account, the derived ultimate strength of the heparin-treated sample was similar to that of the untreated sample (1.9MPa vs 2.0MPa). The stress-strain curves (Figure 4) of the two samples were also similar, demonstrating little change in stiffness after heparinisation.

Suture pull-out test results were also similar between heparinised and control specimens. The mean maximum tear load of the heparinised specimens was 3.92 ±0.26N vs 3.81 ±0.21N (NS) for the control specimens.

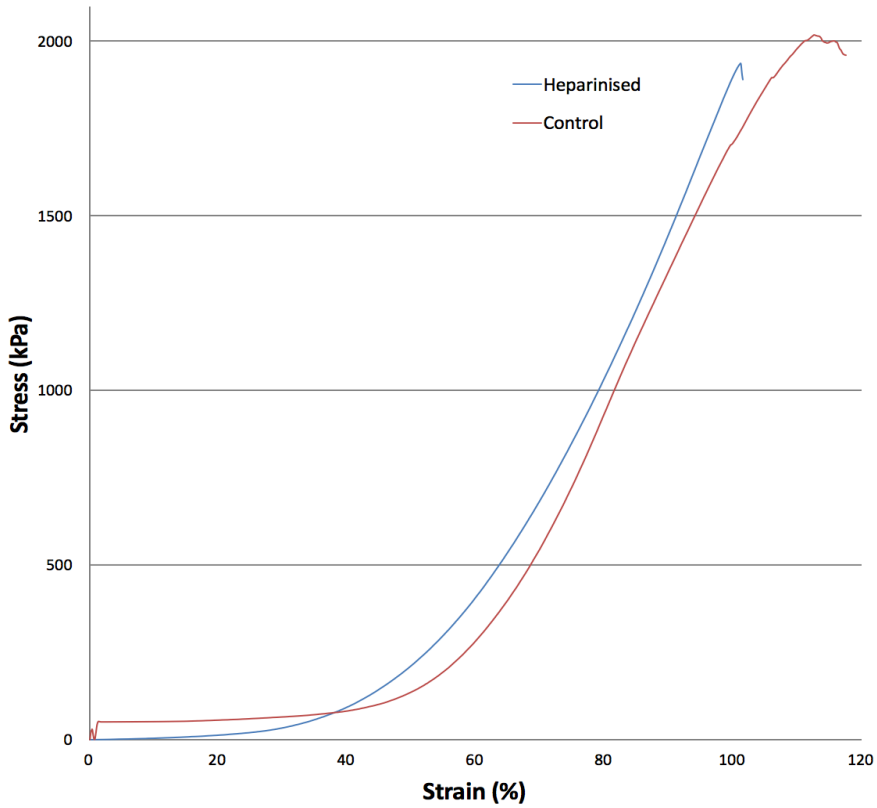



Figure 4 – Comparison of stress-strain relationships of the heparin–treated and untreated (control) decellularised graft samples

### 3.4. Patency

All five of the decellularised grafts were occluded (0% patency) while 2 out of 5 ePTFE grafts remained patent (40% patency) (Table 2).

Table 2
Patency statistics

| *Sheep* | *Right (Decel)* | *Left (ePTFE)* |
|---|---|---|
| *1* | Occluded | Patent |
| *2* | *Euthanised Day 4 post-operative (Sepsis)* | |
| *3* | Occluded | Occluded |
| *4* | Occluded | Patent |
| *5* | Occluded | Occluded |
| *6* | Occluded | Occluded |

The ultrasound assessment of each of the test grafts demonstrated complete occlusion at both the proximal and distal anastomosis (Figure 5). Angiography mirrored these findings.

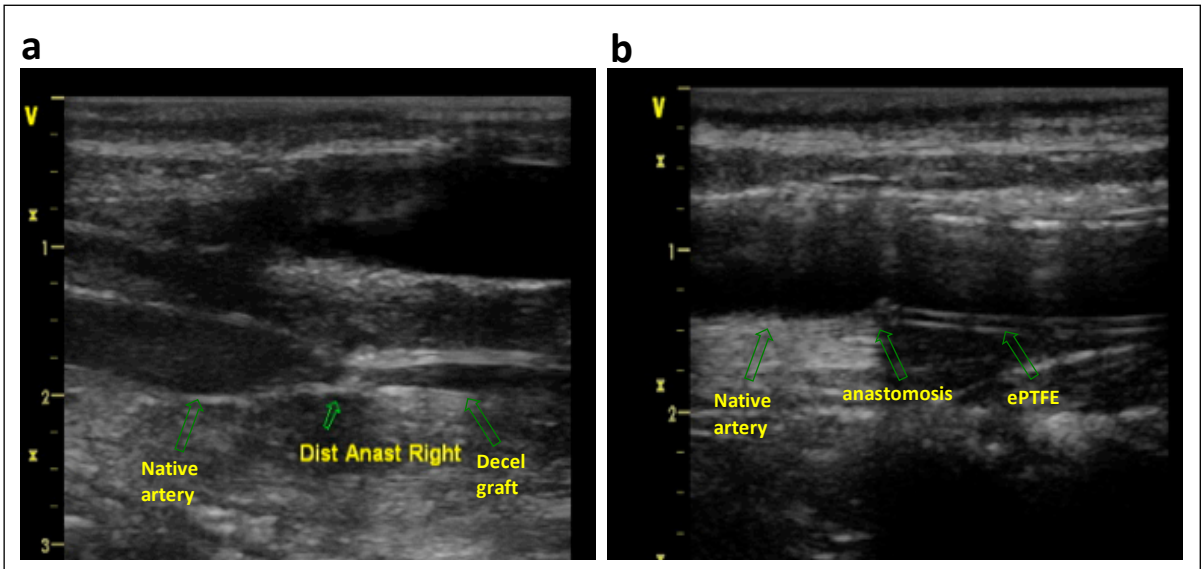



Figure 5 – *In-situ* US image demonstrating a: the interface of the native artery and the decellularised graft. Significant stenosis is seen at the site of anastomosis. In contrast, b: demonstrates the patent ePTFE graft.

3.5. Macroscopic appearance

ePTFE grafts demonstrated familiar post-explant characteristics with minimal surrounding fibrosis and dissection from adjacent tissues with relative ease. No neo-intimal reaction was found within the lumen of the patent grafts (Figure 6a). In contrast, the decellularised grafts were encased in dense fibrotic tissue which was difficult to separate from surrounding tissues. No ectasia or aneurysmal deformation was noted in any of the decellularised grafts. The lumen of all grafts were occluded by concentric organised thrombus (Fig 6b). Longitudinal section demonstrated stricture at the sites of anastomosis (Figure 6c).

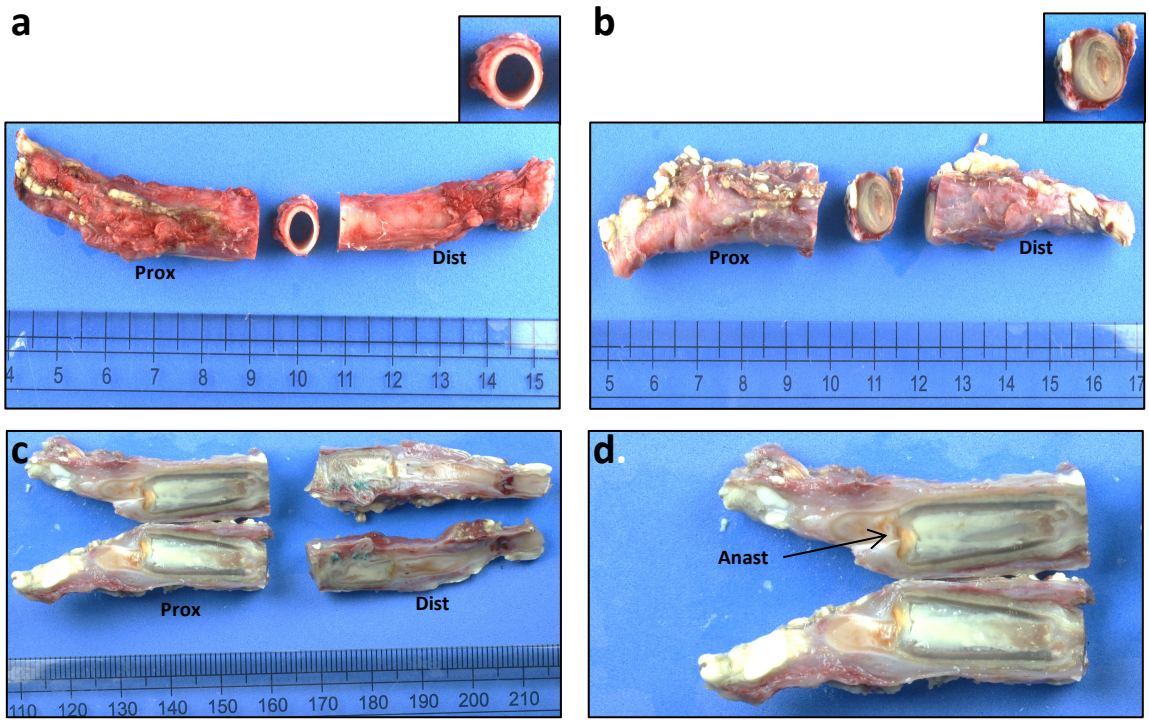



Figure 6: External and luminal appearance of ePTFE and decellularised graft from the same animal. a: ePTFE graft demonstrating patent lumen. b: Decellularised graft with perigraft & luminal neointimal hyperplasia. c: Longitudinal section of decellularised graft and d: demonstrating anastomotic stricture.
Prox: Proximal end, Dist: Distal end, Anast: Anastomosis

### 3.6. Microscopic appearance

Histological analysis of the control group were typical for ePTFE, with pannus ingrowth from the adjacent anastomosis (Figure 7, a&b). A single layer of flat nucleated cells lined the surface of the pannus and extended a short distance onto the luminal surface of the ePTFE representing transanastomotic endothelial ingrowth (Figure 7, e) with the luminal mid-graft remaining acellular. Perigraft foreign body response was limited to a few mononuclear inflammatory cells (Figure 7, f) with minimal cellular ingrowth into the abluminal surface of the graft (Figure 7, c&d).

Due to the complete occlusion of the decellularized grafts, only limited comment could be made on the extent of luminal healing. However, minimal pannus ingrowth would suggest that occlusion was acute (Figure 7, g&h). The luminal surface remained free of cells, with an exposed internal elastic lamina (Figure 7, k) and no abluminal penetration of the grafts with surrounding tissue (Figure 7 i&j) resulting in a completely acellular graft (Figure 7 m&n). The surrounding perigraft foreign body inflammatory response was more intense than the ePTFE and was macrophage and giant cell predominant with less mononuclear cells (Figure 7, l).

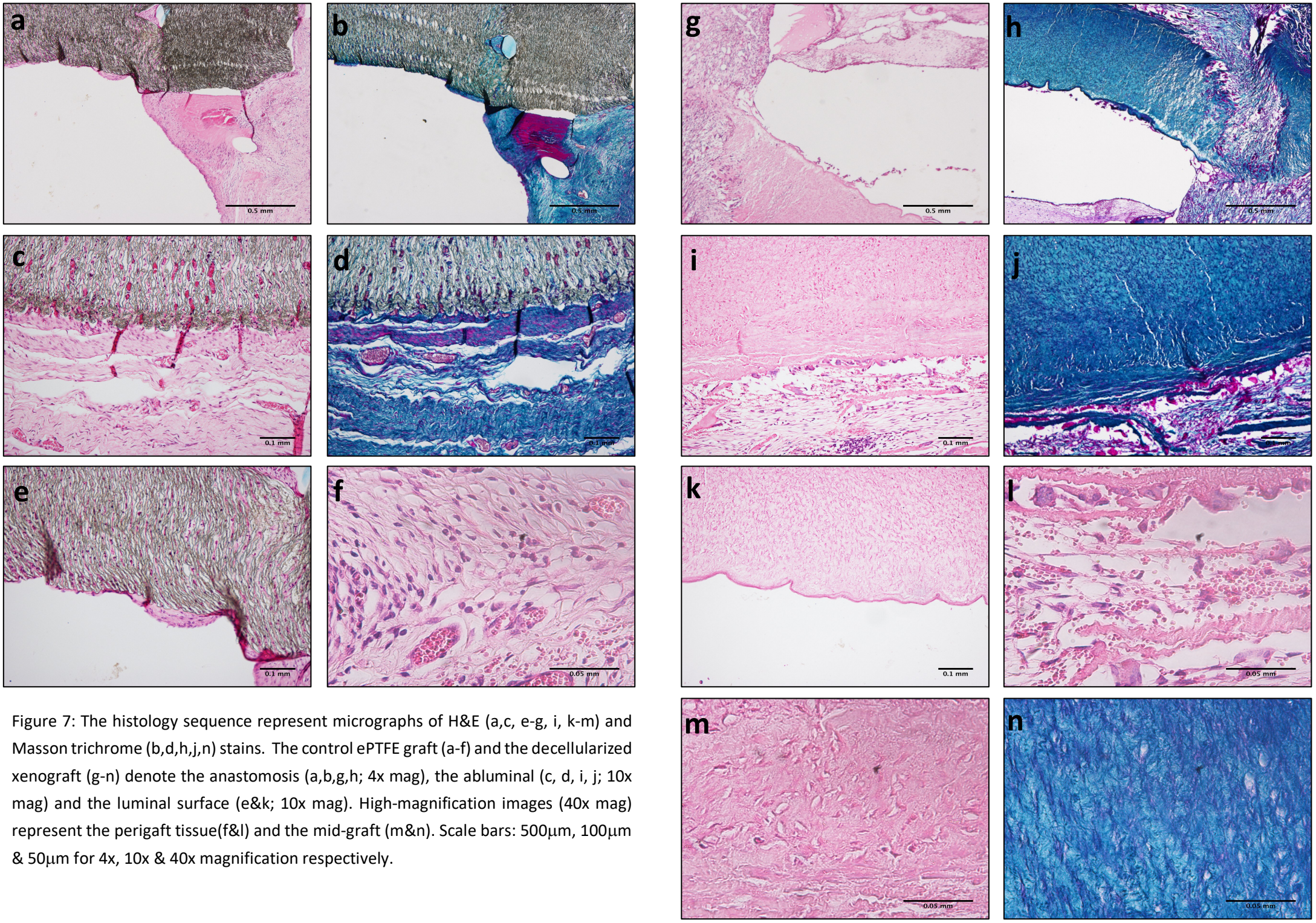



Figure 7: The histology sequence represent micrographs of H&E (a,c, e-g, i, k-m) and Masson trichrome (b,d,h,j,n) stains. The control ePTFE graft (a-f) and the decellularized xenograft (g-n) denote the anastomosis (a,b,g,h; 4x mag), the abluminal (c, d, i, j; 10x mag) and the luminal surface (e&k; 10x mag). High-magnification images (40x mag) represent the perigaft tissue(f&l) and the mid-graft (m&n). Scale bars: 500μm, 100μm & 50μm for 4x, 10x & 40x magnification respectively.

# 4. DISCUSSION

This study aimed to assess the clinical feasibility of chemically stabilised decellularised vascular grafts by testing in an ovine carotid model. None of the test grafts remained patent for the implant period, compared to 40% of the ePTFE grafts. Microscopic analysis revealed thrombosis at the sites of anastomosis without intimal hyperplasia and no sign of luminal endothelisation. The mechanical properties were assessed as being favourable, both in terms of surgical handling and by objective load testing.

The ovine carotid model used in our experiments provides a robust test of vascular grafts as it is similar to human vascular physiology.[18] Studies that have employed this model combined with *ex-vivo* preparation have achieved patency rates comparable to autologous vein grafts. For example, Kaushal et al (2001) seeded decellularised porcine iliac arteries (4mm) with endothelial progenitor cells (EPC) harvested from juvenile sheep. Seven of these grafts were implanted as carotid interposition grafts and all remained patent beyond 4 months.[22] In contrast, groups that do not employ prior *ex-vivo* preparation have reported disappointing results[23–27] which are not superior to ePTFE tested under similar conditions.[19,28,29] For example, in a similar experiment to that conducted in this study, Pavcnik et al implanted 6mm grafts composed of hydrated small intestinal mucosa as bilateral carotid interpositional grafts. Only thirty percent of these grafts were patent at 3 months.[23] The superior results of *ex-vivo* studies do, however, come at a significant cost. In the Kaushal study, producing sufficient quantities of EPCs took 3-weeks to culture and seeding required a complex bioreactor system with pressure pre-conditioning. Although these techniques produce good results, they are not easily scalable and thus not clinically feasible. To find a clinically relevant solution, our group has sought to produce an alternative vascular graft based on *in-vivo* techniques. The decellularisation protocol that was developed produced excellent patency rates in a small animal model[15] but has failed to achieve comparable results in this large animal study. Our results mirror those of similar studies and highlight the difficulty of producing decellularised vascular grafts that achieve long-term patency under clinically relevant conditions.

Heparinisation of synthetic vascular grafts has been used in an attempt to improve patency but has not been shown to significantly improve the patency of ePTFE grafts.[30] Despite this lack of effect in synthetic grafts, heparin bonding has been employed in the preparation of various decellularised grafts,[31] as the anticoagulant effects of heparin bonding on collagen was demonstrated in earlier *in-*

*vitro* studies.[32] In this study, a Jeffamine-based protocol was utilised to increase the heparin content of the decellularised grafts and, compared to passive soaking, a significant increase in the heparin content of the grafts using this protocol was demonstrated.

Using Jeffamine to bond heparin to collagen has the added effect of forming cross-links between collagen molecules, thereby increasing its resistance to degradation[15]. The importance of reducing the degradation of both collagen and elastin, the ECM proteins which confer distensibility and strength had been demonstrated in earlier work on other decellularised tissues e.g. cardiac valves.[33,34] By treating the decellularised grafts with PGG to stabilise elastin, our group previously demonstrated decreased aneurysmal dilatation of animal-derived grafts in a small animal model.[15] In this large animal study, both collagen and elastin stabilisation was achieved by incorporating both methods into the decellularisation protocol.

The preservation of elastin and collagen was reflected in the favourable compliance profile demonstrated on mechanical testing of the decellularised grafts in this study. Comparing these results to similar tests of human arteries is problematic for two reasons. First, there is limited experimental data describing the mechanical properties of human carotid arteries. In those studies that have conducted similar experiments, the cadaver specimens are usually harvested from elderly patients and thus the results are confounded by age-related calcification and atherosclerosis.[35,36] Second, explanted specimens lack the response to luminal pressure conferred by smooth muscle cell activity and thus do not accurately reflect *in-vivo* conditions. The mechanical characteristics of arteries *in-vivo* cannot be determined directly, but can be approximated by measuring the degree of expansion in response to luminal pressure during the cardiac cycle. This technique has employed ultrasound (US)[37] and magnetic resonance imaging (MRI) to assess *in-vivo* flow dynamics and to measure the mechanical response of the arterial wall. Although there was no significant difference in the ultimate strength and compliance profiles of heparinised and control samples, compared to these *in-vivo* data, the decellularised grafts from this study demonstrated favourable mechanical characteristics. The ultimate circumferential tensile strength was well within the range of *in-vivo* conditions and the compliance profile of the decellularised grafts in the physiological range was similar to that derived from MRI-based computational models.[38]

Although the mechanical properties of the test grafts were comparable to human arteries, primary patency was not achieved. Macro- and microscopic analysis revealed that all the decellularised grafts

developed complete occlusion by thrombus formation without signs of intimal hyperplasia, indicating that technical factors played a role in the failure of these grafts. A profound inflammatory response (more pronounced than that seen with the ePTFE grafts) with stricture at the anastomotic sites was also noted, which suggests that the immune response to the xenograft could have been a contributing factor to early graft occlusion. Of these factors, compliance mismatch was likely the predominant cause for graft failure in this study. The diameters of the harvested porcine mammary arteries varied widely and in a number of the grafts, there was a significant difference in calibre of the proximal and distal ends. This led to a calibre mismatch which compromised the quality of the anastomoses. In addition to compliance, calibre mismatch has been showed to be a strong predictor of graft failure for both synthetic[39] and autologous venous grafts.[40] While synthetic grafts can be manufactured to the required size, this study found that producing animal-derived decellularised grafts with the appropriate dimensions is more difficult, and is an important consideration when assessing these grafts as a potential 'off-the-shelf' solution.

# 5. CONCLUSION

While the factors which determine graft success are numerous and their interactions complex, numerous studies have highlighted the pivotal role of the endothelium and the mechanical characteristics of alternative vascular grafts in achieving long-term patency. The decellularisation process, which aims to remove antigenic material while maintaining the chemical and physical integrity of the ECM scaffold, is continually being refined.

This study was unable to achieve primary patency of the porcine-derived decellularised vascular grafts in a large animal model. Although the chemical stabilisation of collagen and elastin preserved the mechanical properties of the grafts, thrombotic occlusion occurred early, probably as a result of calibre mismatch. Our findings and those of others suggest that current decellularisation techniques are able to preserve the mechanical profile of animal-derived vascular grafts but an effective means of facilitating *in-vivo* endothelisation has yet to be achieved. Based on the success of experiments which utilise complex *ex-vivo* methods to achieve graft endothelisation, further work should be directed toward developing a scalable solution to this problem. Finally, as our experience has shown that procuring animal-derived grafts with appropriate dimensions is problematic, the clinical feasibility of utilizing these sources needs to be considered and further characterised.

# 6. REFERENCES


1. Go AS, Mozaffarian D, Roger VL, et al. *Heart Disease and Stroke Statistics--2014 Update: A Report from the American Heart Association.* Vol 129.; 2014. doi:10.1161/01.cir.0000441139.02102.80
2. Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *Eur J Vasc Endovasc Surg*. 2007;33 Suppl 1(1):S1-75. doi:10.1016/j.ejvs.2006.09.024
3. Moran A, Forouzanfar M, Sampson U, et al. The Epidemiology of Cardiovascular Diseases in Sub-Saharan Africa: The Global Burden of Diseases, Injuries and Risk Factors 2010 Study. *Prog Cardiovasc Dis*. 2013;56(3):234-239. doi:10.1016/j.micinf.2011.07.011.Innate
4. Ziegler K, Muto A, Eghbalieh S, et al. Basic Data Related to Operative Infrainguinal Revascularization Procedures: A Twenty Year Update. *Ann Vasc Surg*. 2012;25(3):413-422. doi:10.1016/j.avsg.2010.10.010.Basic
5. Veith FJ, Moss CM, Sprayregen S, et al. Preoperative saphenous venography in arterial reconstructive surgery of the lower extremity. *Surgery*. 1979;85(3):253-256.
6. van Hinsbergh VWM. Endothelium--role in regulation of coagulation and inflammation. *Semin Immunopathol*. 2012;34(1):93-106. doi:10.1007/s00281-011-0285-5
7. Salacinski HJ, Goldner S, Giudiceandrea A, et al. The mechanical behavior of vascular grafts: a review. *J Biomater Appl*. 2001;15(3):241-278. doi:10.1106/NA5T-J57A-JTDD-FD04
8. Deutsch M, Meinhart J, Zilla P, et al. Long-term experience in autologous in vitro endothelialization of infrainguinal ePTFE grafts. *J Vasc Surg*. 2009;49(2):352-362. doi:10.1016/j.jvs.2008.08.101
9. Chong DST, Lindsey B, Dalby MJ, et al. Luminal surface engineering, “micro and nanopatterning”: potential for self endothelialising vascular grafts? *Eur J Vasc Endovasc Surg*. 2014;47(5):566-576. doi:10.1016/j.ejvs.2014.02.007
10. Bergmeister H, Strobl M, Grasl C, et al. Tissue engineering of vascular grafts. *Eur Surg*. 2013;45(4):187-193. doi:10.1007/s10353-013-0224-x
11. Pellegata AF, Asnaghi MA, Stefani I, et al. Detergent-enzymatic decellularization of swine blood vessels: insight on mechanical properties for vascular tissue engineering. *Biomed Res Int*. 2013;2013:918753. doi:10.1155/2013/918753
12. Williams C, Liao J, Joyce E, et al. Altered structural and mechanical properties in decellularized rabbit carotid arteries. *Acta Biomater*. 2009;5(4):993-1005.

doi:10.1016/j.actbio.2008.11.028.Altered

13. Isenburg JC, Simionescu DT, Starcher BC, et al. Elastin stabilization for treatment of abdominal aortic aneurysms. *Circulation*. 2007;115(13):1729-1737. doi:10.1161/CIRCULATIONAHA.106.672873
14. Chuang T-H, Stabler C, Simionescu A, et al. Polyphenol-Stabilized Tubular Elastin Scaffolds for Tissue Engineered Vascular Grafts. *Tissue Eng Part A*. 2009;15(10):2837-2851. doi:10.1089/ten.TEA.2008.0394
15. Pennel T, Fercana G, Bezuidenhout D, et al. The performance of cross-linked acellular arterial scaffolds as vascular grafts; pre-clinical testing in direct and isolation loop circulatory models. *Biomaterials*. 2014;35(24):6311-6322. doi:10.1016/j.biomaterials.2014.04.062
16. Bezuidenhout D, Davies N, Black M, et al. Covalent surface heparinization potentiates porous polyurethane scaffold vascularization. *J Biomater Appl*. 2010;24(5):401-418. doi:10.1177/0885328208097565
17. Raghunath K, Biswas G, Panduranga Rao K, et al. Some characteristics of collagen-heparin complex. *J Biomed Mater Res*. 1983;17(4):613-621. doi:10.1002/jbm.820170406
18. Byrom MJ, Bannon PG, White GH, et al. Animal models for the assessment of novel vascular conduits. *J Vasc Surg*. 2010;52(1):176-195. doi:10.1016/j.jvs.2009.10.080
19. Sardelic F, Fletcher JP, Ao PY, et al. Comparison of fluoropolymer passivated Dacron and polytetrafluoroethylene grafts in a sheep model. Cardiovascular surgery (London, England). http://www.ncbi.nlm.nih.gov/pubmed/8049954. Published 1994. Accessed July 23, 2015.
20. Fercana G, Bowser D, Portilla M, et al. Platform Technologies for Decellularization, Tunic-Specific Cell Seeding, and *In Vitro* Conditioning of Extended Length, Small Diameter Vascular Grafts. *Tissue Eng Part C Methods*. 2014;20(12):1016-1027. doi:10.1089/ten.tec.2014.0047
21. Riesenfeld J, Rodén L. Quantitative analysis of N-sulfated, N-acetylated, and unsubstituted glucosamine amino groups in heparin and related polysaccharides. *Anal Biochem*. 1990;188(2):383-389.
22. Kaushal S, Amiel G, Guleserian K. Functional Small Diameter Neovessels using Endothelial Progenitor Cells Expanded Ex Vivo. *Nat Med*. 2001;7(9):1035-1040. doi:10.1038/nm0901-1035.Functional
23. Pavcnik D, Obermiller J, Uchida BT, et al. Angiographic evaluation of carotid artery grafting with prefabricated small-diameter, small-intestinal submucosa grafts in sheep. *Cardiovasc Intervent Radiol*. 2009;32(1):106-113. doi:10.1007/s00270-008-9449-7
24. Malm CJ, Risberg B, Bodin A, et al. Small calibre biosynthetic bacterial cellulose blood vessels:

13-months patency in a sheep model. *Scand Cardiovasc J*. 2012;46(1):57-62. doi:10.3109/14017431.2011.623788

25. Peng H, Schlaich EM, Row S, et al. A novel ovine ex vivo arteriovenous shunt model to test vascular implantability. *Cells Tissues Organs*. 2012;195(1-2):108-121. doi:10.1159/000331415
26. Bergmeister H, Boeck P, Kasimir M-T, et al. Effect of laser perforation on the remodeling of acellular matrix grafts. *J Biomed Mater Res B Appl Biomater*. 2005;74(1):495-503. doi:10.1002/jbm.b.30228
27. Salacinski H, Tiwari A, Hamilton G, et al. Performance of a polyurethane vascular prosthesis carrying a dipyridamole (Persantin) coating on its lumenal surface. *J Biomed Mater Res*. 2002;61(2):337-338. doi:10.1002/jbm.10175
28. Pedersen G, Laxdal E, Ellensen V, et al. Improved patency and reduced intimal hyperplasia in PTFE grafts with luminal immobilized heparin compared with standard PTFE grafts at six months in a sheep model. *J Cardiovasc Surg (Torino)*. 2010;51(3):443-448.
29. Baek I, Bai CZ, Hwang J, et al. Paclitaxel coating of the luminal surface of hemodialysis grafts with effective suppression of neointimal hyperplasia. *J Vasc Surg*. 2012;55(3):806-814. doi:10.1016/j.jvs.2011.09.012
30. Neville RF, Capone A, Amdur R, et al. A comparison of tibial artery bypass performed with heparin-bonded expanded polytetrafluoroethylene and great saphenous vein to treat critical limb ischemia. *J Vasc Surg*. 2012;56(4):1008-1014. doi:10.1016/j.jvs.2012.03.020
31. Li S, Sengupta D, Chien S. Vascular tissue engineering: from in vitro to in situ. *Wiley Interdiscip Rev Syst Biol Med*. 2014;6(1):61-76. doi:10.1002/wsbm.1246
32. Keuren JFW, Wielders SJH, Driessen A, et al. Covalently-Bound Heparin Makes Collagen Thromboresistant. *Arterioscler Thromb Vasc Biol*. 2004;24(3):613-617. doi:10.1161/01.ATV.0000116026.18945.66
33. Bailey M, Xiao H, Ogle M, et al. Aluminum chloride pretreatment of elastin inhibits elastolysis by matrix metalloproteinases and leads to inhibition of elastin-oriented calcification. *Am J Pathol*. 2001;159(6):1981-1986. doi:10.1016/S0002-9440(10)63048-9
34. Isenburg JC, Simionescu DT, Vyavahare NR. Elastin stabilization in cardiovascular implants: improved resistance to enzymatic degradation by treatment with tannic acid. *Biomaterials*. 2004;25(16):3293-3302. doi:10.1016/j.biomaterials.2003.10.001
35. Teng Z, Tang D, Zheng J, et al. An experimental study on the ultimate strength of the adventitia and media of human atherosclerotic carotid arteries in circumferential and axial directions. *J Biomech*. 2009;42(15):2535-2539. doi:10.1016/j.jbiomech.2009.07.009

36. Sommer G, Regitnig P, Koltringer L, et al. Biaxial mechanical properties of intact and layer-dissected human carotid arteries at physiological and supraphysiological loadings. *AJP Hear Circ Physiol*. 2010;298(3):H898-H912. doi:10.1152/ajpheart.00378.2009
37. Khamdaeng T, Luo J, Vappou J, et al. Arterial stiffness identification of the human carotid artery using the stress-strain relationship in vivo. *Ultrasonics*. 2012;52(3):402-411. doi:10.1016/j.ultras.2011.09.006
38. Liu H, Canton G, Yuan C, et al. Using In Vivo Cine and 3D Multi-Contrast MRI to Determine Human Atherosclerotic Carotid Artery Material Properties and Circumferential Shrinkage Rate and Their Impact on Stress/Strain Predictions. *J Biomech Eng*. 2012;134(January 2012):011008. doi:10.1115/1.4005685
39. Sarkar S, Salacinski HJ, Hamilton G, et al. The mechanical properties of infrainguinal vascular bypass grafts: their role in influencing patency. *Eur J Vasc Endovasc Surg*. 2006;31(6):627-636. doi:10.1016/j.ejvs.2006.01.006
40. Yamane Y, Uchida N, Okubo S, et al. Impact of the size mismatch between saphenous vein graft and coronary artery on graft patency. *Gen Thorac Cardiovasc Surg*. 2017;65(1):25-31. doi:10.1007/s11748-016-0694-4

# APPENDIX

*Department research committee (DRC) approval*

*Animal ethics committee (AEC) approval*

*Guide for authors – Journal of Biomedical Research*

UNIVERSITY OF CAPE TOWN

Department of Surgery

Departmental Research Committee
Professor Anwar Suleman Mall
J-45 Room Old Main Building, Groote Schuur Hospital,
Observatory 7925, South Africa
Tel (021) 406 6168/6232/6227 Fax (021) 448 6461
Email: Anwar.Mall@uct.ac.za

26th August 2013

Dr Keno Mentor
Department of Surgery
Division of General Surgery
Groote Schuur Hospital
University of Cape Town

Dear Dr Mentor,

**RE: PROJECT 2013/100**

**PROJECT TITLE: Elastin derived Vascular Scaffolds in a Sheep Model**

The above proposal was reviewed by the Department of Surgery Research Committee and I am pleased to inform you that the committee approved the study.

Please use the above project number in all future correspondence.

Yours sincerely

**PROFESSOR ANWAR S MALL**
**CHAIRMAN: RESEARCH COMMITTEE**

UNIVERSITY OF CAPE TOWN

**Health Sciences Faculty**
**Research Ethics Committee**
**Room E53-24 Groote Schuur Hospital Old Main Building**
**Observatory 7925**
**Telephone** [021] 406 6338 • **Facsimile** [021] 406 6411
**e-mail:** Sumayah.ariefdien@uct.ac.za

**06 November 2013**

**AEC REF NO: 013/023**

**Dr K Mentor**
Department of Surgery
J-floor
OMB

Dear Dr Mentor

**PROJECT TITLE: ELASTIN DERIVED VASCULAR SCAFFOLDS IN A SHEEP MODEL**

Thank you for submitting your study to the Faculty of Health Sciences Animal Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally authorised** the above mentioned study specifically for the use of **6 Merino Sheep** for three years.

***Please note that the annual progress report is due in November 2014.***

**Please quote the REC. REF in all your correspondence.**

Yours sincerely

**PROF GRAHAM LOUW**
**CHAIR, HSF AEC**

sariefdien

Appendix

ISSN 1674-8301
CN 32-1810/R

The Journal of Biomedical Research




# Guide to Authors

## Scope and Aim

The Journal of Biomedical Research (JBR), a peer-reviewed open access publication, intends to provide international biomedical researchers and clinicians with an open forum to disseminate important new information in all aspects of experimental and clinical medicine. JBR is covered by major abstracting and indexing services and welcomes submissions from authors worldwide. Six issues annually feature original research articles, review articles, case reports, and Letter to the Editor. The Journal also accepts perspective articles on medicolegal and socioeconomic aspects of medicine. Manuscripts with clinical and public health implications and manuscripts with innovative ideas and/or approaches that bridge different fields are considered with priority.

**General Requirements**

All submitted manuscripts should contain original research that has not been previously published partly or in full in print or electronic format and are currently not under consideration for publication elsewhere. Manuscripts from any country are welcome, but must be written in English. JBR also publishes reviews which are mainly by invitation. Original articles should summarize results of a complete, rigorously designed study supported by experimental and/or clinical data. The recommended length for a full-length article is 3,000 to 5,000 words, excluding abstract, references, tables, or figures. Case reports are intended to shed light on unusual cases and promote knowledge about unknown or novel disease processes or manifestations, which help contribute to a better understanding of such diseases, or provide up-to-date knowledge about rare and uncommon diseases. Submissions should not normally exceed 2,000 words or three published pages including figures, diagrams and references. Letter to the Editor is usually written to comment on a current controversial issue or express the viewpoint of the writer, and it should be limited to 2,000 words.

**Details of Submission Requirements**

A cover letter from the corresponding author must accompany the manuscript and should briefly describe what is known in the field, the conceptual advances provided by and the significance of the reported findings. The authors are encouraged to suggest appropriate reviewers and are permitted to make a request for exclusion of up to three reviewers. Cover letter should state that the submitted material is original research, has not been previously published, and has not been submitted elsewhere while under review by JBR. The cover letter is confidential and will not be sent to the reviewers. The cover letter should contain the names of all the authors and their complete mailing addresses and identify the correspondence author.

**Manuscript Components**

Manuscript should be arranged in the following order: (a) Title section, including Title, Running Title, Authors, Author Affiliations, and Correspondence; (b) Abstract; (c) Keywords; (d) Introduction; (e) Materials and Methods, (f) Results; (g) Discussion; (h) Footnotes, including acknowledgment and abbreviations; (i) References; (j) Figure legends; (k) Tables; (l) Figures; (m) Supplemental data.
Text components of the manuscript (a-j) should be prepared using Microsoft Word in 12 pt with one point five line-space on one side of A4 (297×210 mm) sheets with all pages numbered. Tables (k) should be prepared in Microsoft Word with single space. Figures (l) should be converted to JPG format. The manuscript can be submitted through the online submission system (www.jbr-pub.org).

**Research Article Format**

**Title** The title should be informative and concise, and reflect the content of the article in terms understandable to a broad readership. It should be no longer than 150 characters (including spaces) and contain no non-standard acronyms or abbreviations.

**Running title** A running title that describes the key meaning of the paper should be provided and kept within 60 characters (including spaces).

**Authorship** Author names should be spelled out in full, with the given name first and the family name last. Affiliations (including department/subunit, institution, city, province/state/region, postal code and country or region) should be provided for each author. If the authors are affiliated to different units, the affiliation sequence should follow the order of the author list, marked with superscript using a, b, and c, etc. following author names. Corresponding author's name, full postal address, telephone and fax numbers and e-mail address should be provided. Any change in authorship after submission must be approved in writing by all authors.

**Abstract** An unstructured abstract consisting of a single paragraph of no more than 150 words is recommended including the purpose of the study or the reason why you intended to do the study, the main methods used in the study, and main data or outcomes, as well as a conclusion in a definite, conclusive, and short statement, but not indefinite, vague, or suggestive sentences. The abstract must be informative rather than indicative. Authors should emphasize the new and important aspects of the study.

**Keywords** Up to six key words, separated by commas, are allowed. They should be taken from those recommended by the Index Medicus Medical Subject Headings (MeSH) browser list at http://www.nlm.nih.gov/mesh/meshhome.html.

**Introduction** The Introduction summarizes the rationale for the study and mainly includes the background of the research through literature citation, the questions to be addressed, the objectives of the research, and the main methods used.

**Materials and Methods**

***Materials***

# Appendix

Reagents: The names of the suppliers for reagent or kit used in the research should be given along with the country of the supplier (e.g., FITC-conjugated rat anti-goat IgG was purchased from Santa Cruz Biotechnology, USA.).

Drug Names: The Recommended International Non-proprietary Name (RINN) should be used for medicinal substances unless the specific trade name of a drug is directly relevant to the discussion.

Species nomenclature: The scientific name [genus, species (in italics) and authority] for all microorganisms and animals should be given. Simple chemical names may be used in certain places, for example, CO2 .

Experimental subjects: The grade of the animals used should be indicated and the certificate number of the animal breeder provided. Rats and mice of at least Grade II can be reported. The sex, age, and body weights of tested animals or humans should be expressed as mean, standard deviation, and total range.

Ethical considerations: When reporting experiments on human subjects or animals, authors should include an ethical statement in the Materials and Methods section. Authors should indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Human experiments may be performed only in accordance with the ethical standards provided by the responsible committee of the institution at which the work was carried out and in accordance with the Declaration of Helsinki (as revised in Edinburgh 2000), available at http://www.wma.net/. If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must explain the rationale for their approach and demonstrate that the institutional review board explicitly approved the doubtful aspects of the study. The patients' names, initials, or hospital names or any information that may lead to the identification of the patients should be omitted. Animal experiments should follow the guidelines for the care and use of animals established by the authors' affiliated institution(s). For enrolled subjects, each should voluntarily sign an informed consent based on the full understanding of the research. When researches were conducted on animals, authors should indicate whether the institutional and national guidelines for the care and use of laboratory animals were followed.

**Methods** should be described in sufficient detail so that other laboratories can reproduce the results and verify the claims. Generally, standard procedures should be referenced, but significant modifications should be described. Appropriate experimental design and statistical methods should be supplied and described wherever necessary for proper interpretation of data and verification of claims. All novel materials and procedures should be described in sufficient detail to allow their reproduction (e.g., DNA constructs, genetic stocks, enzyme preparations, and analytical softwares).

**Units** Please use the metric system for the expression of length, height, weight, mass, area and volume. Temperatures are to be given in degrees Celsius (°C). Use of Systeme International (SI) units is preferred for all hematological and clinical chemistry measurements.

**Quantities, units, and numerical values** (see ISO 31-0: 1992)
SI units (Système International d'Unités) should be used. For example: 25.4 mm (not 1 inch), mol/L (not M or N). When an Arabic number precedes a SI unit, the unit full name should be used, for example, 1 second, 2 minutes, 3 hours, 4 days, day 4 (the fourth day), 5 years, year 5 (the fifth year) . The symbol ‰ ( " per mill " or per thousand) should be avoided. Abbreviations such as ppm and ppb should not be used. No unit is required for relative molecular mass. A solidus (/) should not be followed by a multiplication sign or a division sign unless parentheses are inserted to avoid any ambiguity. In complicated cases, negative powers or parentheses should be used, for example, mol/(L·s), not mol/L/s. Multiple prefixes (e.g., mμg) should not be used. Dosage is expressed as per kg except in mice. Concentration in solution is expressed as per L, not per mL. Values for rpm should be converted into gravity (×g). Absorbance (A) values are preferred to optical density (OD) values.
Symbols are not pluralized (e.g., 7 kg, not kgs). The numerical value such as the ratio of the quantity to the unit (e.g., λ/nm=589) should be indicated. This is particularly useful in graphs and in the headings of columns in tables.
Use 12.4 mm (not 0.0124 m), 5 μmol (not 5×10-6 mol), 3-8 g, 3%-8%, 3 m×8 m×2 m, 8±3 g, and (8±3) nmol/L.
Do not include more digits than are justified by the accuracy of the determinations. For example: a dog weighs 9 kg (not 9000 g, which implies an accuracy of 1 g). In a sample, the number of significant figures is determined by the variation within the sample, that is, one-third of the standard deviation. For example: 8.6±2.9 kg (not 8619±2930 g, nor 9±3 kg). The sign for multiplication of numbers is a cross (×) or a raised dot (·). Leave a space between the numerical value and the unit symbol, e.g., 56°C. Calendar dates may be written in the following format: 5 June, 2006.
For isotopically labelled compounds, use a square bracket directly attached to the front of the name (word) or formula. Examples: [14C]urea, [α-32P]ATP (not AT32P), sodium [14C] formate. However, both [131I]iodoalbumin and 131I-albumin are correct.

**Results** Emphasize or summarize only important observations. Simple data may be set forth in the text with no need for tables or figures. Results can be described using text, tables and figures. The text should complement the materials given in the Tables and/or Figures, and should not be simple, direct repetition of the information in the Tables and/or Figures. Please provide full details of statistical analysis either in the text or in the Tables and/or Figure legends. Please include the type of test, the precise data to which it was applied, the value of the relevant statistics, the sample size and/or degrees of freedom, and the probability level.

**Discussion** The main purpose of the Discussion is to comment on the significance of the results and set them in the context of previous work. Emphasize any new and important findings and relate your results to other studies. Discuss the limitations of your experiments. Hypothesis should be rational and evidence-based. Please avoid repeating information in the Results section.

**Acknowledgments** may briefly include 1) contributors that do not warrant authorship; 2) technical help; 3) material support.

**Abbreviations** Any abbreviations should be defined on first usage in the text. Use SI units only. However, some common names, such as GTP, RNA and PCR, may not be defined. All nomenclature, including gene names and symbols, should be used in a scientifically accurate manner following the nomenclature conventions adopted by the scientific community.

**References** References, which should be more than 30 and less than 50 for original research, must be numbered consecutively in the order of appearance in the text, and listed in numerical order in the reference list. Each reference citation within the main body of the text should be indicated by a superscript Arabic number in square brackets. Authors are responsible for the accuracy and completeness of their references and correct citation in the text. Please pay particular attention to the content, year, volume, page and so on. Journal titles should be abbreviated in accordance with the Index Medicus (www.nlm.nih.gov). The names of all the authors are listed if three or fewer, followed by their initials, and, if there are more than three authors, only the first three authors are listed followed by et al. References published within the past five years are preferred. Do not cite uncompleted work or work that has not yet been accepted for publication as references. All references should be presented using the following format:

Journal articles in English:
[1] Manabe YC, Clark DP, Moore RD, et al. Cryptosporidiosis in patients with AIDS: correlates of disease and surival[J]. *Clin Infect Dis*, 1998,27(3):536-542.

[2] Moisan M, Barbeau J, Moreau S, et al. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms[J]. *Int J Pharm*, 2001,226(1-2):1-21.
[3] Steube KG, Meyer C, Drexler HG. Constitutive protein expression of monocyte chemotactic protein-1 (MCP-1) by myelomonocytic cell lines and regulation of the secretion by anti- and proinflammatory stimuli[J]. *Leuk Res*, 1999,23(9):843-849.

Articles not in English:
[1] Chachin M, Ohmura T, Hayashi N, et al. Pharmacological and clinical profile of telmisartan, a selective angiotensin II type-1 receptor blocker[J]. *NipponYakurigaku Zasshi (in Japanese)*, 2004,124(1):31-39.
Volume with supplement:
Avis NE, Deimling GT. Cancer survivorship and aging[J]. *Cancer*, 2008,113(S12):3519-3529.
Volume with parts:
Ozben T, Nacitarhan S, Tuncer N. Plasma and urine sialic acid in non-insulin dependent diabetes mellitus[J]. *Ann Clin Biochem*, 1995,32(Pt3):303-306.

Books:
Levinsky NG. Fluid and electrolytes. In: Thorn GW, Adams RD, Braunwald E, Editors. Harrison's Principles of Internal Medicine, 8th edition. New York: McGraw-Hill, 1977:364-375.

**Tables** Tables should be a three-line graph and cited consecutively in the text. All numbers should be written in the same decimal place. Not applicable data should be represented by a " - " . Pay attention to the difference between " zero " and " not done " as an entry in the table. Tables also should be practically self-contained and self-explanatory. All tables should be cited in the text and numbered consecutively with Arabic numerals. Ensure that all symbols or abbreviations are explained. Label each table at the top with an Arabic numeral followed by the table title. Insert explanatory material and footnotes below the table. Designate footnotes using lowercase superscript letters (a, b, c) reading horizontally across the table. Abbreviations that are used in a table should be defined in the footnotes. Please prepare the tables in MS Word with a single-space format. Do not embed tables as graphic files, document objects, or pictures.

**Figures** Figures (graphs, charts, photographs, and illustrations) should be practically self-explanatory and labeled professionally. Dimensional drawings and diagrams should include only the essential details with as little lettering as possible. The magnification for micrograph should be indicated by scale bars, inscriptions should be legible, and the symbols and letters should be consistent among the panels. Figures should be explained in the text, and should appear in the order cited. Each figure should be a single file. Photographs must be of the highest quality with good contrast. Color photographs are welcome. Photomicrographs should be cropped to show only the essential parts and an inset at higher magnification is usually more informative than a single large one. Scale bars (e.g., 1 mm) should be put inside the photomicrographs. For initial submission, JPG format is preferred. Adequate figure resolution is essential to a high-quality print of your paper. Raster line art should carry an absolute minimum resolution of 600 dpi, and for grey scale and color artworks, a minimum resolution of 300 dpi is required.

**Figure legends** The legends should be typed below the reference of the manuscript. Staining methods and magnification should be indicated for a graph of tissue sections. Every figure should contain a title, and the figure legend should follow the title closely and provide enough information to make the figures understandable without frequently referring to the text. Do not describe methods in figure legends unless they are necessary for interpreting the results conveyed by the figure. All symbols and abbreviations that are used in the figure should be defined in the legends.

**Supplemental Materials** Supplemental materials that are directly related to the conclusion are welcomed. However, unrelated data may be removed by editors. Depending on the nature of supplemental data, they can be in plain text, MS Word, MS Excel, JPEG, TIFF, and video. We encourage authors to submit supplemental documents in PDF format if applicable.

**Potential Conflicts of Interest**
Authors are responsible for disclosing all financial and personal relationships that might bias their work. Authors should state explicitly whether potential conflicts do or do not exist in the manuscript on a conflict-of-interest notification page that follows the title page, providing additional detail, if necessary, in a cover letter that accompanies the manuscript.

**Letters to the Editor**
The Journal accepts Letters to the Editor, which report original data or may discuss published articles. Submission requirements for Letters are the same as for regular manuscripts. Letters should be less than 1000 words in length, and cannot include more than 12 references and two figures (including tables). A single Figure or Table can be made but should contain no more than 8 separate panels labeled from (a) to (h). One panel may be composed of multiple figures with annotation around individual panels to make viewing easier. An abstract and subheadings should not be included in Letters. All letters to the Editor are subject to editing and possible abridgement. The number of references for Letter to the Editor should be no more than eight.

**Reporting Clinical Trials**
Randomized controlled trials should be presented according to the CONSORT guidelines. At manuscript submission, authors must provide the CONSORT checklist accompanied by a flow diagram that illustrates the progress of patients through the trial, including recruitment, enrollment, randomization, withdrawal and completion, and a detailed description of the randomization procedure. The CONSORT checklist and template flow diagram can be found on http://www.consort-statement.org.

**Self-archiving Policy**
Authors are allowed to self-archive their accepted articles on their own websites or in any repository, provided it is only made publicly available concurrently or after official publication of their articles. The authors are allowed to use the publisher's final version of the article for the purpose of self-archiving or deposit. Furthermore, the authors should give acknowledgement to the original source of publication.