Thrombogenecity of decellularized and re-endothelialized baboon arteries

By

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Declaration

I, Mmakgabu Martha Khemisi, declare that the master's research dissertation that I herewith submit to the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education. I declare that I am aware that the copyright is vested in the University of the Free State. I also hereby declare that I am aware that the research may only be published with the dean's approval.

Mmakgabu Martha Khemisi

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"This dissertation is dedicated to my late father, Thabiso Simon Khemisi, for he is my rock and motivation in life."

"The lord is my shepherd, I shall not want… He restores my soul and leads me in the paths of righteousness… Yea, though I walk through the valley of the shadow of death, I will fear no evil; for you are with me; your rod and you stuff, they comfort me…and I will dwell in the house of the Lord forever".

Psalm 23

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Abbreviation list

ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AHA	American Heart Association
AMP	Adenosine mono-phosphate
AT	Anti-thrombin
ATP	Adenosine triphosphate
BH_4	tetrahydrobiopterin
CA	Carlifornia
Ca^{2+}	Calcium
CaM	calmodulin
CAMs	Cell adhesion molecules
CD	Cell of Differentiation
cGMP	cyclic guanosine monophosphate
CO_2	Carbon dioxide
Coll	Collagen
COX	Cyclooxygenase
CVD(s)	Cardiovascular Diseases
DAPI	4’,6-diamidino-2-phenylindole
Dil-Ac-LDL	Dil-Acetylated Low Density Lipoprotein
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EC(s)	Endothelial Cell(s)
ECM	Extracellular Matrix
ecto-ATPDase	ecto-Adenosine diphosphohydrolase
ecto-NTPDase	ecto-Nucleoside triphosphate diphosphohydrolase
EDTA	Ethylene diamine tetra-acetate acid
EFs	Elastic fibers
EGM-2	Endothelial Growth Medium-2
ELAMs	Endothelial leukocyte adhesion molecules
eNOS	Endothelial nitric oxide synthase

EPC	Endothelial Progenitor Cell
ePTFE	expanded-Polytetrafluoroenthylene
FBGC	Foreign body giant cells
FBS	Bovine Fetal Serum
FDA	Food and Drug Administration
GAGs	Glycosaminoglycans
GC	Guanylate cyclase
H&E	Haemotoxylin and Eosin
hEGF	human endothelial growth factor
HEPES-BSS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - Basal Salt Solution
hFGF-B	human fibroblasts growth factor-Basic
HSPGs	Heparan sulphated proteoglycans
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule-1
ILE	Internal elastic lamina
IND	Intermodal distance
LDL	Low density lipoprotein
MHC	Major Histocompatibility Complexes
MT	Masson Trichrome
N	Nuclear
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPCO	North Australian Pastoral Company
N-ECs	Nuclear of endothelial cells
N-FBs	Nuclear of fibroblasts
NO	Nitric oxide
N-SMCs	Nuclear of smooth muscle cells
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PBS	Phosphate Buffered Saline
PE	Pulmonary Embolism
PGH_2	Prostaglandin
PGI_2	Prostacyclin

PGIS	Prostacyclin synthase
Prot C	Protein C
Prot S	Protein S
P-sel	P-Selectin
PU	Polyurethane
R3-IGF-1	Recombinant insulin-like growth factor-1
ROS	reactive oxidized species
SA	South Africa
SABS	South African Bureau Standard Division
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
SMC(s)	Smooth muscle cells
TA	Tunica adventitia
TE	Tissue Engineering
TEBV	Tissue engineered blood vessel
TEM	Transmission Electron Microscopy
TEVG(s)	Tissue engineering of vascular grafts
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TI	Tunica intima
TM	Thrombomodulin
TM	Tunica media
tPA	Tissue Plasminogen Activator
USA	United States of America
VEGF	Vascular endothelial growth factor
VLDL	Very low density liprprotein
VSMCs	Vascular smooth muscle cells
VVG	Verhoeff-Van Gieson
vWF	von Willebrand factor
WHO	World Health Organization
WPB	Weibel-Palade bodies

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1. Introduction

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality in the world (World Health Organization (WHO), 2011). An estimated 17.3 million deaths were due to this non-communicable disease in 2008 (WHO 2011). Over 80% of these deaths took place in low and middle income countries (WHO 2009), where close to 200 deaths occurred daily in South Africa (SA) (Maredza *et al.* 2011). The CVD burden in SA is estimated to increase among all age groups and is predicted to become the prime contributor to overall morbidity and mortality (Maredza *et al.* 2011). The American Heart Association (AHA) further estimated that by 2030 more than 23 million people in the world will die annually from CVDs (AHA 2010).

Cardiovascular disease is a broad class of diseases that primarily affects the heart and blood vessels. Vascular diseases form part of cardiovascular disease and primarily affect blood vessels (WHO 2011). Atherosclerosis is the main cause of vascular diseases responsible for more than 25% of all deaths worldwide (WHO 2011). Atherosclerosis results from hardening and narrowing of the inner lining of the blood vessel as a result of plaque formation inside the vessel walls (Steyn 2007). The consequence of vascular disease is poor functioning of the tissue, tissue damage (ischemia) and worst case – tissue death as a result of inadequate blood flow to the affected tissue.

Current therapies for vascular diseases often require replacement of diseased vessels with vascular grafts. However, many patients do not have vessels suitable for grafting due to pre-existing vascular diseases, size mismatch or the vessels have been used in bypass procedures (Cho *et al.* 2005; Zhou *et al.* 2012; Barron *et al.* 2003). Commercially available synthetic grafts made from expanded polytetraflouroethlyne (ePTFE) or Dacron are currently used for reconstruction of large arteries, such as the aorta or the iliac artery. These synthetic grafts are however not suitable for reconstruction of smaller diameter (< 6 mm) arteries. This is due to the low patency rate caused by thrombogenecity and limited re-endothelialization *in vivo* (Sapsford *et al.* 1981; Whittermore *et al.* 1989). According to Mitchell and Niklason (2003), autologous arteries or veins are still considered to be the best substitutes for small diameter vessels and are in high demand for bypass procedures. Hence extensive research that focuses on the development of novel small diameter vascular grafts has been conducted until recently. However there is still no adequate alternative to the autologous vessels (Zhou *et al.* 2012; Kakisi *et al.* 2005). Therefore there is still a clinical need for an alternative supply of vessels that can be used to replace diseased arteries.

Regenerative medicine and tissue engineering studies have become fields of interest in medical research in order to address this problem. Tissue engineering offers the potential of providing vessels that can be used to replace diseased and damaged native blood vessels. The

use of decellularized biological scaffold material from both xenograft and allograft origin are used in constructing tissues and organs in order to restore or establish normal function (Gilbert *et al.* 2006; Jordan *et al.* 2012; Ning-tao *et al.* 2007). These studies aim to fabricate living autologous grafts with the capacity for growth, repair and remodelling (Khait *et al.* 2008; Ning-tao *et al.* 2007). These biological scaffolds materials (including those from blood vessels) have already been successfully used in pre-clinical animal studies and in human applications (Chen *et al.* 2004; Valentin *et al.* 2010; Chen *et al.* 1999; Parekh *et al.* 2009; Lantz *et al.* 1993; Conklin *et al.* 2002; Dahl *et al.* 2003; Lichtenburg *et al.* 2006; Lin *et al.* 2004).

The use of decellularized biological scaffold materials represents an attractive possibility for use especially in vascular grafts construction. However, thrombogenecity is a major concern as these graft materials contains no cells, thus exposing collagen fibres to blood. Studies showed that a decellularized vessel scaffold attract and induce platelet activation and thrombosis when directly exposed to blood (Conklin *et al.* 2002; Ning-tao *et al.* 2007; Kasimir *et al.* 2006; Sarkar *et al.* 2007). After some time these vessels calcify and/ or degenerate due to the absence of an endothelial lining (Schoen and Levy 2005; Kasimir *et al.* 2006). The introduction of surface coating of small diameter grafts with angiogenic growth factors to enhance migration and proliferation of ECs *in vivo* has shown some promising results, but does not solve the problem completely (Sgarioto *et al.* 2012; Sales *et al.* 2007; Kerdjoudj *et al.* 2007; Balcells and Edelman 2002; Kipshidze *et al.* 2000; Sipehia *et al.* 1996). As a result no small diameter grafts exists even today.

Recently a more promising approach for construction of small diameter vascular grafts is the recellularization of decellularized biological scaffold material with autologous vascular endothelial cells prior to implantation. Endothelial cell (EC) seeding of decellularized vessels is an attractive proposition as the endothelial layer incorporates many of the anti-thrombogenic properties of blood vessels. However successful construction of small diameter vascular grafts still remains a great challenge. As endothelialization of vascular grafts is limited by the inability of endothelial cells to remain attached to the scaffold after exposure to flow (Sgarioto *et al.* 2012). As a result more investigations are still needed to optimize endothelial seeding methods *in-vitro* in order to provide a suitable support structure especially for endothelial cells.

Our study aims to successfully re-endothelialize decellularized baboon arteries. Thereafter investigate thrombogenecity of these arteries after perfusing them with baboon blood.

2. Literature review

This literature review will start with an overview of the cardiovascular system and tissue organization of the blood vessels.

2.1. Overview of the cardiovascular circulation system

The cardiovascular system was first described completely and in detail by the English physician William Harvey (1578-1657) (**figure 2-1: A**) in the 17th century. In his published book titled "*Exercitatio Anatomica de Muto Cordis et Sanguinis in Animalibus*" which translates "*An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings*" he described the motion of blood in the body as circular. He demonstrated that blood is forced by the action of the left ventricle of the heart into the large diameter, low resistance conducting arteries. These large arteries are able to carry blood away from the heart under high pressure. Arteries transport blood that carries oxygen and nutrients to the systemic circuit (**figure 2-1: B**) to nourish the body. When a large artery reaches the organ it is supplying, it branches into small arteries and then arterioles. Arterioles are the primary resistance vessels that offer high resistance, causing a marked drop in pressure as blood flows through them (Sherwood 2007:350). These arterioles help regulate blood flow into various organs by regulating the flow of blood into capillary vessels. The capillaries are a meshwork of vessels that connect the arteries to the veins. They are thin walled and allow the exchange of nutrients, water, oxygen and waste material between the surrounding tissues and blood. The deoxygenated blood which is "effete" as he refers to it is then returned to the right ventricle of the heart through the large distensible capacitance veins. Veins contain one way valves that prevent backflow of blood and move blood towards the heart. The right ventricle then pumps blood into the pulmonary circuit (**figure 2-1: B**) to become oxygenated again and returned back to the left ventricle of the heart, thus completing the vascular circuit. Dr William Harvey's discovery led to today's basic knowledge of the cardiovascular system as a closed loop in which blood is circulating.

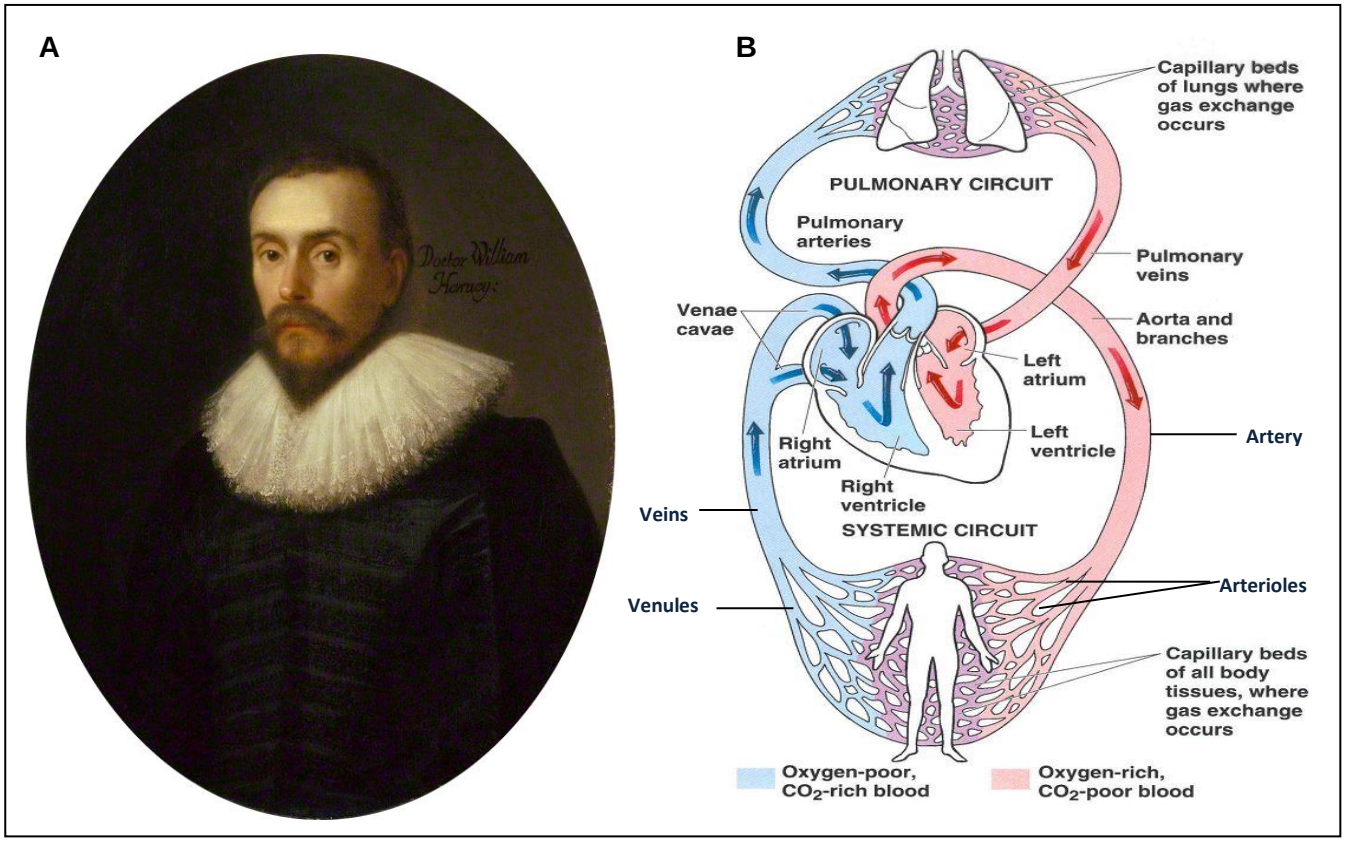

Figure 2-1: (A) Dr William Harvey (1578-1657) (http://www.npg.org.uk/collections/search/largerimage.php?LinkID=mp02074&page=1&role=sit&rNo=0). (B) The cardiovascular System showing the pulmonary circuit and the systemic circuit (http://www.phschool.com/science/biology_place/biocoach/cardio2/intro.html).

2.1.1. Tissue organization of the blood vessels

All vessels are lined with a thin layer of smooth, flat endothelial cells that are continuous with the endothelial lining of the heart (Ross and Pawlina 2006:372). Walls of a blood vessel consist of three basic layers, tunica intima, media and adventitia (Saladin 2012:750). The different types of blood vessels differ structurally and functionally (**figure 2-2)**. Arteries and veins differ in the thickness of their layers due to their distinct functions. The tunica intima lining is composed of a monolayer of squamous endothelial cells that is in contact with blood. The intima is separated from tunica media by the internal elastic lamina. The tunica media is the middle layer composed of the smooth muscle cells, collagen and elastic tissue. The media strengthens the vessels and prevents blood pressure from rupturing them. It also produces vasomotion – allowing the vessel diameter to increase or decrease by dilating and constricting the vessel. The outer limit of the media is separated from the adventitia by the external elastic lamina. The outermost layer, tunica adventitia (externa) is composed mainly of loose fibrous connective tissues. Adventitia (externa) anchors the

vessel and provides passage for small nerves, lymphatic vessels, and smaller blood vessels that supply the tissues of the larger vessel. Smaller vessels (vasa vasorum) pass through the adventitia into the outer one-half to two-thirds of the media to perfuse and nourish the vessel wall. The next section will discuss the vascular endothelium.

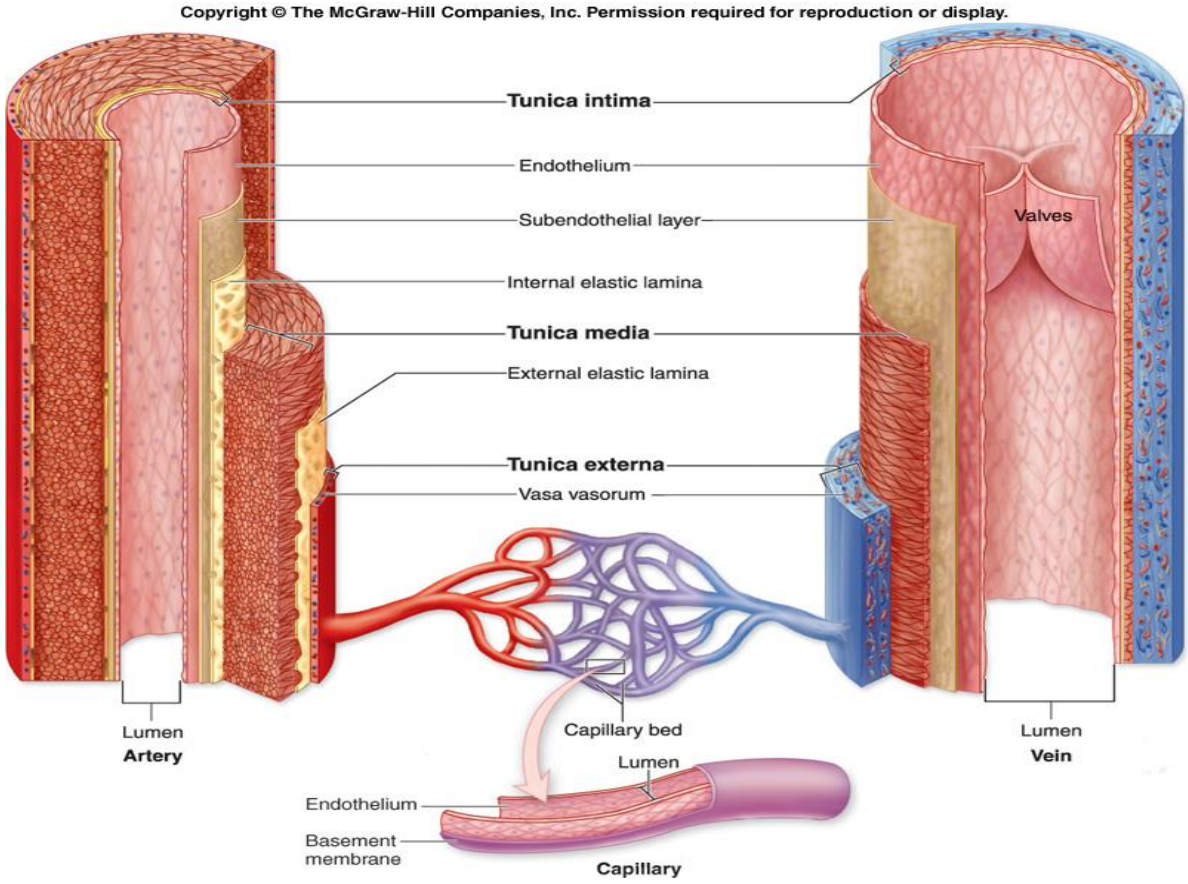

Figure 2-2: **Tissue organisation for the different types of blood vessels (http://mrcatlee.weebly.com /circulatory-system.html).**

2.2. Vascular endothelium: the important regulator of vascular homeostasis

When William Harvey discovered the motion of blood and the functions of the heart and blood vessels during his vivisections, he must have noticed that blood had coagulated in the bodies of the dead but remained fluid in the bodies of the living. Decades later, we have a better understanding of the active role of ECs in maintaining fluidity of blood within blood vessels. The ability to culture ECs *in-vitro* has also allowed us to understand more about the physiology and biochemistry of these cells. Normal functioning of the vascular system is mostly modulated by vascular ECs which form a continuous monolayer that lines the lumen of all blood vessels. At first, the vascular endothelium was regarded as just a passive barrier between the circulating blood and the vascular

wall. However through research, we know that vascular ECs play a major in the regulation of the vascular system. Endothelial cells (ECs) are active participants in a variety of interactions between circulating blood and underlying connective tissues. They are responsible for maintaining a selective permeability barrier and a non-thrombotic surface. They modulate blood flow and regulate cell growth and immune responses. They also maintain the extracellular matrix and are involved in lipoprotein metabolism (Ross and Pawlina 2006:375). **Table 2-1** summarizes the properties of endothelial cells.

The vascular endothelium also helps regulate vascular homeostasis while maintaining the patency of all blood vessels. It plays a major role in regulating blood flow by regulating vascular tone through the synthesis and release of vasoactive substances. These substances include nitric oxide (NO), prostacyclin, platelet activating factor and endothelin-1. **Table 2-2** describes in more details the vasoactive substances synthesized and released by endothelium. Vascular endothelium also secretes substances that stimulate angiogenesis and regulate proliferation of vascular smooth muscle cells (VSMCs).

The emphasis of this section is however on the ability of the vascular ECs to provide a non-thrombogenic environment to the circulating blood. Several EC-mediated mechanisms have been shown to be responsible for maintaining a non-thrombogenic environment in healthy blood vessels. The vascular ECs have the ability to maintain a haemostatic balance through the synthesis and secretion of anti-coagulant and anti-thrombotic factors. Thus ECs have the ability to inhibit inappropriate coagulation of the blood under normal physiological conditions. The next section explains the anticoagulant properties of vascular endothelium.

Table 2-1: A summary of properties and functions of ECs (Ross and Pawlina 2006:375).

Major properties	Functions	Active molecules involved
Maintains a selective permeability barrier	Allows simple diffusion, Actively transport molecules, Pinocytosis – pinocytotic vesicles, Receptor mediated endocytosis	Oxygen, carbon dioxide Glucose, amino acids, electrolytes Water, small molecules, soluble proteins Low density lipoprotein, cholesterol, transferrin, growth factors, antibodies, major histocompatibility complexes (MHC)
Maintains a non-thrombogenic environment	Secretes anticoagulants Secretes antithrombotic agents Secretes pro-thrombotic agents	Tissue factor pathway inhibitor (TFPI), Thrombomodulin, heparan sulphated proteoglycans Prostacyclin (PGI_2), tissue plasminogen activator (tPA), Nitric oxide (NO) Tissue thromboplastin, von Willebrand factor, plasminogen activator inhibitor
Modulate blood flow and vascular resistance	Secretes vasodilators Secretes vasoconstrictors	Endothelial-derived relaxation factor, NO, PGI_2 Endotheli-1, angiotensin converting enzyme, platelet activating factor (PAF)
Regulates cell growth	Secretes growth factor-stimulating factors Secretes growth inhibiting factors	Platelet-derived growth factor, hemopoietic colony-stimulating factors, fibroblast growth factor Heparin, transforming growth factor β
Regulates immune responses	Regulates leukocyte migration by expressing adhesion molecules Regulates immune functions	Selectins, intergrins, CD marker molecules Interleukin molecules (IL-1, -6, -8), MHC molecules
Maintains the extracellular matrix	Synthesizes the basal lamina Synthesizes glycocalyx	Type IV collagen, lamina, Proteoglycans
Involved in lipoprotein metabolism	Produces free radicals to modify lipoproteins	LDL, cholesterol, VLDL

Table 2-2: A list of vasoactive substances synthesized and released by the endothelium (Cines *et al.* 1998). The table shows principal regulatory compounds synthesized by the endothelium, their effects on the vasculature and other processes, their mode of secretion, and the nature of their chemical composition and precursor compounds.

Substances	Principal effect	Other effects	Secretion	Compounds	Precursor compound
Nitric oxide (NO)	Vasodilation	Maintains basal tone of blood vessels; inhibits leukocyte adhesion; inhibits platelet activation, secretion, adhesion and aggregation; inhibits smooth muscle cell migration and proliferation	Paracrine/ Constitutive and induced by thrombin, adenosine diphosphate (ADP), bradykinin, Substance P, muscarinic agonists, shear stress, cyclic strain, cytokines	Heterodiatomic free radical	L-arginine
Prostacyclin (PGI_2)	Vasodilation	Retard platelet aggregation and deposition	Paracrine/induced at sites of vascular perturbation	Elcosanoid	Arachidonic acid
Platelet activating factor (PAF)	Vasoconstriction	Promotes leukocyte adhesion at cell surface	Juxtacrine/induced	Phospholipids	Arachidonic acid
Endothlin-1 (ET-1)	Vasoconstriction	Mitogen for smooth muscle cell; modulates effects of numerous compounds	Paracrine/induced by hypoxia, shear stress and ischemia	21 amino acids peptide	Preproendothelin -1n(203 amino acids)

2.2.1. Anti-coagulant properties of vascular endothelial cells

Endothelial cells (ECs) in a resting state express neutral phospholipids that do not support coagulation (Smith 2009). In addition, ECs have the ability to synthesize and release anti-coagulant factors that actively prevents platelet activation and thrombin generation. These factors also act together with anti-coagulant factors found in the circulating blood to inhibit inappropriate coagulation. **Figure 2-3** explains this process.

Firstly, the intact vascular endothelium has a strong negative charge that repels circulating platelets at physiological pH to maintain an anticoagulant environment (Hoak *et al.* 1981). Tissue factor (TF) expressed on EC membrane is the primary initiator of coagulation (Báchli 2000; Smith 2009). Controversial believe is that TF is inactive in resting ECs as these ECs only express neutral phospholipids and not pro-coagulant surfaces, (Butenas 2012, Mackman *et al.* 2007, Smith 2009). However activated FVII (Factor VIIa), the only coagulation protein that circulates in blood in its active form, can rapidly bind to TF initiating coagulation. Therefore to inhibit this action, resting ECs also express tissue factor pathway inhibitor (TFPI) (Smith 2009). Tissue factor pathway inhibitor (TFPI) prevents initiation of coagulation by blocking the actions of factor VIIa – TF complex. It also functions as an upstream inhibitor of thrombin by forming a complex with FXa and FVIIa. It then binds to TF forming a quaternary complex TFPI-FXa-FVIIa-TF to prevent further participation of these proteins in the generation of additional thrombin (Butens 2012).

Another anticoagulant factor produced by ECs and expressed on the luminal surface is heparan sulphated proteoglycans (HSPGs) (Mertens *et al.* 1992). The HSPGs chains bind anti-thrombin III (AT), a proteinase inhibitor that is produced by the liver and circulates in blood to inactivate thrombin. Anti-thrombin III (AT) can also inactivate FXa and FIXa to prevent any new additional thrombin from being generated during coagulation to maintain hemostatic balance (Smith 2009).

The ECs also synthesize and express thrombomodulin (TM), a membrane glycoprotein that binds and inactivates the circulating thrombin molecule. The inactivated thrombin molecule cannot convert the circulating fibrinogen into fibrin. The inactivation of thrombin also prevents activation of platelets. If thrombin is generated - even in small amounts, it binds to platelet receptors causing them to express pro-coagulant surfaces. The thrombin-TM complex also activates protein C (Prot C), a serine protease that inhibits coagulation factors V and VIII and thus thrombin generation. However Prot C requires the presence of its cofactor protein S (Prot S) (the free form) that is synthesized by the endothelium in order to inactivate these factors. The Prot C – Prot S complex also inactivates plasminogen activator inhibitor (PAI) which up regulates lysis of any fibrin that is

formed. Furthermore Prot C provides an anti-inflammatory activity by inhibiting mononuclear cell adhesion to endothelial cells.

Furthermore, to prevent the activation of platelets, ECs express ecto-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) also known as ecto-ADPase/CD39 on their surface membranes (Marcus *et al.* 1997). Platelets become activated in response to adenosine diphosphate (ADP) activity (Hoak *et al.* 1981; Schneider *et al.* 1997). Therefore ecto-ATPDases prevents activation of platelets by converting ATP (adenosine triphosphate) and ADP to inert adenosine mono-phosphate (AMP).

Endothelial cells (ECs) also synthesize and release von Willebrand factor (vWF) a protein that facilitate the interaction of platelets with the basement membrane. However ECs stores vWF within their storage granules, known as Weibel-Palade bodies (WPB). This prevents the inappropriate interaction of vWF with platelets.

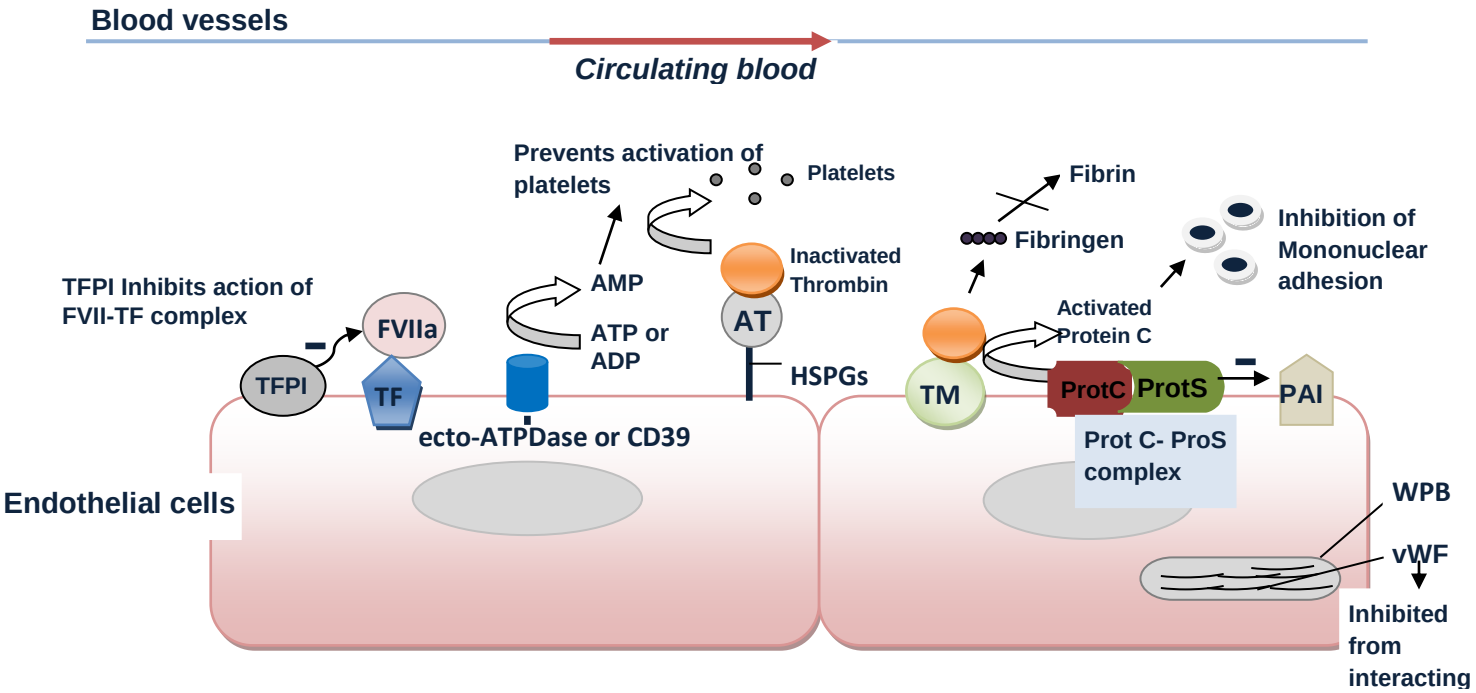

Figure 2-3: Anti-coagulant effects of vascular endothelial cells. The anti-coagulant properties of endothelial cells are maintained through various mechanisms that involve the expression of TFPI, ecto-NTPDase/CD39, HSPGs, TM and TM-activated protein C pathways.

2.2.2. Anti-thrombotic properties of vascular endothelial cells

Healthy vascular ECs secrete endothelial-derived nitric oxide (NO), prostacyclin (PGI_2) and tissue plasminogen activator (tPA) to protect the vascular wall against thrombosis (Gryglewski *et al.* 2002; Gryglewski *et al.* 2001; Ohtake 1997; Wu and Liou 2005). These molecules have an inhibitory effect on platelet aggregation and leukocyte adhesion to undamaged ECs, and also promote thrombolysis.

Prostacyclin, also known for its vasodilator effects, inhibits platelet aggregation, leukocyte adhesion and proliferation of vascular smooth muscle cells (VSMC) (Noda *et al.* 2007). It also inhibits the invasion of activated platelets into the vessel wall (Gryglewski *et al.* 1976). Prostacyclin, a prostanoid, is formed when the arachidonic acid is released from the plasma membrane of endothelial cells by phospholipases, see **Figure 2-4** (Ricciotti and FitzGerald 2011). It is also an anti-inflammatory mediator generated through the metabolic actions of cyclooxygenase (COX) and prostacyclin synthase (PGIS) (Smith *et al.* 1983).

When COX binds to an endothelial cell marker PGIS, prostaglandin H_2 (PGH_2) is converted to prostacyclin I_2 (PGI_2). Once generated, PGI_2 is released to exert its effects locally on neighbouring ECs, VSMCs as well as circulating platelets. However, PGI_2 is rapidly inactivated by nonenzymatic processes to a hydrolysis product (6-keto-PGF_{1}-alpha) (Wu and Liou 2005). Substances like thrombin, bradykinin, serotonin, platelet-derived growth factor, interleukin-1, oxidised low-density lipoprotein (LPP) and shear stress can also stimulate the secretion of PGI_2 (Miyata *et al.* 1994, Caughey *et al.,* 2001).

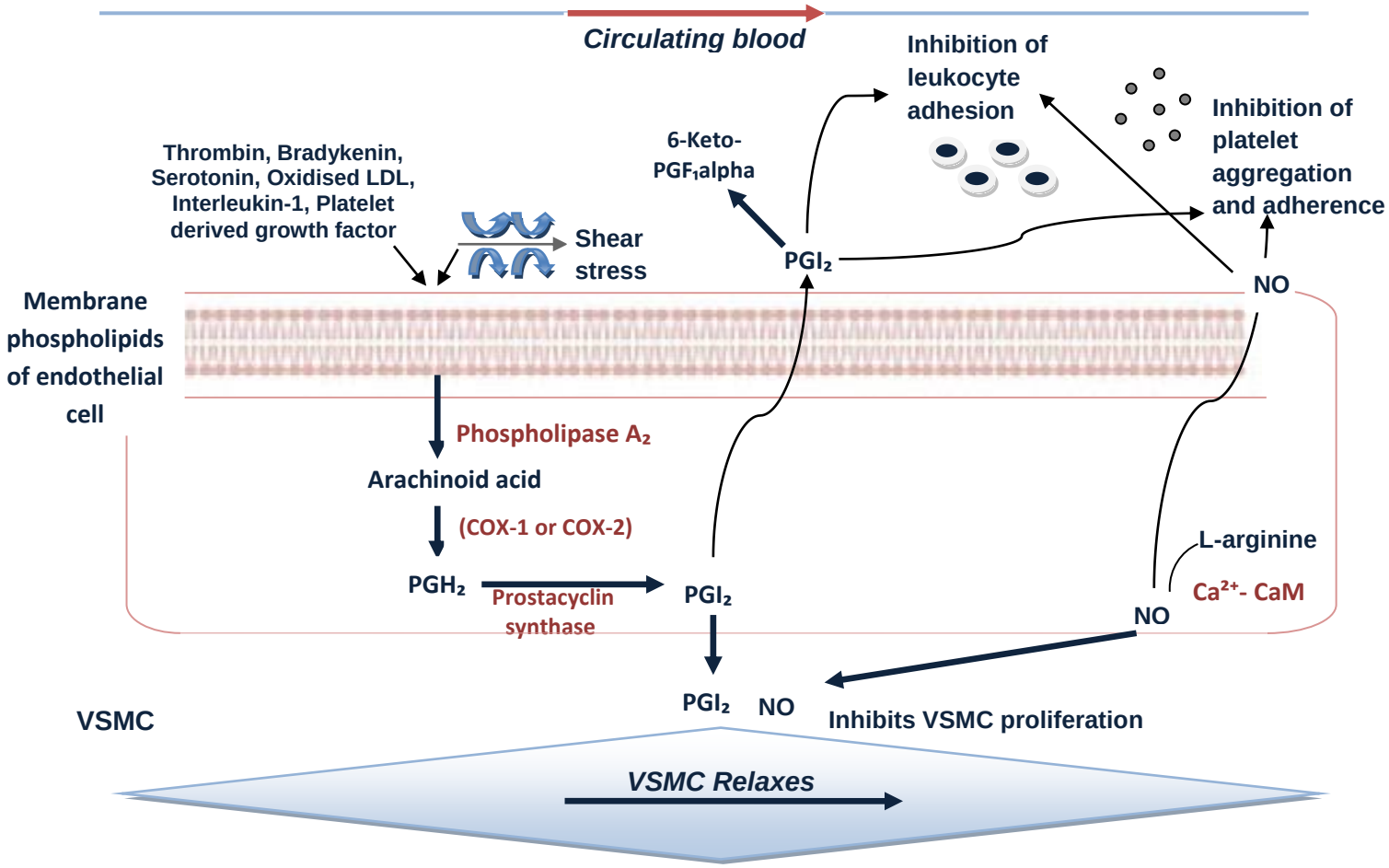

Figure 2-4: Anti-thrombotic effects of vascular endothelial cells. Both prostacyclin (PGI_2) and nitric oxide (NO) released by endothelial cell inhibits leukocyte adhesion, platelet aggregation and adhesion to normal resting endothelial cells. They also inhibit vascular smooth muscle cell (VSMC) proliferation. Prostacyclin is rapidly degraded to hydrolysis product 6-Keto PGF_1alpha.

Endothelial cells (ECs) also constantly express tissue plasminogen activator (tPA) which constantly activates circulating plasminogen to plasmin. Plasmin is a fibrinolytic enzyme that prevents inappropriate clotting throughout the vasculature. Therefore the fibrins that are constantly being formed are being dissolved by fibrinolytic activity of plasmin. This helps maintain the haemostatic balance of the blood vessel.

A pro-angiogenic factor, vascular endothelial growth factor (VEGF) stimulates angiogenesis and exerts anti-fibrin effects (Ferrara 2009; Sahni and Francis 2000). It is produced by macrophages and platelets in the vascular system. When released, VEGF stimulates endothelial cells to release tPA which results in plasmin activation. However VEGF also stimulates the release of plasminogen activator inhibitor (PAI) by endothelial cells, which counteracts the action of plasminogen activator (Hoeben *et al.* 2004).

2.2.3. The importance of endothelial-derived Nitric Oxide

Nitric oxide is mostly considered as the protector of the vascular wall, since it promotes vascular relaxation (Ignarro *et al.* 1987; furchgott 1996), inhibits smooth muscle cell (SMC) migration and proliferation (Cornwell *et al.* 1994), inhibits platelets adhesion and aggregation (De Graaf *et al.* 1992) and leukocyte adhesion (Gauthier *et al.* 1995; Kubes *et al.* 1991) on the vascular endothelium.

The synthesis of nitric oxide is continuously generated within vascular ECs by the enzyme endothelial nitric oxide synthase (eNOS) (Palmer *et al.* 1988). Endothelial nitric oxide synthase (eNOS) is composed of 2 globular domains, a reductase domain and oxygenase domain, connected by a flexible protein strand. In the presence of calcium (Ca^{2+}) dependent binding of calmodulin (Craig *et al.* 2002), the eNOS reductase domain binds NADPH (Nicotinamide adenine dinucleotide phosphate) to generate electrons needed for NO synthesis. These electrons are transferred across the flexible protein strand to the oxygenase domain's catalytic center, which is responsible for the production of NO. The catalytic center binds and oxidizes the L-arginine amino acid to L-citrulline and NO. Endothelial nitric oxide synthase (eNOS) also requires the presence of the cofactor tetrahydrobiopterin (BH_4) to form NO from L-arginine (Behrendt and Ganz 2002; Xia *et al.* 1998). The BH_4 cofactor directs the electron transfer from the eNOS enzyme to L-arginine (Heitzer *et al.* 2000).

Substances like acetylcholine, bradykenin, serotonin, thrombin, Substance P and shear stress also stimulates the secretion of NO by the endothelium (Harris *et al.* 2001; Ignarro *et al.* 1987; furchgott 1996). When NO is released by ECs, it becomes expressed on the EC membrane surface to exert its effects. The other fraction of the produced NO diffuses to the VSMCs to promote SMC relaxation. The NO achieves this by activating guanylate cyclise (GC) in the SMC leading to an increased production of cyclic guanosine monophosphate (cGMP) which relaxes the muscle. It also inhibits the entry of contraction-inducing calcium ions (Ca^{2+}) into SMCs (Sherwood 2007:349). Thus NO protects endothelium against vascular injury, inflammation and thrombosis, all key events that are involved in the progression of atherosclerosis (Behrendt and Ganz 2002). **Figure 2-5** depicts the effects of NO in protecting the vascular endothelium.

Abnormalities surrounding the synthesis of NO may contribute to the pathogenesis of vascular diseases (i.e. atherosclerosis (Cooke and Dzau 1997)). When normal production of NO is reduced as a result of either accelerated degradation by reactive oxidant species or altered eNOS expression at a posttranslational level, vascular ECs may become dysfunctional.

The synthesis of NO is regulated by various cofactors and substrates, subcellular targeting, protein-to-protein interactions and phosphorylation (Harris *et al.* 2001).

Binding of Calmodulin (CaM) to its specific binding site on the eNOS molecule increases the release of NO. The deficiency or absence of BH_4 cofactor can also results in uncoupling of L-arginine with eNOS, resulting in the production of superoxide instead of NO by eNOS (Vasquez-Vivar *et al.* 1998). The production of superoxide molecules can rapidly inactivate NO molecules, reducing the bioavailability of NO especially under high oxidative stress.

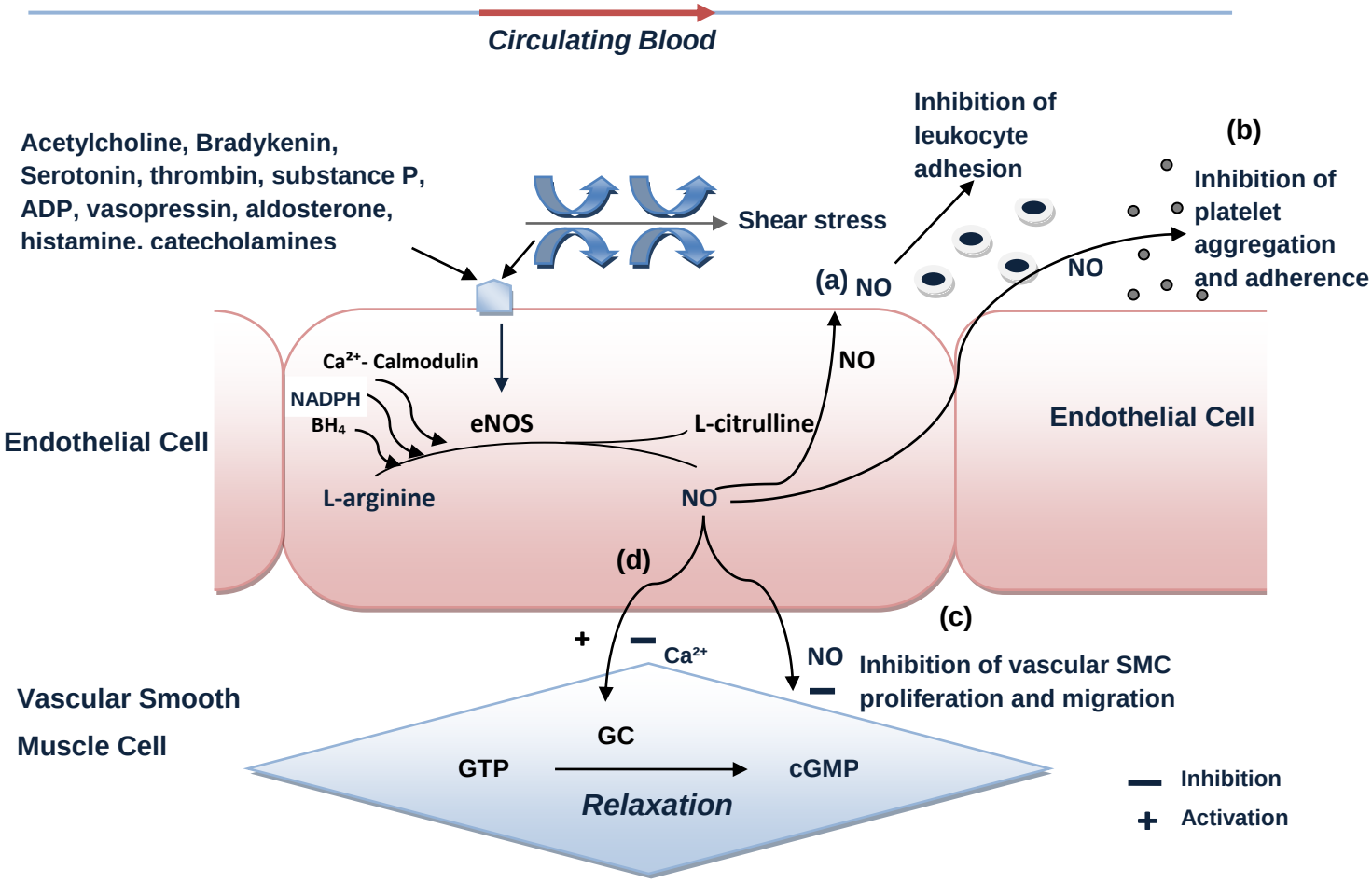

Figure 2-5: The protective effects of nitric oxide (NO) (edited from Behrendt and Ganz 2002). Nitric oxide inhibits (a) leukocyte adhesion, (b) platelet aggregation and adhesion on the vascular endothelium, (c) vascular SMC proliferation and migration and (d) promotes SMC relaxation. Additionally, NO can protect against vascular injury and re-establish a normal vascular environment.

2.2.4. Pro-thrombotic effects of activated endothelial cells

Just as well as ECs are able to secrete substances that help protect against inappropriate coagulation, they are also able to secrete substances that promote healing mechanisms when the vessel is injured. Several studies show that ECs can rapidly shift the haemostatic balance from anti-thrombotic to a pro-thrombotic state (Bombeli *et al.* 1997). This may be due to pertubative factors, such as modified lipoproteins, oxidative stress and infectious agents in the blood that alter the anticoagulant properties of ECs. As a result ECs synthesize and release substances to express a pro-coagulant surface membrane. The pro-coagulant membrane surface is able to bind coagulation proteins and cofactors found in the circulating blood in order to initiate coagulation (Colman *et al.* 2006).

When ECs are activated they express phosphatyldserine (PS) and phosphatidylethanolamine (PE) on their external surfaces (Smith 2009; Bombeli *et al.* 1997). In resting ECs, these phospholipids are localized on the inner surface of the EC membrane in resting ECs. The expression of PS-containing pro-coagulant membrane immediately activates TF expressed on EC membrane (Mackman *et al.* 2007; 12. Butenas 2012). Once the circulating blood is exposed to an activated TF-bearing endothelial cell, FVIIa in blood rapidly binds to the exposed TF. The TF-FVIIa complex then activates additional FVII to FVIIa, allowing for even more TF-FVIIa complex activity (Smith 2009). This complex further activates FIX and FX. The ECs also express or release pro-thrombotic substances such as cofactor FV, platelet activating factor (PAF), von Willebrand factor (vWF) and plasminogen activator inhibitor (PAI). Further activation of other coagulation proteins upstream then follows. **Figure 2-6** explains the tissue factor pathway of blood coagulation.

The activated enzymes, together with their cofactors ultimately lead to the activation and generation of small amounts of thrombin. Thrombin is regarded as the “prima ballerina” of hemostasis, as it plays multiple roles in hemostasis. It is present in an inactive form as prothrombin in the circulating blood. Thrombin activates platelets by binding to platelet receptors. It cleaves coagulation factors FXI to FXIa, activates FV to FVa and FVIII to FVIIIa on the platelets surface. Thrombin ultimately converts fibrinogen to fibrin. The fibrin strands are stabilized by activated FXIII to an insoluble fibrin matrix, forming a clot. This clot seals off a broken vessel in cases of vessel injury, preventing any further loss of blood. Activated ECs also release endothelin to promote vasoconstriction, in order to reduce blood flow through the defect site of the vessel.

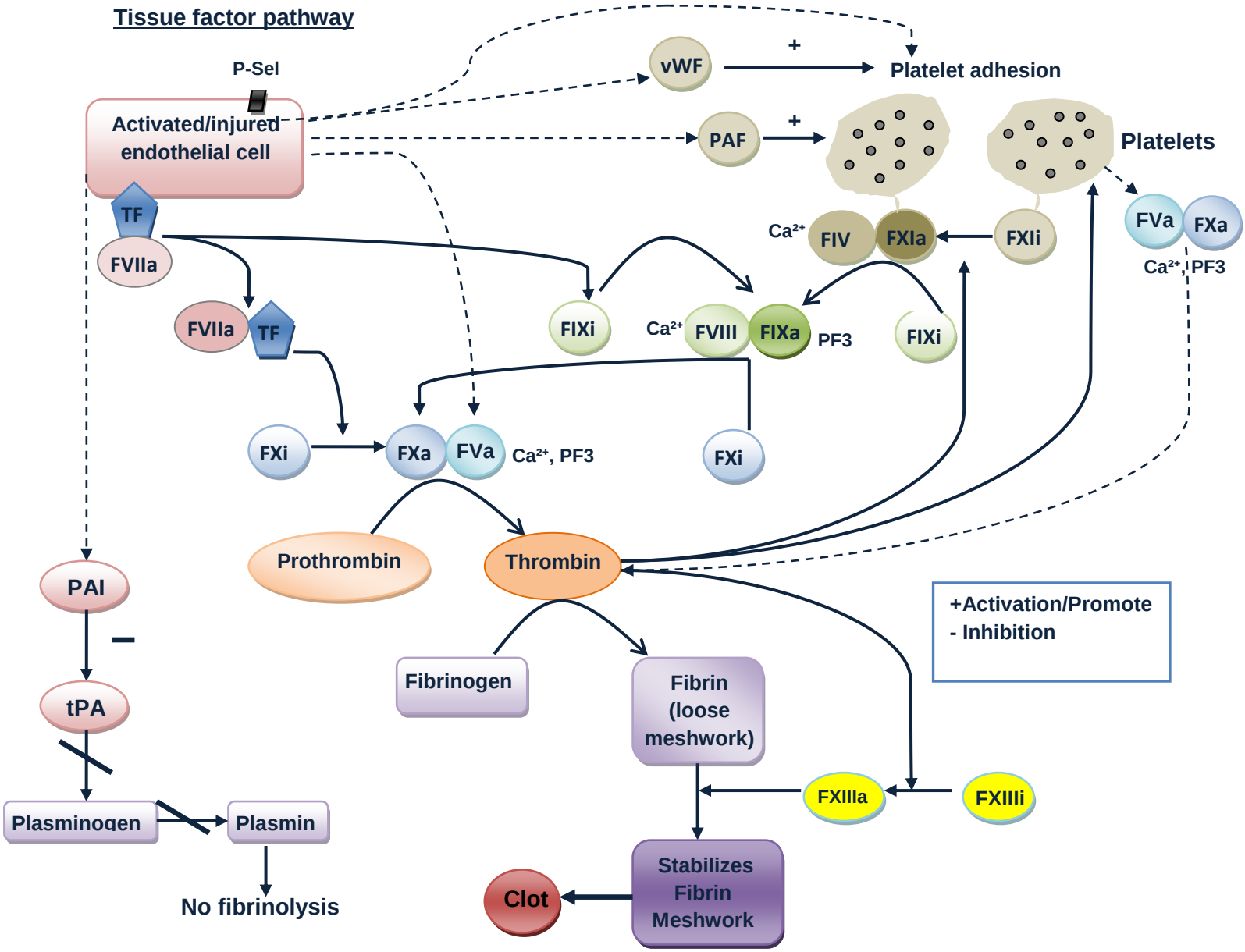

Figure 2-6: Coagulation cascade. The tissue factor pathway is initiated by the binding of FVII to TF, which results in the autoactivation of FVII (FVIIa). The FVII-TF complex also activates FIX and FX that lead to the formation of thrombin. Thrombin activates FXI on the surface of platelets. FXIa activates FIX generating more thrombin and accelerating fibrin formation. Activated endothelial cell also releases the following: FV- a cofactor for FXa to cleave prothrombin to thrombin, platelet activating factor (PAF) which activates platelets, von Willebrand factor (vWF) that facilitates platelet adherence, and plasminogen activator inhibitor (PAI) that prevents activation of plasmin (fibrinolytic enzyme) by inhibiting plasminogen activator (PA). Activated platelets in turn release FXI, FVa and PF3 which play a part in the generation of thrombin.

Activated ECs also express a cell adhesion molecule P-selectin (P-sel) on their surface membranes (Furie and Furie 2004). This molecule promotes binding of more platelets activated by PAF released by ECs. The stored vWF within the damaged EC is also released and immobilised on the surfaces of damaged ECs. The released vWF enables activated platelets to adhere to the exposed collagen. The vWF binds to activated platelets through the interaction of its A1 domain (vWFA1) with platelet glycoprotein Ibα receptor. The adhered platelets aggregate to form a plug at the defect site in order to reduce blood flow through the injured vessel wall. However, the platelet plug releases thromboxane A_2, serotonin and epinephrine that further constrict the vessel wall to reinforce vascular spasm (Sherwood 2007:397). The activated platelets also secrete adenosine diphosphate (ADP) to promote further platelet aggregation. The ADP secreted in turn stimulates adjacent normal ECs to release NO and prostacyclin. These two chemicals prevent platelet aggregation and adhesion to the undamaged endothelium, in order to localize the thrombus to the place of injury.

2.2.5. Endothelial dysfunction in vascular diseases

Endothelial dysfunction is commonly referred to as an impairment of the endothelium and includes the presence of widespread abnormalities in endothelial integrity and homeostasis (Quyyumi 2003). Endothelial dysfunction also contributes to the development of nearly all vascular diseases. Arterial thrombosis and atherosclerosis are the most prevalent causes of vascular diseases that arise from endothelial dysfunction (WHO 2011). These two entities are referred to as atherothrombotic disease, because of their relevant biochemical interactions to each other (Furie and Furie 2008; Jackson 2011; Ross 1999). The pathogenesis of atherothrombotic disease involves a series of events that include endothelial dysfunction, dysregulated adhesive interactions between platelets, endothelium and leukocytes and infiltration of inflammatory cells into the vessel wall (Jackson 2011). The diseased vessels in turn allows an impaired blood flow through them as a result of inappropriate clot developments and progressive atherosclerotic lesions (Sullivan *et al.* 2000; Gawaz *et al.* 2005). Chronic inflammation of ECs results in dysfunctional ECs that may cause the progression of cardiovascular diseases.

An inflamed vascular endothelium as a result of accumulating reactive oxidised species (ROS) and modified lipoprotein particles (cholesterol) in the intima leads to an increased expression of adhesive molecules (vWF and P-selctin (P-Sel)) on the endothelium surface to support platelet rolling and adhesion (Jackson 2011). Binding of intercellular adhesion molecule-1 (ICAM-1) or fibrinogen complexes with alpha-v beta-3 ($\alpha_v\beta^3$) endothelium also promotes platelet adhesion. Adhered platelets in turn secrete numerous bioactive substances that further alter the chemotactic and adhesive properties of endothelial cells (Jackson 2011).

Thus a chronic inflammatory response causing EC activation is initiated by pertubative substances in blood (Jackson 2011; Pober *et al.* 2009). The activated ECs then lose their antithrombotic properties and increase secretion of pro-thrombotic substances, leading to a hypercoagulable state. The permeability of the inflamed endothelium also increases. Furthermore, the vascular tone is impaired by an increased secretion of endothelial-derived vasoconstrictor substances. The inflamed endothelium also expresses chemokines, inflammatory cytokines, and lipid mediators (Nielsen 1998). It also express an increased number of adhesion molecules, such as von Willebrand factor, P-selectin molecules, endothelial leukocyte adhesion molecules (ELAMs), intergrins and other cell adhesion molecules (CAMs) on their surfaces (Furie and Furie 2004; O'Brien *et al.* 1993; Albelda and Buck 1990). The expressed adhesion molecules support adhesion and rolling of activated platelets and inflammatory cells on the endothelium. The activated platelets and inflammatory cells then adhere and accumulate at the site of injury and release bioactive substances that alter the chemotactic and adhesive properties of the endothelium (Jackson 2011).

Monocytes and leukocytes infiltrate the wound site of the inflamed vessel wall (intima) and become foam cells (Sena *et al.* 2013). Cytokines released by the ECs and the infiltrating white blood cells stimulates the smooth muscle cells to proliferate and migrate towards the blood vessel lumen (Bjorkerud and Bjorkerud 1996). This process causes thickening of the vessel wall, forming a plaque consisting of proliferating smooth muscle cells, macrophage-derived foam cells and various types of lymphocytes within one sub-endothelial space (Sullivan *et al.* 2000). Continued influx of mononuclear cells and deposition of matrix components give rise to the fibroproliferative progression of the plaque. A collagen-rich fibrous cap develops over the plaque to help stabilize it, propagating the development of atherosclerotic lesions. During unstable rapid progression of the atherosclerosis plaque, a necrotic lipid core develops from apoptosis of macrophages and other plaque cells (Jackson 2011). Thinning and erosion of the fibrous cap ultimately result in plaque rapture, exposing the thrombogenic matrix proteins to the circulating blood. The coagulation system becomes activated and thrombotic plaque lesions within the artery start to form. Repeated cycles of plaque injury and thrombus formation may lead to progressive stenosis of the vessel lumen.

The thrombotic lesions may occlude the vessel, obstructing blood flow leading to diminished amounts of oxygen and nutrients reaching the target organ (Lassila 1993). The consequence of this is poor functioning of the vessel, including tissue ischemia or total occlusion of the vessel as seen in acute myocardial infarction or stroke (Ross 1999). The exposed thrombogenic plaque contents leads to the formation of blood clots that may cause deep vein thrombosis (DVT) (Wakefield *et al.* 2008). The clots may also dislodge (embolus) and circulate in the blood to other organs, causing pulmonary embolism (PE) (Goldhaber and Morrison 2002).

2.3. Risk factors associated with endothelial dysfunction

Endothelial dysfunction thus is the hallmark and predictor of most cardiovascular diseases (Vanhoutte *et al.* 2009). Most studies rely on studying the normal functioning of vascular ECs by assessing the endothelium's ability to promote vasodilation. This is done by measuring the ability of the normal endothelial cells to release NO.

The two most common methods used to assess endothelial dysfunction in humans *in vivo*, include: (a) measuring the diameter of the coronary arteries before and after infusion with acetylcholine to assess the production of NO-mediated vasodilation response, and (b) less invasively, measuring the systemic arteries diameter in response to high flow conditions (shear stress), which leads to endothelial-dependent dilation (Puranik and Celermajer (2003). *In vitro* studies make use of NO assay kits that measures the NO levels in cultured ECs by measuring NO_2^-/NO_3^- spectrophotometrically (ScienCell's Nitric Oxide assay kit, ScienCell™ Research Laboratories, Corte Del Cedro, Carlsbad, California). In normal vascular ECs, active nitric oxide synthase (eNOS) is constitutively generating NO in blood vessels. The generated NO undergoes rapid degradation to nitrite (NO_2^-) and nitrate (NO_3^-) which can be used to quantitate NO production.

This section discusses the diseases that risk factors have been reported to cause endothelial dysfunction by down regulating NO synthesis and release. These include diseases such as diabetes, hypertension, and pulmonary hypertension coronary artery disease, and heart diseases. For example, a study conducted by Thorogood *et al.* (2007) in South Africa (SA), found that high prevalence of hypertension, obesity in women, and subclinical atherosclerosis are the most contributing factors to vascular diseases, especially in rural areas. In the 1990's, a research study by Kalm and Tollman (1999) also reported a high rate of deaths from stroke in SA. These findings indicate that SA is facing an emerging epidemic of vascular diseases. Therefore the progression of cardiovascular diseases can be minimised or treated with living a healthy lifestyle that include physical activity, healthy balanced diet and correct treatment options.

Figure 2-7 schematically discuss the role of disease risk factors in endothelial dysfunction that lead to the pathogenesis of cardiovascular event.

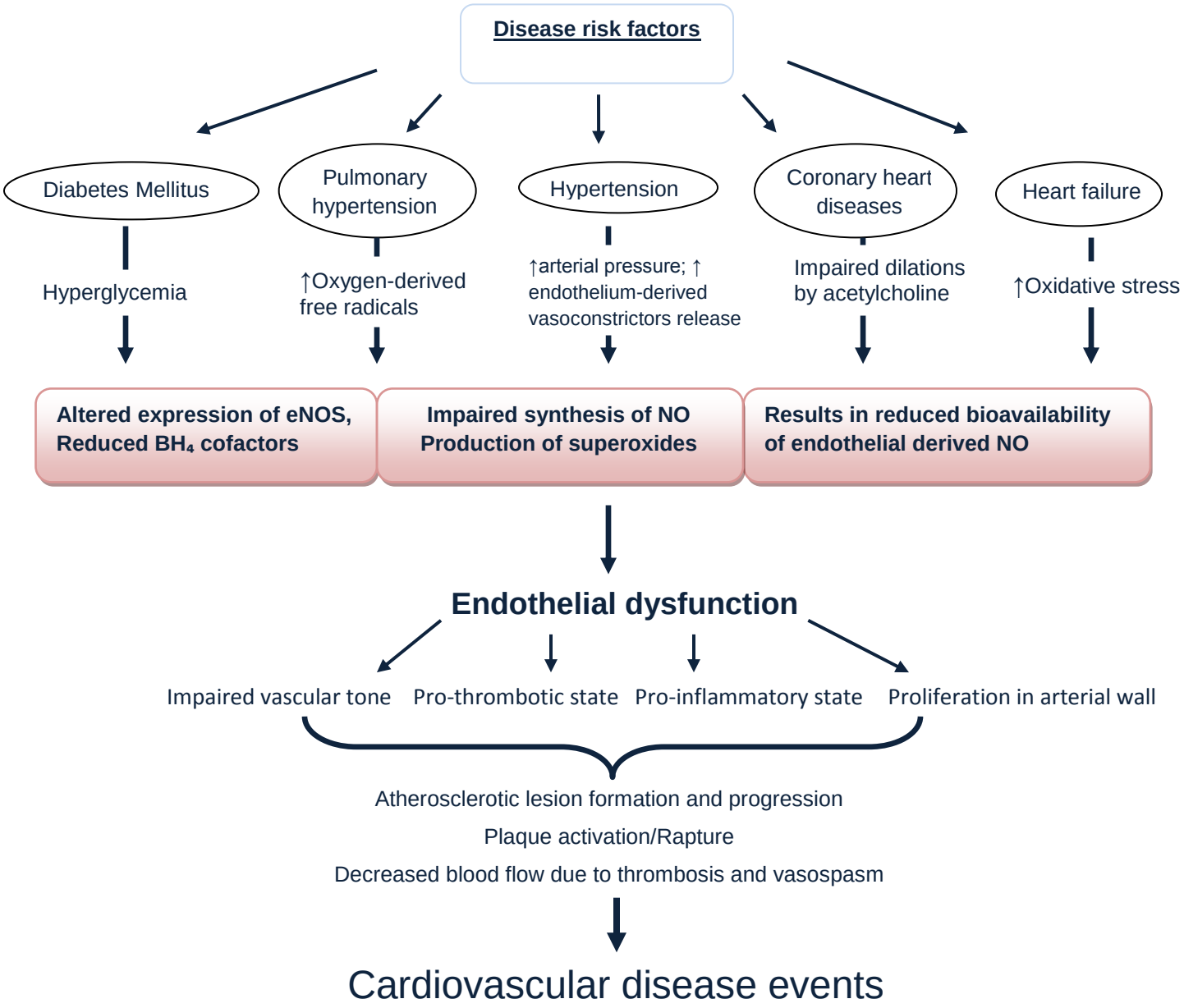

Figure 2-7: The role of disease risk factors in endothelial dysfunction leading to pathogenesis of cardiovascular disease event. Disease risk factors adversely affect endothelial-dependent vasodilation impairing the homeostatic functions of ECs. The response of the endothelium to the cumulative effects of risk factors contributes mechanistically to the development, progression and clinical expression of atherosclerosis. Thus endothelial function may serve as a measure for cardiovascular risk factor.

Diabetes Mellitus

The hyperglycaemic content in diabetes alone is thought to cause impairment in the endothelium-dependent vasodilation, blunting the secretion of NO by ECs. Other studies suggest that ECs may be producing more vasoconstrictor prostanoids than NO which contributes significantly in endothelium dysfunction (Vanhoutte *et al.* 2009). The following underlying mechanisms are thought to be responsible for reduced bioavailability of NO in diabetes:

a. Reduced bioavailability of BH_4 content. This results in the release of eNOS and production of superoxides (Cai *et al.* 2005; Alp *et al.* 2003; Pannirselvam *et al.* 2002).
b. The increased production of superoxide molecules that break down NO and increase the presence of peroxynitrate (Duncan *et al.* 2007; Pacher & Szabo 2006; Gao *et al.* 2008)
c. Increased activity of arginase, which competes with eNOS for the common substrate arginine (Romero *et al.* 2008; Vanhoutte 2008)

Hypertension

The chronic increase of arterial pressure blunts off the endothelium-dependent vasodilators that might also cause premature ageing of the vasculature (Perticone *et al.* 2005). The blunting of endothelium-dependent vasodilation in spontaneous hypertension may be due to an increased release of vasoconstrictor prostanoids by ECs, rather than a reduced release of NO (Yasuro *et al.* 1999; Koga *et al.* 1988). Furthermore, the reduced response to endothelium-dependent stimuli *in vivo* may be due to high circulating levels of Asymmetric dimethylarginine (ADMA) (Perticone *et al.* 2005).

Pulmonary hypertension

Chronic hypoxia resulting in pulmonary hypertension reduces the endothelium-dependent vasodilation of pulmonary arteries. This may be due to the over production of oxygen-derived free radicals that reduces the activity of eNOS (Vanhoutte 2009), and then leads to reduced bioavailability of NO.

Coronary disease

Individuals at increased risk of coronary heart disease are characterized by impaired peripheral dilatations in response to acetylcholine (Vanhoutte 2009; Tousoulis and Davies 1998). Therefore endothelial dysfunction predicts the severity of the outcome, especially myocardial infarction and stroke (Rossi *et al.* 2008; Vanhoutte 2009).

Heart failure

The coronary and peripheral arteries of humans with ventricular hypertrophy or heart failure are dilated (Vanhoutte 2009). This may be due to the increased oxidative stress that leads to down-regulation of eNOS and reduced NO bioavailability. Furthermore, the ability of vascular smooth muscle cells to relax may also be impaired, blunting off the endothelium-dependent responsiveness (Gill *et al.* 2007). The impairment of the vascular endothelium thus also predicts the severity of the outcome of chronic heart failure in patients (Meyer *et al.* 2005).

2.4. Therapies used for vascular diseases

Diseased or damaged vessel in the event of vascular disease, such as atherosclerosis often require replacement with a vascular graft. Vascular grafts are used to replace, bypass or maintain the function of the damaged or occluded diseased vessel. The types of vascular grafts that are currently used in clinical settings include both biological and synthetic grafts.

Autologous arteries or veins (mostly the saphenous vein) are usually used in cardiac and peripheral bypass surgery procedures. However, many patients do not have suitable blood vessels for grafting due to pre-existing vascular diseases or amputation of the vessels in previous surgery (Cho *et al.* 2005). As a result the patient is restricted to modest treatment modalities, with the results often leading to myocardial infarction or limb amputation (Ratcliffe 2000).

There are however commercially available synthetic grafts that can be used when the use of autologous grafts is contraindicated. These synthetic vascular grafts are used for reconstruction of medium and large sized diameter (> 6mm) vessels. Unfortunately they are considered not suitable for replacement of small diameter vessels (< 6mm) due to thrombogenecity and limited re-endothelialization *in vivo* (Sapsford *et al.* 1981; Whittermore *et al.* 1989).

Autologous arteries or veins are still regarded as best substitutes for small diameter vessels and are in high demand in bypass procedures (Mitchell and Niklason 2003). Attempts in using small diameter autologous vessels have however failed due to graft infection, thrombogenecity and intimal hyperplasia resulting in occlusion (Zhou *et al.* 2012; Kakisi *et al.* 2005). **Table 2-3** summarizes the vascular substitutes that are currently used for different vascular regions.

Table 2-3: A summary of vascular substitutes in clinical use according to body regions (Chlupáč *et al.* 2009).

Vascular regions		Vascular substitute choice	
		1st choice graft	2nd choice graft
Large diameter arteries (≥ 8 mm)	Aorta, arch vessels, iliac and common femoral arteries	Prosthesis (Dacron and ePTFE)	Allograft, deep venous autograft
Medium sized diameter arteries (6 – 8 mm)	Carotid, subclavian, common femoral, visceral and above the knee arteries	Prosthesis or auto graft (equal)	Prosthesis or autograft
Small diameter arteries (≤ 8 mm)	Coronary, below the knee, tibial and peroneal artery	Arterial or venous autograft	Composite graft, vein interporsition prosthesis (ePTFE, Dacron) allograft, biosynthetic
Venous reconstructions	Superior and inferior vena cava, ilico-femoral veins, portal vein, visceral vein	Saphenous spiral vein graft, deep venous autograft	Allografts, ePTFE, Dacron, biografts
Hemodialysis arterio-venous access	Upper > lower extremity	Native material	ePTFE, PU, xenografts, biografts, TEBV (Tissue engineered blood vessel) (clinical trials)

The next section outlines the different biological and synthetic vascular grafts that are currently in use. The emphasis is on the characteristics and drawbacks of these vascular grafts, including the physiological response to the materials when used for replacement.

2.4.1. Biological vascular grafts

Biological grafts include autologous grafts, allografts and xenografts (Lichtenberg *et al.* 2006). Autologous grafts are blood vessels harvested from the patient's own body. Allografts are blood vessels harvested from a donor and transplanted to genetic dissimilar patient. Xenografts or heterografts are vessels transplanted from one species origin to another species. Biological grafts

can be obtained from different sources, such as human umbilical vein, saphenous vein, aortic, internal mammary, radial, hypogastric and pulmonary arteries or bovine/porcine arteries. Biological grafts have good biocompatibility and high patency rates (Lantz *et al.* 1993). However, they consist of dead tissues with low levels of collagen available for cross-linking. This results in them having poor durability and strength. Attempts made to overcome this problem include reinforcing biological grafts with loose supporting polyester mesh.

Autologous arteries are regarded as the gold standard for vascular replacement due to their inherent physiological properties. Saphenous vein, radial artery and the mammary artery are mostly used for autologous bypass surgeries (Chlupáč *et al.* 2009; Beghi *et al.* 2002). The greater saphenous vein is preferentially used for bypass procedures including distal revascularization (Hölzelnbein *et al.* 2013; Regalado *et al.* 2009; Locati *et al.* 1996). Smaller saphenous vein (Chang *et al.* 1992), deep leg veins (Ali *et al.* 2009) and other veins of the arm (Calligaro *et al.* 1997) and the right gastroepiploic artery (Sasaki 2008) have also been used for coronary and peripheral bypass surgeries. These blood vessels have also been used in infected aortic graft replacement, visceral revascularisation and even primary lower limb bypass.

The most durable and best performing homograft conduits have been shown to be internal mammary, radial and hypogastric arteries (Nimish *et al.* 2004). Glutaraldehyde preserved human umbilical vein grafts were used in the past for peripheral bypass surgery (Dardik *et al.* 2002). The preserved human umbilical vein grafts showed improved resistance to infection. These grafts are no longer considered suitable for use as they have demonstrated poor long term patency rates and low limb salvage rates (Farber and Major 2004; Johnson and Lee 2000; Budd *et al.* 1990).

Cryopreservation of artery and vein grafts from human tissue banks has also been explored. Cryopreservation focuses on long-term preservation of arteries and veins obtained from human allograft (Randon et al. 2010; Albers et al. 2004). Studies on cryopreservation of blood vessels as an option for vascular replacement have been conducted since from the 1960s. However, not enough significant clinical data was drawn from these studies (Randon *et al*. 2010). Protocols used for cryopreserving the arterial grafts were either not described, or the protocols involved procedures that damages cells and tissues of the artery. The use of anticoagulant therapies, improvements in harvesting techniques and preservation fluids for cryopreserved vascular grafts also did not improve the patency of these grafts in most studies (Galambos *et al.* 2005; Buckley *et al.* 2000). Cryopreservation studies were then abandoned due to difficulties observed in preserving allografts, late grafts deterioration and aneurysm formation (Chlupáč *et al.* 2009; Farber and Major 2004; Albers *et al.* 2004). The availability of synthetic grafts also contributed to the abandonment of vascular cryopreservation studies.

Recently tissues are being cryopreserved to manage aortic prosthetic graft infection for primary revascularization of lower extremities and revascularization in solid organ transplants (Matia *et al.* 2008; Kieffer *et al.* 2004; Dardik *et al.* 2002). The current cryoprotectants used include dimethyl sulfoxiude and chondroitin sulfate. Disadvantages of cryopreserved vascular grafts however include aneurysm degeneration, plaque formation and late rejection leading to thrombosis.

A summary of biological vascular grafts currently in clinical use are provided in **Table 2-4**.

Table 2-4: A summary of biological vascular grafts currently in clinical use (Chlupáč *et al.* 2009).

BIOLOGICAL VASCULAR GRAFTS				
	Autografts		**Allografts**	**Xenografts (heterografts)**
	Arterial	*Venous*	*Arteries and Venous*	*Porcine or bovine arteries and veins*
Advantages	Closest approximation, less diameter mismatch, excellent function (internal mammary artery anatomically nearby)	Durable and versatile, good results, infection resistance, relative availability	Off the shelf availability, better resistance to infection, transplant-recipient patients, decellularized allogenic and xenogeneic grafts can serve as scaffold for cell seeding in tissue engineering of vascular grafts.	
Disadvantages	Availability, vasospasm (radial artery), donor site morbidity	Availability, harvest injury, vein graft disease	Antigenicity, graft deterioration, early graft occlusion, chronic rejection, intake of drugs, infection risk	
Healing	Intimal thickening, myointimal hyperplasia (radial artery)	Endothelial desquamation, vein dilation, wall thickening, arterializations, re-endothelialization	Endothelial denudation, immune response, fibrous formation	

2.4.2. Synthetic vascular grafts

Two types of synthetic grafts are currently available for clinical use. Also referred to as mechanical grafts (Kannan *et al.* 2005), these grafts are developed from synthetic polymers such as Dacron® and expanded-polytetrafluoroenthylene (ePTFE). These grafts material have been proven to be most suitable and possess superior properties for use as vascular grafts for large diameter vessels (Harrison 1958). They are mostly used in bypass surgeries above the knee with satisfactory results (Ballotta *et al.* 2003). Both Dacron and ePTFE are good in providing strength and durability and also show good patency rates in high blood flow regions with larger diameter (> 6 mm) (Johnson and Lee 2000). The recent emerging synthetic graft material of interest is polyurethane. Polyurethane grafts are more compliant than ePTFE, and have surfaces that promote cell attachment. Each material will be discussed below.

Dacron®

Dacron® grafts are made from knitted or woven polyester (**figure 2-8-1 and 2-8-2**). Knitted grafts (**Figure 2-8-1)** have larger pores compared to woven grafts **(figure 2-8-2)** to allow tissue in-growth. They are commonly used for larger vessel replacement in regions of high blood flow. Dacron® grafts originally required pre-clotting with recipient's own blood before implantation. This is to reduce blood loss, aid clotting and stimulate tissue ingrowth. However they are currently produced with sufficient connective tissue coating proteins such as albumin, gelatin and collagen coating to avoid blood loss. Polyester material is also very susceptible to infection and bacteria can easily adhere to it. Therefore some polyester materials are coated with an antibacterial agent to limit bacterial growth.

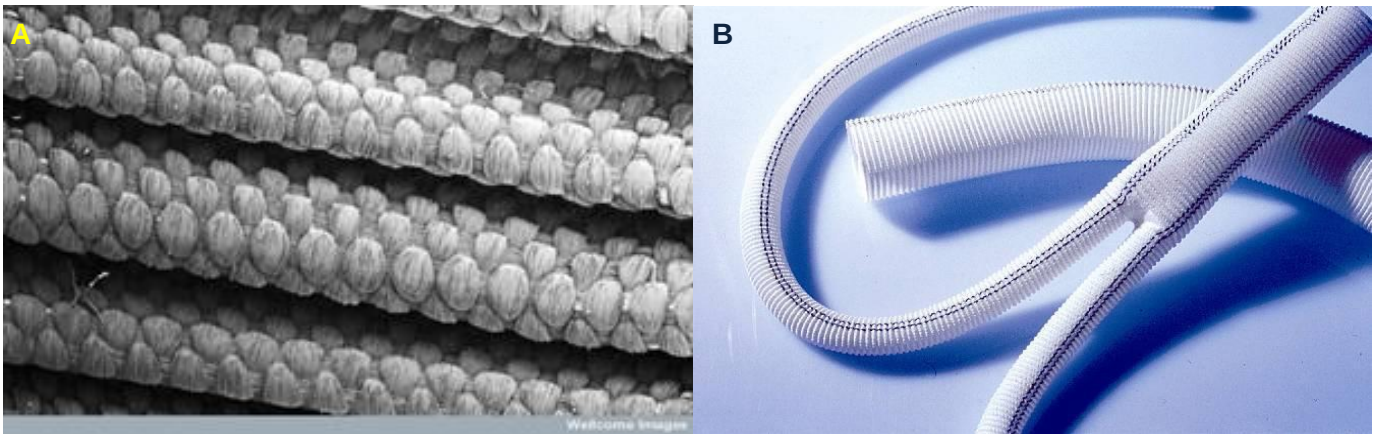

Figure 2-8-1: (A) SEM of a woven Dacron® material with large folds of Dacron material (http://wellcomeimages.org/indexplus/page/Prices.html). (B) Image of a woven Dacron® vascular graft (http://www.tiptekmedikal.com.tr/templates/urunview/topresim.php?Id=5953980&ResId=bs196914).

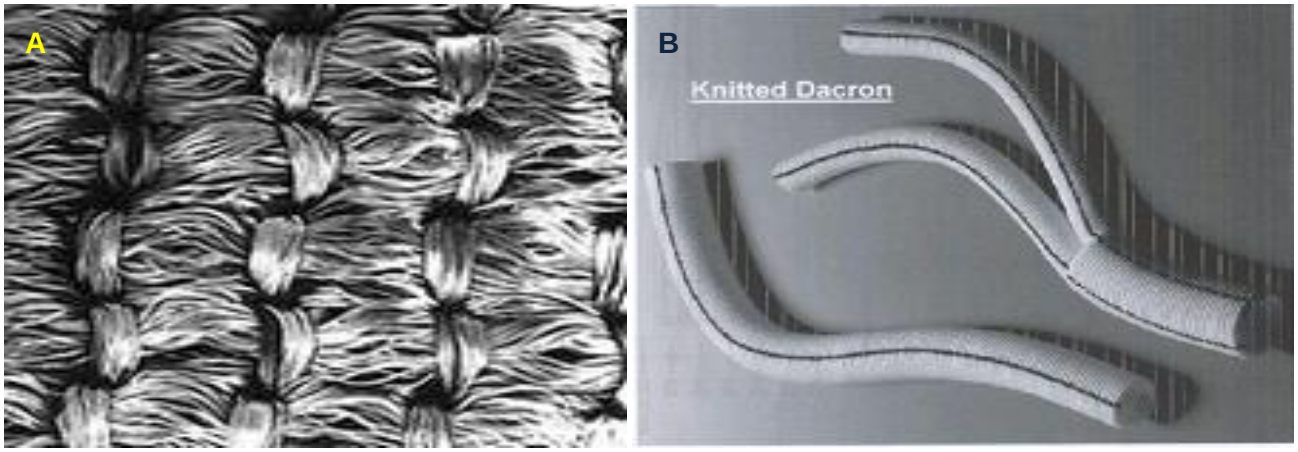

Figure 2-8-2: (A) SEM of a knitted Dacron material which provides dilation (http://www.terumo-cvs.com/about/competencies/vascular_grafts/index.shtml). (B) Image of a knitted Dacron vascular graft (http://chemistry.pixel-online.org/files/ed_pack/03/introduction02/image006.jpg).

Expanded-polytetrafluoroenthylene (ePTFE)

Expanded-polytetrafluoroenthylene (ePTFE) grafts are made from micro-porous material (**Figure 2-9**). This material is aligned by thin and irregular shaped nodes and a dense meshwork of fine fibril stretching between the nodes. The ePTFE grafts are most commonly used for peripheral bypass procedures. The porosity of ePTFE material allows for tissue in-growth to heal the graft. However this process may eventual lead to occlusion of the lumen. The ePTFE graft can also bleed through the suture lines during implantation and leak if punctured after implantation. This can cause seromas that can be the site for infection and bleeding can often be difficult to stop.

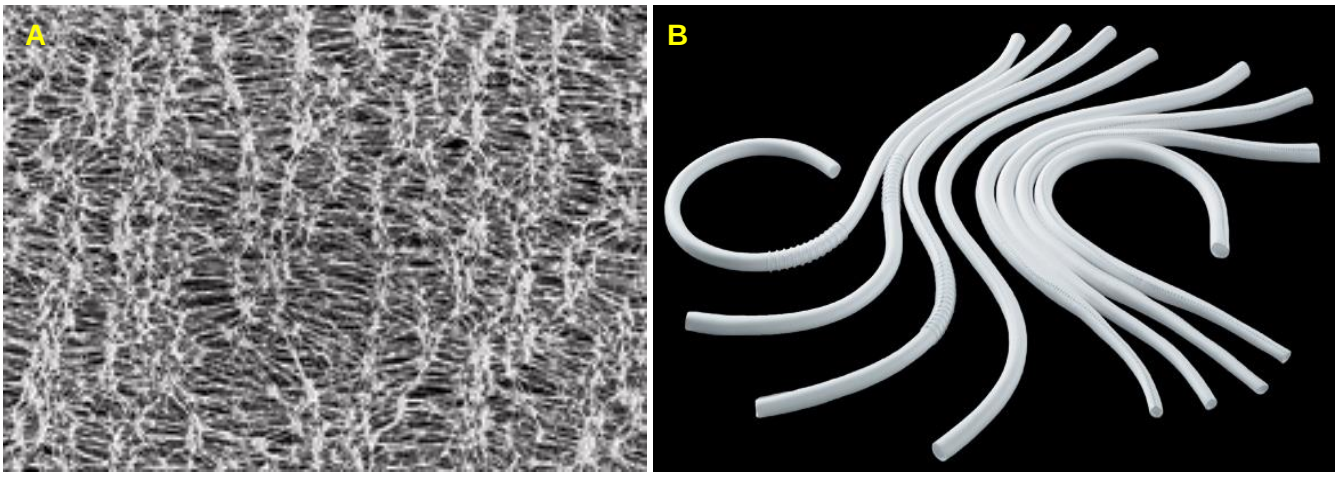

Figure 2-9: (A) SEM of expanded PTFE material (http://www.mdtmag.com/articles/2009/08/use-expanded-ptfe-membranes-medical-filtration). (B) ePTFE graft (http://www.goremedical.com/vgstretch).

Polyurethane grafts (PU)

Polyurethane (PU) is made from a large family of elastic polymers containing a urethane –NH-(CO)-O- group (Chlupáč *et al.* 2009) (**figure 2-10**). It consists of copolymers made from three monomers: the crystalline monomer which is hard and accounts for the rigidity of the material, the amorphous segments which are soft and provides flexibility and the third monomer serves as a chain extender (Zdrahala 1996). The PU grafts contain either fibrillar or foamy microstructures. Both these structures tend to lack communicating spaces for potential capillary ingrowth (Chlupáč *et al.* 2009). For example, the luminal surface of the fibrillar PU is covered by a thin layer of fibrin and the outer surface encapsulated by scar formation containing foreign body giant cells (FBGC) when implanted *in vivo*, thus limiting any capillary ingrowth. The foamy PU on the other hand has a microporous feature with pore sizes of 15 µm that can allow little capillary ingrowth. When the pore size is increased up to 175 µm, capillary ingrowth can be observed and the inflammatory reaction of FBGC diminished (Zilla *et al.* 2007).

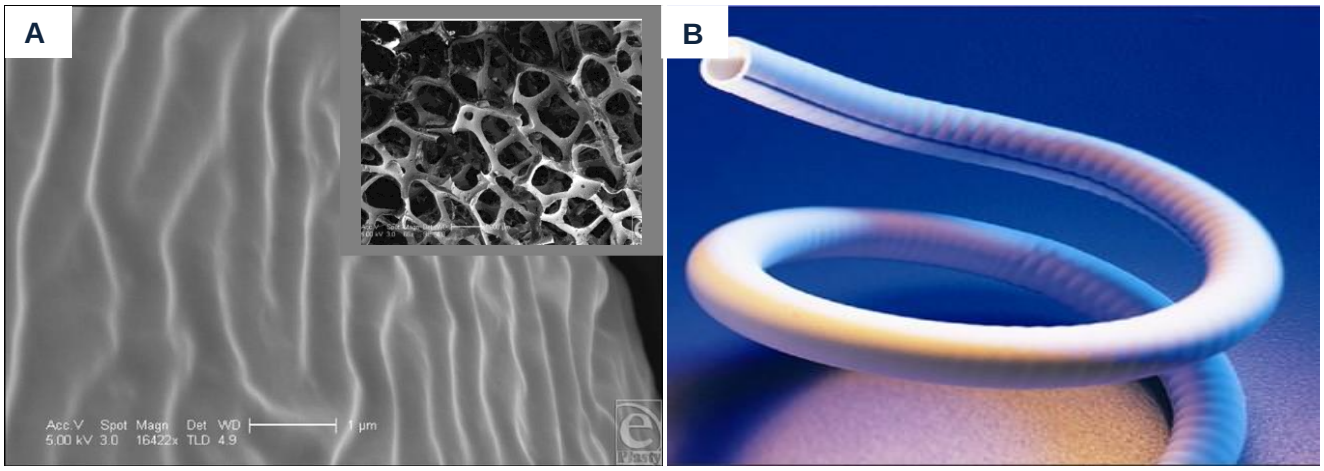

Figure 2-10: (A) SEM of a Polytech MicroPolyurethane Polyurethane graft surface at 16422x magnification (insert) at high magnification of 66x (Barr *et al.* 2009). (B) Image of a polyurethane graft (A Vectra® Access Graft, http://www.vascularnews.com/vn-archives/new-products-vn23/bard5).

The first PU was manufactured in the 1930s and has been available for biomedical applications since the 1960s (Boretos and Pierce 1967). This PU material had a disadvantage of hydrolytic biodegradation when used in clinical trials (Zhang *et al.* 1997). This led to the development of a polyether-based hydrolysis-resistance PU that received FDA (Food and Drug Administration) approval in 2000. However the disadvantage of this PU was susceptibility to oxidation (Chlupáč *et al.* 2009). The latest PU manufactured is polycarbonated based and is hydrolytically and oxidatively

stable. This type of PU has more advantages when compared to Dacron® and ePTFE grafts (Jeschke *et al.* 1999; Seifalian *et al.* 2003), including its ability to promote faster luminal endothelialisation (Chlupáč *et al.* 2009). The PU grafts have been successfully used as haemodialysis access grafts (Ravari *et al.* 2010; Glickman *et al.* 2001), but there no evidence for their use in human peripheral bypass surgeries (Dereume *et al.* 1993). Thus further investigations are still required before they can be recommended for use as synthetic vascular prostheses.

Table 2-5 compares the different synthetic vascular grafts by discussing the advantages, disadvantages and healing properties of each.

Table 2-5: A summary of vascular grafts in clinical use (IND - Intermodal distance) **(Chlupáč *et al.* 2009).**

SYNTHETIC VASCULAR GRAFTS						
	Dacron®		**ePTFE**		**Polyurethane**	
	Woven	*Knitted*	*Low porosity* (<30 µ m IND)	*High porosity* (>45 µm IND)	*Fibrillar*	*Foamy*
Advantages	Better stability, lower permeability and less bleeding	Greater porosity, tissue ingrowth and radial distensibility	Biostability, no dilation over time	Biostability, better cell ingrowth	Compliance, good hemodynamics and biocompatibility, less thrombogenecity	
Disadvantages	Reduced compliance and tissue incorporation, low porosity, fraying at edges, infection risk	Dilation over time, infection risk	Stitch bleeding, limited incorporation, infection risk, perigraft seroma formation	Late neointimal desquamation in 90 µm IND, infection risk	Biodegradation in first generation, infection risk, carcinogenic possibility?	
Healing	Inner fibrinous capsule, outer collagenous capsule, scarce endothelial islands	Fibrin luminal coverage, very sporadic endothelium, trans-anastomotic endothelia-lisation in animals	Luminal fibrin and platelet carpet, connective tissue capsule with foreign body giant cell, no transmural tissue in growth	Magrophages and polymorpho-nuclear invasion, capillary sprouting, fibroblast migration, certain angiogenesis, thicker neointima, endothelialisation in animals	Thin inner fibrin layer, outside foreign body cells, limited in growth	Better ingrowth with bigger pores

2.5. Limitations of therapies for vascular diseases

Most surgeons prefer using autologous grafts for vessel replacement due to their superior patency rates, accessibility and low infection rates when compared to synthetic grafts. However many patients do not have suitable vessels for grafting (Herrera *et al.* 2009; Kerdjoudj *et al.* 2007). For peripheral bypass procedures 30 – 40% of patients lack the appropriate saphenous vein due to limb amputation, diseased vessels or hypoplasia (Faries *et al.* 2000). In addition, use of saphenous veins in bypass procedure have been shown to only have a 4 year patency rate of 40 -70 % (Donaldson *et al.* 1991). Saphenous vein grafts are also prone to atherosclerotic lesions and intimal hyperplasia occurring throughout the length (Sarjeant and Rabinovitch 2002). Furthermore, any surgical vessel harvest is associated with unavoidable donor site morbidity (Swenne *et al.* 2006). Biological vascular grafts from allogenic and zenogenic origin are also associated with the risk of pathogen transfer and immune reaction.

Synthetic grafts are only used when the use of autologous grafts as replacement is unadvisable. These synthetic grafts perform well when used as replacements for large diameter vessels (> 6 mm). However they start failing when used as small diameter (< 6 mm) replacement grafts. Thrombosis is the major limiting factor for using small-diameter (< 6 mm) synthetic grafts for replacement. This is due to the absence of endothelial lining on the inner surface and also their lack of cell attachment factors present in native scaffolds (Kasimir *et al.* 2006).

Synthetic grafts provide the *Vroman's* effect to the circulating plasma proteins and coagulation factors found in blood following implantation (Andrade and Hlady 1986; Vroman 1962). Glycoprotein IIb-IIIa found on platelets can become absorbed on the graft surface and lead to the activation of platelets (Spijker *et al.* 2003). Glycoprotein IIb-IIIa also acts a receptor for fibrinogen and vWF. Once vWF and fibrinogen are bound, platelets aggregation and activation follows, including fibrin generation. Activated platelets in turn secrete thromboxanes and β-thrombomodulin, and may lead to thrombus formation (Suzuki *et al.* 1996). Platelets adhesion is also influenced by the wet-ability of the surface material (Vroman 1962). Hydrophilic materials lead to higher rates of platelet adhesion, while hydrophobic materials lead to greater rates of platelets activation. When the *Vroman* process is propagated exponentially, thrombosis results. The high hydrophobicity of synthetic grafts (especially the ePTFE grafts) also limits endothelialisation *in vivo* (Kerdjoudj *et al.* 2007). Thus patency rates of small-diameter synthetic grafts are still unacceptable when compared to autologous grafts.

Furthermore, synthetic grafts do not possess all the characteristics of an ideal vessel substitute. These include compliance mismatch which influences patency of the vascular graft (Abbott *et al.* 1986). Compliance mismatch between the transplanted synthetic graft and the natural vessel

causes trauma to the natural vessel by disruption of the endothelium. The damaged vascular endothelium responds by initiating thrombosis leading to intimal hypertrophy. The polyurethane grafts have good compliance compared to other synthetic grafts and surfaces that promote endothelial seeding (Salacinski *et al.* 2001; Tiwari *et al.* 2002). However they have a tendency to degrade leading to aneurysm formation and have an inherent thrombogenecity (Szycher 1981).

Synthetic grafts have also been reported to have poor hemodynamic flow at the anastomosis (Greenwald and Berry 2000). This leads to fluctuations in shear stress and injury due to suturing and stress concentration at the anastomosis. The rigidity of synthetic grafts also causes hyperplasia at the proximal anastomosis. This is due to the increased pressure at the anastomosis, which increases stress at the walls of natural vessels that in turn increases cell proliferation. Mismatch of the mechanical properties between the replacement graft and the natural vessel may also provoke tissue reaction leading to anastomotic hyperoplasia.

Expanded-PTFE grafts have an increased calcification rate since Ca^{2+} accumulates on the polymer surface (Park *et al.* 2001). The Dacron® graft was also found to gradually deteriorate due to material fatigue, leading to fragmentation of individual fibres and subsequent biodegradation of the material (Berger and Sauvage 1981). These synthetic grafts degenerate after some time leading to intrinsic structural failure, compromising their quality as a biomaterial (Van Damme *et al.* 2005). Therefore synthetic materials are not optimal in terms of regeneration.

Synthetic grafts are also prone to infection with incidences varying from 1% -7% (Zetrenne *et al.* 2007; Herrera *et al.* 2009) and have unfavourable healing process. The standard treatment for graft infection has been graft removal and replacement of the vessel. However these treatments are associated with amputation rates of up to 70% and mortality rates ranging from 10% -30 % (Herrera *et al.* 2009).

Lastly, synthetic grafts lack growth capabilities that are necessary, especially in paediatric patients (Lichtenberg *et al.* 2006; Kakisis *et al.* 2005; Hopkins 2003).

The last section discusses the current efforts and improvements that are used to develop small diameter vascular grafts that are suitable for implantation.

2.6. Current efforts and improvements to develop suitable small diameter vascular grafts

For decades, extensive research has focused on the development of novel small diameter vascular grafts. The ideal characteristics of vascular graft are as follows:

1. Biocompatible: the vascular graft must be completely non-thrombogenic and non-immunogenic. Both these characteristics are provided by an intact endothelial lining.
2. Good nature-like mechanical properties: The vascular grafts must be strong enough to withstand considerable internal pressure before bursting and allow the sutures to hold under tension while still being compliant.
3. Long lasting durability and normal hemodynamics: it should be able to resist stress, show normal blood flow under external forces, and able to constrict or relax in response to neural or chemical stimuli.
4. Infection resistant.
5. It should be able to be manufactured in a relatively short time, available in all sizes, and be affordable.
6. And most importantly the ability to grow and remodel itself once it has been implanted

(Jordan *et al.* 2012; Thomas *et al.* 2003),

Several improvements to small diameter vascular grafts have been made in the laboratory settings, but remain an unmet clinical need. Most concerning is the unavailability of small-diameter vascular grafts for replacement. Therefore, there is still great interest in the development of small diameter grafts that have long-term patency rates. Several approaches to overcome the limitations and complications connected with the use of small caliber vascular grafts have been undertaken. The next subsections focus on the antithrombotic therapy used in using vascular therapy, the infections involved in vascular graft implantation and the tissue engineering approaches that are currently followed.

2.6.1. Antithrombotic and anticoagulant therapies

Several studies have focused on the use of antithrombotic and anticoagulant therapies *in vitro* and *in vivo* to reduce thrombosis of synthetic small diameter vascular grafts (Tatterton *et al.* 2012). Many of these studies used animal models that showed antithrombotic efficacy against synthetic graft materials (Aldenhoff *et al.* 2001; Hanoaka 1994). In a study by Liu *et al.* (2008), Dacron grafts treated with L-arginine (a NO precursor) in an attempt to reduce synthetic graft thrombogenecity and improve long-term patency rates. These grafts were incubated with blood *in vitro* in an attempt to improve blood-graft compatibility. This study showed that there was a 25%

reduction in protein absorption and a 67% reduction in thrombus formation in the L-arginine treated grafts compared to Dacron alone. In another study, hirudin-mobilized and monomer-grafted PTFE grafts were compared to controls using fresh blood thrombogenic assay (Onder *et al.* 2011). Hirudin is a potent thrombin inhibitor. This study reported reduced clot formation on the surfaces of hirudin-mobilized PTFE grafts. The immobilized hirudin also demonstrated continued antithrombotic effects for at least 40 days.

Many *in vivo* animal studies assessed the performance and limitations of antithrombotic therapies (Hanoaka 1994; Wyers *et al.* 1999; Aldenhoff *et al.* 2001). However, very few antithrombotic therapies to date have translated into clinical use as there is a distinct lack of human *in vivo* studies. Many antithrombotic effects were seen over a short timescale in *in-vivo* studies (Goldman *et al.* 1983). According to Tatterton *et al.* (2012) more human *in vivo* studies are still required to assess the efficacy of such therapies.

2.6.2. Vascular graft infection

Reports concerning vascular graft infection have demonstrated that not even high concentration of antibiotics can completely eradicate bacterial contamination (Mirzaie *et al.* 2007). Therefore constructing vascular grafts that avoid bacterial adhesion to the scaffold surfaces is of high importance. To achieve this vascular graft surfaces are coated with antibiotics. However, a study by Earnshaw *et al.* (2000) found that rifampicin antibiotic bonded to Dacron® did not reduce the incidence of vascular graft infection. Silver coated collagen impregnated Dacron® graft is suggested to offer an alternative approach in the treatment of vascular grafts.

2.6.3. Tissue engineering approaches

Tissue engineering of vascular grafts (TEVGs) is currently being explored as an alternative to develop improved small diameter vascular grafts. The current tissue engineering (TE) approach addresses three important components: the scaffold (either synthetic or biological – to provide the initial graft shape and strength), the matrix (important for facilitating cell attachment, proliferation and maintenance of the phenotype) and the source of autologous ECs (Kerdjoudj *et al.* 2007; Baguneid *et al.* 2006). Currently TE focuses on harvesting human vascular endothelial cells and human smooth muscle cells *in vitro* and growing biologic blood vessels by using acellular matrices, such as decellularized scaffolds, followed by cell seeding of the scaffold material. **Figure 2-11** shows an overview of the tissue engineering approach in constructing vascular grafts.

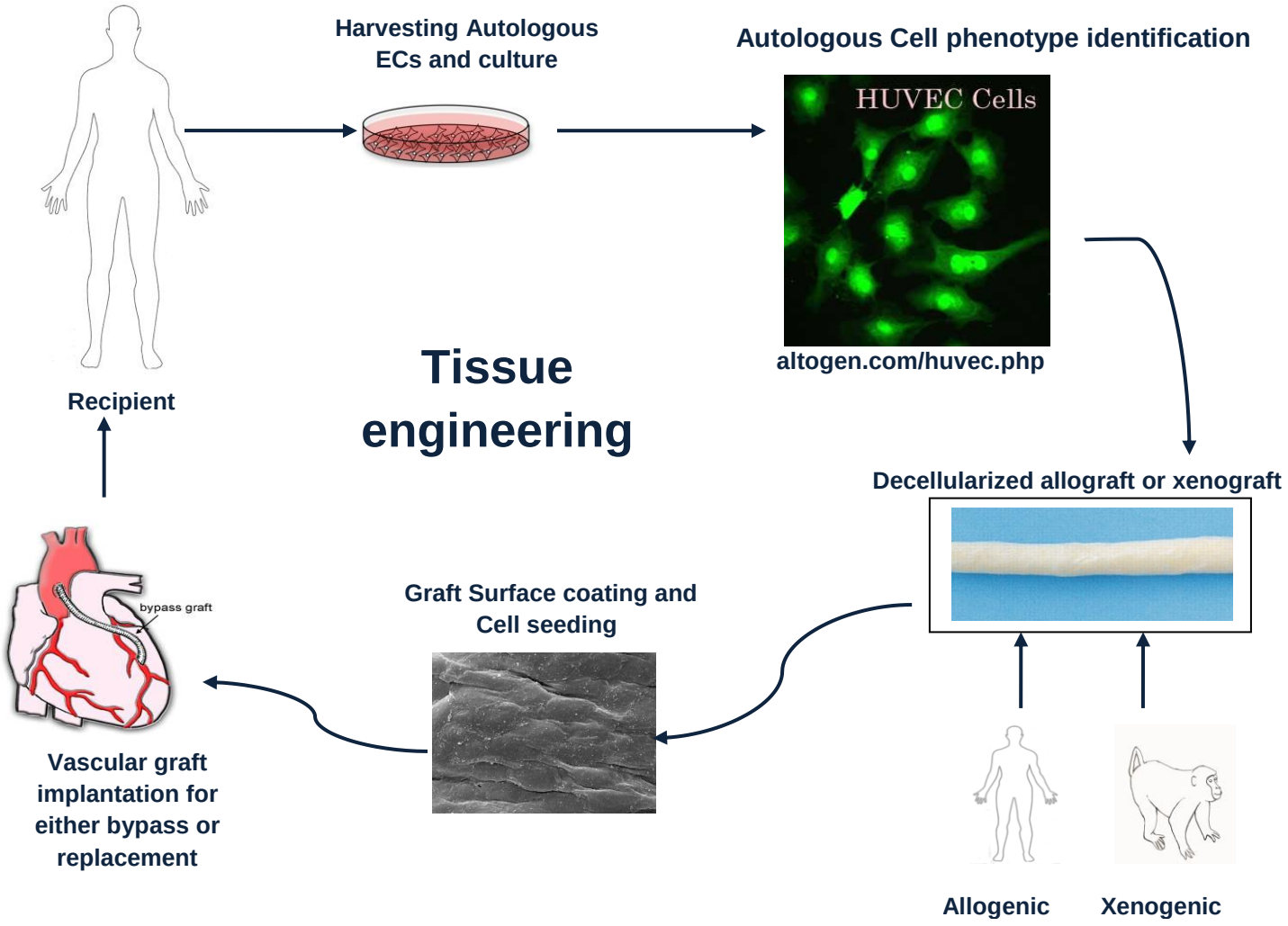

Figure 2-11: An overview of tissue engineering approach in constructing vascular grafts suitable for replacement.

The search to create a more biocompatible tissue engineered vascular grafts (TEVG) which can express physiological functions that closely resemble native blood vessels has led to the process of decellularization of tissues. Recently medical scientists are investigating the use of decellularized biological scaffold material of both xenograft and allograft origin in constructing vascular tissues in order to restore or establish normal tissue function (Gilbert *et al.* 2006; Jordan *et al.* 2012; Ning-tao *et al.* 2007). Studies on TEVGs aim to fabricate living autologous grafts with the capacity for growth, repair and remodelling (Khait *et al.* 2008, Ning-tao *et al.* 2007). Several studies have shown that TEVGs possess many of the characteristics consistent with arteries formed *in vivo* (Schaner *et al.* 2004; Conklin *et al.* 2002; Bader *et al.* 2000 and Wilson *et al.* 1995). They also have good functional performance properties and similar histology as native blood vessels. These decellularized biological scaffolds are already in the shape of the tissue structure that is to be replaced, in this case a blood vessel. The decellularized scaffolds are also thought to regulate cell

proliferation and phenotype development, and can facilitate tissue repair and regeneration (Ning-tao *et al.* 2007). The seeding of autologous endothelial cells onto the decellularized scaffold also allows for growth, repair and regeneration of tissues through tissue engineering approaches (Jordan *et al.* 2012; Kerdjoudj *et al.* 2007).

Tissue engineering aims to overcome the limitations of conventional prosthesis by using autologous living decellularized grafts in order to obtain extracellular matrix (ECM) scaffolds. Decellularized blood vessels are also a promising vascular graft substitutes as they do not exhibit the same limitations as synthetic grafts in terms of compliance (Conklins *et al.* 2002). The regeneration of these vascular tissues through a tissue engineering approach can furthermore overcome the consequences of immunosuppressant and chronic rejection faced by recipients (Elkins *et al.* 2001, Song and Ott 2011). At the same time, this will help to reduce vascular graft shortage and the prolonged waiting time for individuals who need valve or vessel replacement.

The advantages of TEVGs is that they have shown to be responsive, non- thrombogenic, and can self-repair (Shin'oka and Imai 2001). They are able to promote growth, are metabolically active and complete healing integration can be observed. However the disadvantage of TEVGs is the demanding fabrication time. They require specialized canters (i.e. bioreactor cell laboratory) and can be cost consuming. The first tissue engineering process discussed below is the decellularization of vascular tissues.

2.6.3.1. Decellularization of vascular tissues

The goal of decellularization is to efficiently remove all cellular and nuclear materials while preserving the integrity of the ECM (Gilbert *et al.* 2006). This means minimizing any adverse effects on the composition, biological activity and mechanical integrity of the remaining ECM. The ECM consists of a complex mixture of structural and functional proteins such as adhesion proteins (fibronectin and laminin), collagen, elastic fibres, growth factors and glycosaminoglycans (GAGs). These components do not induce any adverse response by the host when the ECM is grafted, since they are well tolerated by different species (Gilbert *et al.* 2006). Thus when there is complete removal of cellular materials from a tissue, the inflammatory response or immune mediated rejection of the tissue by the recipient is limited as no cellular antigens are left behind.

The most common and effective methods used for decellularization of tissues involve a combination of specific physical, enzymatic or chemical treatments (Gilbert *et al.* 2006). Sufficient cell removal is dependent on the biochemical composition, tissue density and mechanical behaviour of the scaffold. Cellular material and the ECM of different tissues are arranged in variable degrees of compactness (Gilbert *et al.* 2006). Therefore the ECM must be adequately exposed to the

chaotropic agents during the decellularization process to allow complete removal of all cellular materials. Decellularization techniques might not remove 100% of cellular components from a tissue (Crapo *et al.* 2011; Gilbert *et al.* 2006). However methods which remove most or all of the visible cellular material provide biologic scaffold materials that are safe for implantation. **Table 2-6-1** and **2-6-2** shows the physical, enzymatic and chemical methods used for decellularization of tissues.

Table 2-6-1: An overview of (A) physical and (B) enzymatic methods used for decellularization of tissues (Badylak *et al.* 2011; Gilbert *et al.* 2006).

A. PHYSICAL METHODS		
	MODE OF ACTION	**EFFECTS ON ECM**
Mechanical agitation / Sonication	Can cause cell lysis, but more commonly used to facilitate chemical exposure and cellular material removal	Aggressive agitation or sonication can disrupt ECM as the cellular material is removed
Mechanical force	Pressure can burst cells and tissue removal eliminates cells	Mechanical force can cause damage to the ECM
Freeze/thaw techniques (Snap freezing)	Intracellular ice crystals disrupt the cellular membrane	ECM can be disrupted or fractured during rapid freezing

B. ENZYMATIC METHODS		
	MODE OF ACTION	**EFFECTS ON ECM**
Trypsin	Cleaves peptide bonds on the C-side of Arginine and Lysine	Prolonged exposure can disrupt the ECM structure, removes laminin, fibronectin, elastin and GAGs
Exonucleases	Catalyze the hydrolysis of the terminal bonds of ribonucleotide and deoxyribonucleotide chains	
Endonucleases	Catalyze the hydrolysis of the interior bonds of ribonucleotide and deoxyribonucleotide chains	Difficult to remove from the tissue and could invoke an immune response

Table 2-6-2: An overview of chemical methods (C) used for decellularization of tissues (Badylak *et al.* 2011; Gilbert *et al.* 2006).

C. CHEMICAL METHODS		
	MODE OF ACTION	**EFFECTS ON ECM**
alkaline/acid	Solubilise cytoplasmic components of cells; disrupts nucleic acids	Removes GAGs
Nonionic detergents Triton X-100	Disrupts lipid-lipid and lipid-protein interactions, while leaving protein-protein interactions intact	Mixed results; efficiency depends on tissue, remove GAGs
Ionic detergents sodium dodecyl sulphate (SDS)	Solubilise cytoplasmic and nuclear cellular membranes; tends to denature proteins	Removes nuclear remnants and cytoplasmic protein; tends to disrupt native tissue structure, remove GAGs and damages collagen
sodium deoxycholate		More disruptive to tissue structures than SDS
Triton X-200		Yields efficient cell removal when used with zwitterionic detergents
Zwitterionic detergents		
Tri(n-butyl)phosphate	Organic solvent that disrupts protein-protein interactions	Variable cell removal; loss of collagen content, although effect on mechanical properties was minimal
Sulfobetaine-10 and -16 (SB-10, SB-16)		Yielded cell removal and mild ECM disruption with triton X
Hypotonic and hypertonic solutions	Cell lysis by osmotic shock	Efficient for cell lysis, but does not effectively remove the cellular remnants
CHAPS (3-[(3-cholamidopropyl)dimethyl ammonio]-1-propanesulfonate)	Exhibit properties of non-ionic and ionic detergents	Efficient cell removal with ECM disruption similar to that of triton X-100
Ethylene diamine tetra-acetic acid (EDTA), Ethylene glycol tetraacetic acid (EGTA)	Chelating agents that bind divalent metallic ions, thereby disrupting cell adhesion to ECM	No isolated exposure, typically used with enzymatic methods (e.g. trypsin)

Generally, the decellularization protocols begin by applying physical treatments or ionic solutions to the tissue to lyse the cell membranes. Freezing of tissue has been shown to be effective as it allows intracellular crystals that disrupt cellular membranes to form and cause cell lysis (Jackson *et al.* 1991; Roberts *et al.* 1991; Jackson *et al.* 1987; Jackson et al. 1987). Some research studies recommend treating the vessel tissue with a hypertonic or hypotonic solution to lyse the cells within a tissue (Dahl *et al.* 2003; Goissis *et al.* 2000; Vyavahare *et al.* 1997).

To separate cellular components from the ECM, enzymatic treatment such as trypsin/EDTA is often used (Gilbert *et al.* 2006). Trypsin is thought to adequately break bonds between cell membrane and the ECM at pH 8 at 37°C to remove cellular materials. Other studies recommend treating the vessel with trypsin/EDTA while applying agitation for 24 hours for efficient decellularization (Schenke-Layland *et al.* 2003). The use of trypsin (including different concentrations and time exposure thereof) does not always give same results for all tissue types from different sources. Trypsine/EDTA can also reduce the laminin and fibronectin content of the ECM, and prolonged exposure can greatly decreases the elastin content and GAGs over time (Gilbert *et al.* 2006).

Non-ionic or zwitterionic detergents are used to solubilise the cytoplasmic and nuclear cellular components by using detergent treatments. Triton X-100 is the most widely used non-ionic detergent for its effectiveness in removing nuclear material. However, Triton X-100 is not effective in preserving the components of the ECM during the decellularization of tissues (Gilbert *et al.* 2006; Woods and Gratzer 2005; Dahl *et al.* 2003; Cartmell and Dunn 2000). It causes complete loss of GAGs and also decreases the laminin and fibronectin content of blood vessel ECM. The last step of decellularization is the removal of cellular debris from the tissue to avoid any adverse host response. As already mentioned, any cellular remnants left on decellularized vessel tissue can elicit an immune response by the host.

Removal of chemical residues after the decellularization processes is important in limiting host response when the scaffold is implanted *in vivo* (Gilbert *et al.* 2006). When high concentrations of chemicals are used during the decellularization process, a toxic effect on the host cells may occur. Also some of the commonly used enzymes (trypsin, RNase and DNase) derived from bovine sources can potentially invoke an adverse immune response by the host. There are no assays available that can quantify the presence of residual chemicals in decellularized tissues currently. Thus more research still needs to be done to develop such assays.

Another alarming problem associated with decellularization of tissues is the presence of bacteria which can contaminate the remaining ECM. Bacterial growth on decellularized vessels can further interrupt endothelialisation of the ECM during TE processes. Fortunately studies have

developed protocols for using antibiotic solutions to eradicate this problem. Most antibiotic solutions used include penicillin, streptomycin and amphotericin B (29, Ketchedjian *et al.* 2005; Hilbert *et al.* 2004; Affonso *et al.* 2004). However if antibiotic residues remain in the scaffold material after decellularization, the material cannot be considered useful for TE as it is then considered as a drug and not a medical device (Gilbert *et al.* 2006).

Following the decellularization processes, the efficiency of decellularization is assessed by several verification methods (adapted from: Gilbert *et al.* 2006 and Crapo *et al.* 2011). These include: (1) Histology analysis (using hematoxylin and eosin (H&E) staining) to assess for any nuclear structures left behind, (2) fluorescent staining (either DAPI or Hoechst) can also be used for detecting the presence of DNA material on the decellularized tissue scaffold, (3) immunohistochemical methods to determine for intracellular proteins such as actin and vimentin, and (4) microscopic methods (SEM and TEM) are also applied to examine for the presence of cellular remnants or cytoplasmic debris. Thereafter the morphological integrity and preservation of the functional and structural components of the ECM needs to be assessed. Histology analysis (H&E staining) is mostly used to evaluate the morphology of the ECM. It is used to determine if the necessary components of the ECM are still retained and preserved and were not compromised by the decellularization processes. Mechanical testing methods, such as strength testing of the tissue, are used to evaluate the mechanical properties of the ECM and assess the integrity of the structural proteins within the scaffold. The most used method is tensile testing, which stretches the tissue in order to determine the maximum braking point. The stiffness, elasticity modulus, stress and strain of the decellularized tissue is then compared to the native tissue to determine significant differences.

The above mentioned methods provide important information on the effectiveness of the decellularization methods. However complete decellularization of tissues and the protocols used is dependent of the specific tissue of interest. There are many different methods to effectively decellularize tissues (Gilbert *et al.* 2006), but they also include robust protocols. These protocols can substantially affect the composition and mechanical behaviour of the remaining biologic scaffold. For example, chemical treatments could compromise the mechanical properties of the ECM scaffold, making it more susceptible to degradation *in vivo*. According to Gilbert *et al.* (2006), this can result in rapid decrease in strength and affect the load bearing capacity of the scaffold. Thus optimizing tissue processing methods for decellularization could improve the outcome of tissue engineering and clinical success. There are already natural occurring ECM scaffolds derived from decellularization protocols that have received regulatory approval for use in human patients. These include the human dermis (Alloderm®, LifeCell Corp), porcine heart valves (Synergraft®, CryoLife Inc) and porcine urinary bladder (ACell Inc).

In addition, various decellularization techniques have been documented (Gilbert *et al.* 2006; Badylak *et al.* 2011), and decellularization methods that allow a cell-free scaffold to be obtained are available (Kasimir *et al*. 2006; Reider *et al.* 2004; Netelenbos *et al.* 2002; Pillarisetti *et al.* 2002). However, factors such as tissue density and organization, biologic and geometric properties desired for the end product dictates the efficiency of the applied decellularization protocol and needs to be considered (Crapo *et al.* 2011). In addition, applying the same decellularization protocol on the same specific tissue of interest obtained from different species can also affect the efficiency of cell removal (Grauss *et al.* 2003; Cho *et al.* 2005; Scheke-Lyland *et al.* 2003; Netelenbos *et al.* 2002; Pillarisetti *et al.* 2002). However, it is unclear what the effect of the applied protocol has on the efficiency of cell removal.

2.6.3.2. ECM as a graft material

The use of the ECM in regenerative medicine and tissue engineering applications is increasing. It is being used to facilitate the constructive remodelling of different tissues in clinical studies and applications (Badylak *et al.* 2011). Extracellular matrixes (ECM) are harvested from a variety of allogeneic or xenogeneic tissue sources. These include the dermis (Chen *et al.* 2004), skeletal muscles (Valentin *et al.* 2010), urinary bladder (Chen *et al.* 1999; Parekh *et al.* 2009), small intestine (Lantz *et al.* 1993), blood vessels (Conklins *et al.* 2002; Dahl *et al.* 2003), heart valves (Lichtenburg *et al.* 2006) and the liver (Lin *et al.* 2004) from several different species. The ECM represents an environment in which cells of each tissue and organ secrete their products in response to changes in the microenvironment. The ECM thus provides cues that affect cell migration, proliferation, and differentiation (Crapo *et al.* 2011). It regulates cell phenotype for the development of tissues, guides organ development, and regulates tissue repair and physiologic regeneration (Song and Harald 2011). ECM has also been shown to influence cell mitogenesis and chemotaxis and induce constructive host tissue remodelling responses (Xu *et al.* 2010; Parekh *et al.* 2009; Valentin *et al.* 2010). Thus preservation of this naturally occurring three dimensional biological scaffold (ECM) is highly desirable during decellularization processes.

2.6.3.3. Thrombogenecity of the decellularized scaffold material

Thrombogenecity is a major problem in decellularized vascular grafts when in contact with blood (Kesimir *et al.* 2006). These grafts contain no cells (including ECs), thus exposing the collagen fibre to blood (Smith 2009). Decellularized blood vessels have been shown to attract and induce platelet activation (Ning-Tao *et al.* 2007; Kasimir *et al.* 2006; Sarkar *et al.* 2007), and after

some time these vessels calcify and/ or degenerate due to the absence of endothelial lining (Schoen and Levy 2005; Kasimir *et al.* 2006). The presence of vascular endothelial cells is thus necessary for the normal functioning of vascular grafts and also to prevent thrombosis formation.

2.6.3.4. Endothelialization of vascular grafts

In order to improve long term patency of synthetic small diameter vascular grafts, endothelial seeding has been proposed. Combining the synthetic material and biologically active cells in order to create haemo-biocompatible vascular grafts is promising. Surface endothelialization will reduce thrombogenecity as we know that ECs possess antithrombotic properties that make the luminal surfaces blood vessels completely non-thrombogenic.

Endothelial cells can be isolated from different sources and easily harvested *in vitro*. Endothelial cells (ECs) can be derived from endothelial progenitor cells (EPCs) found in umbilical cord blood (Schmidt *et al.* 2005), peripheral blood (Boyer *et al.* 2000), and bone marrow (Cho *et al.* 2005; Kusuma and Gerecht 2010). Endothelial progenitor cells (EPCs) are more restricted in their proliferative and differentiating capacity into functional ECs and cannot give rise to other cell types *in vitro* (Badylak *et al.* 2011; Asahara *et al.* 1997). Once seeded, ECs form a continuous monolayer that selectively separates blood from the arterial wall and also performs vast array of mechanisms already discussed in section **2.2**. Thus, this makes endothelial seeding approach an important technique in constructing haemo-compatible grafts that can perform the same way as blood vessels.

Difficulties observed in achieving spontaneous endothelialisation in humans led to the investigation of endothelial cell (EC) seeding *in vitro*. Endothelial cells (ECs) play an integral part in angiogenesis by having the ability to proliferate in order to provide additional cells that form new vessels (Krennings *et al.* 2007; Brown *et al.* 2009). These cells can also reorganize themselves to form the necessary three dimensional vessel structures and migrate towards angiogenic stimuli (Krenning 2009; Popa *et al.* 2006; Van der Strate *et al.* 2007). Seeding of endothelial cells (ECs) on decellularized vessels is an attractive proposition as the endothelial layer incorporates many of the anti-thrombogenic properties of blood vessels. Furthermore, EC cultures are viable and show excellent metabolic activity and proliferation capabilities and are able to form a contiguous monolayer on the blood vessel scaffolds (Ning-tao *et al.* 2007). The EC monolayer also show cobblestone morphology with the capacity for growth and self-repair. Furthermore, cultured ECs have also shown to express high levels of endothelial oxidase synthase and tissue plasminogen activator markers that are found similarly to those obtained from human umbilical cord vein (Schmidts *et al.* 2005).

The concept of using autologous ECs for seeding synthetic grafts lumen before implantation was first experimentally implemented by Herring *et al.* in 1978. A one-stage technique to endothelialize the synthetic grafts using EC-derived from canine veins was developed. This technique makes use of freshly harvested ECs from venous origin or microcirculation and then seeded on synthetic grafts immediately before implantation (Herring *et al.* 1978). This seeding technique was reported not successful as ECs detached after implantation due to blood flow exposure. The problem was thought to be the low seeding density of ECs that failed to produce a confluent monolayer and a low shear stress (Herring *et al.* 1978; Park *et al.* 2000). These vascular grafts produced low patency results and did not overcome the problem of thrombogenecity.

The disappointing results of the one-stage technique led to the development of a more promising technique. The two-stage technique, which produced better results (Magometschnigg *et al.* 1992; Deutsch *et al*. 1999). The two-stage technique involves harvesting and culturing of autologous ECs followed by seeding the vascular grafts *in vitro* before implantation (Heyligers *et al.* 2005). This seeding technique ensured high patency rates of small-diameter grafts (Deutsch *et al.* 1999). The confluent monolayer of the endothelial lining can be obtained, as better seeding densities are possible (Heyligers *et al.* 2005). Enough numbers of ECs can also be obtained as cells are allowed to multiply *in vitro* using cell culture techniques prior to seeding. Thus the success of endothelial seeding critically depends on the methods used to re-endothelialize grafts surfaces.

2.6.3.5. Endothelialisation by surface coating

Endothelialisation of vascular grafts is often limited by the inability of ECs to remain attached to the scaffold after exposure to flow (Sgarioto *et al.* 2012). This often leads to intimal hyperplasia in the long term, resulting in low patency rates. To enhance antithrombogenecity of scaffold materials, studies have focused on coating luminal surfaces of TEVGs with cell adhesion mediators to improve cell attachment (Sgarioto *et al.* 2012; Sales *et al.* 2007; Kerdjoudj *et al.* 2007; Balcells and Edelman 2002; Kipshidze *et al.* 2000; Sipehia *et al.* 1996). These cell adhesion mediators have shown to enhance cell proliferation, differentiation and migration (Sales *et al.* 2007; Balcells and Edelman 2002). The current cell adhesion mediators commonly used include fibronectin, fibrin glue, collagen type 1, gelatin, laminin and heparin. For biological scaffold material, fibronectin, fibrin glue, collagen type 1 and serum coatings have been used this far. Fibrinogen attachment protein has been found to be most effective in facilitating cell attachment and retention in both biological and synthetic materials (Kerdjoudj *et al.* 2007). Fibronectin is a glycoprotein found on the basement of native blood vessels to which ECs attach (Sipehia *et al.* 1996). Collagen type 1 on the other hand is known to be the major matrix component of endothelial wall of blood vessels (Ricard-

blum 2011; Shekhonin *et al.* 1985). Together, collagen and fibronectin co-exist in the ECM and have interactions with ECs that are essential for many ECM-dependent cell functions (Mao and Schwarzbauer 2005).

Surfaces coating of scaffolds has produced encouraging results in improving EC retention in TEVG surfaces. Heparin coated decellularized carotid arteries showed improved retention of seeded ECs on the graft surfaces under flow (Conklin *et al.* 2002). By adding basic fibroblast growth factor to the coated grafts, 60% of seeded ECs remained attached to the coated scaffold after 3 hours of exposure to flow. The basic fibroblast growth factor coating increased proliferation of seeded ECs significantly. Double coating with collagen type 1 and fibronectin combined provides better support structure for EC proliferation, attachment and retention as compared to single protein coating (Sgarioto *et al.* 2012). Several other studies have been conducted using pre-coating of luminal surfaces with different cell attachment mediators (Assmann *et al.* 2013; Zhou *et al.* 2009). Surface coating of graft material not only improved EC monolayer but also represent a novel approach to fast endothelialisation leading to better graft biocompatibility.

3. Aim and objectives

The aim of this study was to successfully re-endothelialize decellularized baboon arteries. Furthermore, to determine the thrombogenecity of the vessels by perfusing the newly tissue engineered vascular grafts with blood.

The first objective of this study was to assess the degree of decellularization on the decellularized baboon arteries by evaluating their morphology for any remaining nuclear or cellular material. Secondly, to successfully culture commercially available human umbilical cord vein endothelial cells (HUVECs) and obtain enough viable ECs to seed the luminal surface of decellularized baboon arteries. Thirdly, to re-endothelialize the decellularized grafts surfaces with HUVECs. The last objective was to investigate the potential of thrombogenecity in the decellularized and re-endothelialized baboon arteries by perfusing the newly engineered arteries with baboon blood and compare them to the native perfused blood vessel.

4. Methodology

4.1. Ethics Approval

The study was performed in accordance with the “Guide for the Care and Use of Animals for Scientific Purposes” published by the South African Bureau (SABS) Standard Division (2008). Ethics approval was obtained from the Interfaculty Animal Ethics Committee of the Health Sciences Faculty at the University of the Free State (NR 17/2014).

4.2. Study design: Explorative study

Two male baboons (Species name: Papio Ursinus) weighing approximately 10 kg were used for this study. The 2 baboons were euthanized as part of another study in our Department that had already obtained ethics approval (ethics number: NR 06/12). Twelve medium-sized muscular arteries with diameter ranging from 3 - 5mm and a minimum length of 6 cm were acquired from different regions of the 2 euthanized baboon bodies. Six arteries were collected from each baboon. Artery acquisition was performed at the Animal Experimental Unit of the Health Sciences Faculty at the University of Free State. The main source of blood vessels collected was from arteries branching from the aorta: the right (R) and left (L) carotid, R and L radial and R and L femoral arteries. Four arteries of each type were collected and grouped together to form 3 groups: the carotid artery group, the radial artery group and the femoral artery group. All baboon arteries acquired were decellularized by the Department of Cardiothoracic Surgery. Cell culturing of HUVECs was performed under optimal conditions in our cell culture laboratory at the Department of Haematology and Cell Biology. Proliferation rate of the cultured cells was assessed using MTT cell viability and proliferation assay after subculture. After cell culture work, 3 decellularized arteries (1 from each group) were randomly selected and seeded with HUVECs. The remaining decellularized baboon arteries remained unseeded to serve as controls. The confluent endothelial monolayer of seeded decellularized arteries was assessed using scanning electron microscopy (SEM) examination. The seeded cells were detached from the grafts surfaces of small sections and tested for viability (metabolic activity and proliferation) using the MTT assay. Afterwards, all arteries were perfused with baboon blood collected in 3.2% sodium citrate tubes. Blood samples were collected from 4 healthy baboons (10 – 15 kg) by standard venipuncture of the femoral vein performed by the head of Animal Experimental Unit. A perfusion experiment was performed to determine possible thrombus formation on the luminal surfaces of all arteries. Thereafter, arteries were sent for SEM analysis to evaluate them morphologically. Histology, TEM and SEM evaluations were performed by the Department of Anatomical Pathology and the Microscopic Unit of the University of the Free State. **Table 1** shows the different groups of baboon arteries used.

Table 4-1: Total number of baboon arteries collected for experimental procedures. Six decellularized arteries were subjected for seeding with cultured HUVECs, the other 6 were not.

2 euthanized male baboons used for artery collection		
12 blood vessels collected – 6 blood vessels from each baboon		
Location/group	Experimental (Decellularized)	
	Seeded	Unseeded
Carotid	2	2
Radial	2	2
Femoral	2	2

4.3. Experimental procedure

4.3.1. Decellularization of baboon arteries

4.3.1.1. Euthanasia of baboons

Two baboons (≈10 kg) were euthanized at the Animal Experimental Unit of the faculty of Health Sciences at the University of the Free State. Before euthanasia, each animal was anaesthetized with Rompun (0.1ml/10kg) together with Ketamine hydrochloride (10 mg/kg) to give deep general anaesthesia. Thereafter a saturated solution of potassium chloride was administered for euthanasia and baboon arteries collected. Potassium chloride was used to prevent damage to the vessel wall that can be caused by other pharmaceuticals, such as pentobarbital sodium.

4.3.1.2. Artery acquisition

A total of 12 medium-sized muscular arteries were acquired from the euthanized baboons within 1 hour after euthanization. As mentioned, six arteries were collected from each baboon. The acquired arteries had a diameter ranging from 3 – 5 mm and a minimum length of 6 cm. During incision, care had to be taken to insure that the vessels were not injured, since this would compromise the mechanical properties of the vessels.

The acquired arteries were washed 3 times with sterile phosphate-buffered saline (PBS, pH 7.4; Invitrogen, Carlsbad, CA) to remove residual blood clots. For comparison with native untreated arteries, 2 small sections (0.5 cm) were cut from all acquired baboon arteries for histology, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Thereafter arteries were stored at 4º C for 24 hours in 100ml PBS supplemented with an antibiotic cocktail of penicillin (200 U/ml), streptomycin (200 µg/ml), amphotericin B (10 µg/ml), ciprofloxacin (50 µg/ml) and cefuroxin (750 µg/ml) (ScienCell Research Laboratories, Carlsbad, CA) prior to decellularization.

4.3.1.3. Decellularization

All acquired arteries were transported in sterile PBS to the Department of Cardiothoracic Surgery for decellularization. To prepare the arteries for decellularization, excess connective tissue was removed using scissors or blades. Thereafter the arteries were subjected to the decellularization procedure. Decellularization of baboon arteries was accomplished using the acid and detergent based method revised from the published protocol of Dohmen *et al.* (2002) with slight modifications. In short, the arteries were decellularized using a combination of 1% sodium deoxycholic acid, 0.05% sodium dodecyl sulphate (SDS) and 0.05% triton-X100 at 37ºC. This was then followed by extensive rinsing period in normal saline. Small sections (0.5 cm) were taken from each artery after decellularization and send for histology, SEM and TEM investigations. The remaining segments of the decellularized arteries were stored in 100 ml PBS supplemented with an antibiotic cocktail of penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (10 µg/ml) at 4ºC.

4.3.1.4. ECM evaluation

To determine the decellularization efficiency, the small arterial sections (0.5 cm) were examined using histology analysis. Histology analysis was employed to view the vessel structure on a microscopic level. Staining the vessels highlighted specific components to enable comparison between the histology of a native artery to the histology of a decellularized artery. Paraffin embedded sections were stained with hemotaxylin & eosin stain to detect any cellular and nuclear material. Masson's Trichrome (MT) stain was used to evaluate the presence of collagen fibres. The Verhoeff-Van Gieson (VVG) stain was used to evaluate the presence of elastic fibres.

Transmission electron microscopy (TEM) analysis were performed to determine the presence and condition of the basement membrane on the luminal surface of decellularized arteries. The histology and TEM examinations on the samples were both performed by the Department of Anatomical Pathology, faculty of Health Sciences at the University of the Free State.

Scanning electron microscopy (SEM) was also performed to examine the morphological differences (on the luminal surface) between the decellularized and native arteries. The SEM examinations were performed by the Center of Microscopy of the University of the Free State.

4.3.2. Culturing of endothelial cells (ECs)

Human Umbilical Vein Endothelial Cells (HUVECs) are usually used to obtain ECs for seeding vascular grafts (Sgarioto *et al.* 2012; Dahan *et al.* 2011; Gui *et al.* 2009; Kasimir *et al.* 2006; Kasimir

et al. 2005). The advantage of using umbilical vein ECs is the high growth potential of these ECs. For our study HUVECs were obtained from Whitehead Sscientific (Clonestics™ HUVEC systems; Lonza Walkersville, Inc., Maryland, USA). These cells are already tested positive for the presence of von Willebrand factor, CD31 and CD105 endothelial markers, acetylated Dil-Ac-LDL uptake and were negative for alpha smooth muscle actin marker. Thus verification and validation studies were not needed in this study.

4.3.2.1. Cell Culture

The cell culture work was performed in a laminar flow bench under sterile conditions to prevent contamination. All equipment, hand gloves and reagent bottles were sterilized with 70% ethanol (99.9 % ethanol, Saarchem, Krugersdorp, Gauteng, South Africa) diluted in double distilled water. Culturing of HUVECs and reagent preparations were performed according to the manufacturer's instructions (Lonza Walkersville, Inc., Maryland, USA).

a. Coating of culture flasks

Eight 25 cm^2 culture flasks (Lasec (Pty) Ltd., Cape Town, South Africa) were coated with 5 µg/ml fibronectin (Human plasma superfibronectin, Sigma-aldrich™ Inc., Saint Louis, USA) diluted in 1 ml PBS (w/o Ca^{2+} and Mg^{2+}, Gibco®, Grand Island, New York, USA). The coated flasks were incubated at 37˚C for 2 hours before cell culturing. Fibronectin is needed to facilitate HUVEC's attachment to the flask surface area. Thereafter the unbound fibronectin was discarded and the flask surfaces washed twice with 1 ml of PBS and used immediately for cell culture.

b. Establishing a cell culture from a primary culture

Endothelial growth medium containing the basal medium and growth supplements was used (endothelial growth medium kit, Clonestics™ EGM-2 Bullet kit Medium, Lonza Walkersville, Inc., Maryland, USA). Growth supplements included: hydrocortisone, human recombinant epidermal growth factor (hEGF), fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor-Basic (hFGF-B), human recombinant insulin-like growth factor (R3-IGF-1), ascorbic acid, heparin, and gentamicin/amphoterin-B. First the supplements were thawed at room temperature and added to the basal medium within 72 hours. The supplemented endothelial growth medium was then allowed to equilibrate in a 37°C, 5% CO_2 humidified incubator (NAPCO water-jacketed CO_2 incubator, Thermo Fisher Scientific, Shandong, China) for at least 30 minutes.

One vial (1 ml) of HUVECs (Clonestics™ HUVEC systems; Lonza Walkersville, Inc., Maryland, USA) was thawed from storage in liquid nitrogen in a 37°C water bath for ≈ 1 minute and then moved into the laminar flow hood area. The cell count and viability of 20µl cells was determined using a TC10™ Automated Cell Counter (Bio-Rad Laboratories, Inc., Singapore) with 20µl trypan blue (Gibco®, Grand islands, New York, USA). The HUVECs vial contained approximately 500 000 viable cells/ml. Cells were then diluted in 40 ml of supplemented endothelial growth medium. The recommended seeding density for HUVEC is 2, 5 x 10^3 viable cells/cm^2 (Clonetics™, Lonza Walkersville, Inc., Maryland, USA). Therefore 5 ml of the cell suspension was added to each 25 cm^2 culture flask to obtain the recommended concentration. The cells were then cultured at 37°C in a 5% CO_2, 95% humidified incubator for at least 24 hours without any disturbances before the medium was changed. Cells were subcultured when they reached 80% confluence after 6 days. An inverted Nikon light microscope was used to observe the cells.

c. Subculturing

Cells were subcultured with a Reagentpack™ from Lonza (Walkersville, Inc., Maryland, USA) containing Trypsin/EDTA, Trypsin Neutralising Solution and HEPES-BSS (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - Basal Salt Solution). First the cells were washed with 5 ml HEPES-BSS at room temperature. HEPES-BSS was used for extra buffering of cells and to allow the trypsine to work effectively. This was then followed by adding trypsin using 2 ml of Trypsin/EDTA solution to detach the cells for six minutes by tapping the flask against the hand every 30 seconds. The cell layer was examined microscopically to observe the detachment process. After 90% detachment, 4 ml of trypsin neutralizing solution was added to neutralize the trypsin/EDTA. The detached cells were then transferred to a sterile 15 ml conical tube (Lasec (Pty) Ltd., Cape Town, South Africa). Two ml of HEPES-BSS was added to the flask to collect the residual cells, which was also added to the centrifuge tube. The culture flask was again examined under a microscope to make sure that the harvest process was successful. There was less than 5% of cells left behind indicating a successful harvest. The conical tube was then centrifuged at 220 x g for 5 minutes to pellet the cells. Thereafter, the supernatant was discarded leaving at least 100-200 µl. The pellet was then re-suspended in 3 ml of growth medium and diluted with HEPES-BSS to achieve the desired concentration of cells/ml (1, 25 x 10^4 viable cells/ml) and recounted. The cells were cultured again into prepared culture flasks and incubated at 37°C, 5% CO_2 humidified incubator. The medium was changed the day after subculturing and every second day for 6 days until cells were 80% confluent (Clonetics™, Lonza Walkersville, Inc., Maryland, USA). When a confluent monolayer of ECs was observed, another passage was performed in 3 large 75 cm^2 culture flasks (Lasec (Pty) Ltd., Cape Town, South Africa).

Human umbilical vein endothelial cells (HUVECs) can only be passaged three times (Clonetics™, Lonza Walkersville, Inc., Maryland, USA) for studies of this type before being used for seeding experiment. This is because growth rate, biological responsiveness and function deteriorate with subsequent passages (Clonetics™, Lonza Walkersville, Inc., Maryland, USA). Thus ECs were passaged only 2 times, as they are already purchased passaged ones as a primary culture. After the second passage, the cells were available for seeding of the decellularized baboon arteries.

d. Cell viability and proliferation rate

To determine the proliferation rate of the cultured ECs after confluency before seeding experiment, an MTT cell viability and proliferation rate assay was performed. It is very important in this study to evaluate a cell population's response to external factors such as growth factors and antibiotics (ScienCell™, Corte Del Cedro, Carlsbad, CA). The MTT assay kit (MTT Cell Viability and Proliferation Assay Kit, ScienCell™, Corte Del Cedro, Carlsbad, CA), verifies cell viability and proliferation by measuring the metabolic activity of cellular enzymes. It is a calorimetric test that measures the activity of cellular enzymes to reduce the pale yellow tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour. This assay measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may under defined conditions, reflect the number of viable cells (ScienCell™, Corte Del Cedro, Carlsbad, CA).

In the MTT assay, cells were plated and cultured in a clear-bottom 96 well ELISA cell culture plate (NUNC). A cell suspension of 1 x 10^6 cells/ml was used to prepare serial dilutions of 1 x 10^3 cells per ml in fresh culture medium. Hundred microliter (100 µl) of the dilutions were then plated into wells of a 96 well plate in triplicates. Three control wells of medium alone were included to provide the blanks for absorbance reading. Cells were then incubated for 24 hours at 37ºC in a CO_2 incubator to allow them to recover from handling. The next day, a newly prepared MTT solution (5mg/ml in distilled water) was allowed to equilibrate at room temperature. Then 10 µl of the MTT solution was added to each well of the 96 well plate, and mixed well gently by rocking the plate side to side. The cultures with MTT were incubated at 37ºC for a further 2 – 4 hours, after which black crystals started to form in the live cells. An MTT solubilisation buffer (100 µl) was added to each well to stop the reaction and to help dissolve the crystals. An orbital mixer was used to further enhance the dissolution. The solubilised crystals were then spectrometrically quantified by measuring the absorbance with an ELISA plate reader (Synergy HT, BioTek instruments, Vermont, USA) within an hour. A test wavelength at 570 nm and a reference wavelength at 690 nm were used. Thereafter, a linear relationship was observed between OD570nm – OD690nm and the number of HUVECs. This assay was performed in the dark and the intensity of the colour is proportionate to the amount of viable cells. The MTT assay was performed after the second passage prior to the seeding experiment.

4.3.3. Seeding of decellularized scaffolds

Three decellularized baboon arteries were seeded with cultured ECs. The seeding of the decellularized baboon arteries was accomplished using the method revised from the published protocol of Dohmen *et al.* (2002) with slight modifications. The selected arteries were chosen based on the efficiency of the decellularized process. A specialized static bioreactor (see **figure 4-1**) was used for seeding of artery constructs with ECs. The static bioreactor consists of a flask made of bioinherent material in which the vessel construct can be sutured. This type of specialized bioreactor was provided by our Department of Cardiothoracic Surgery.

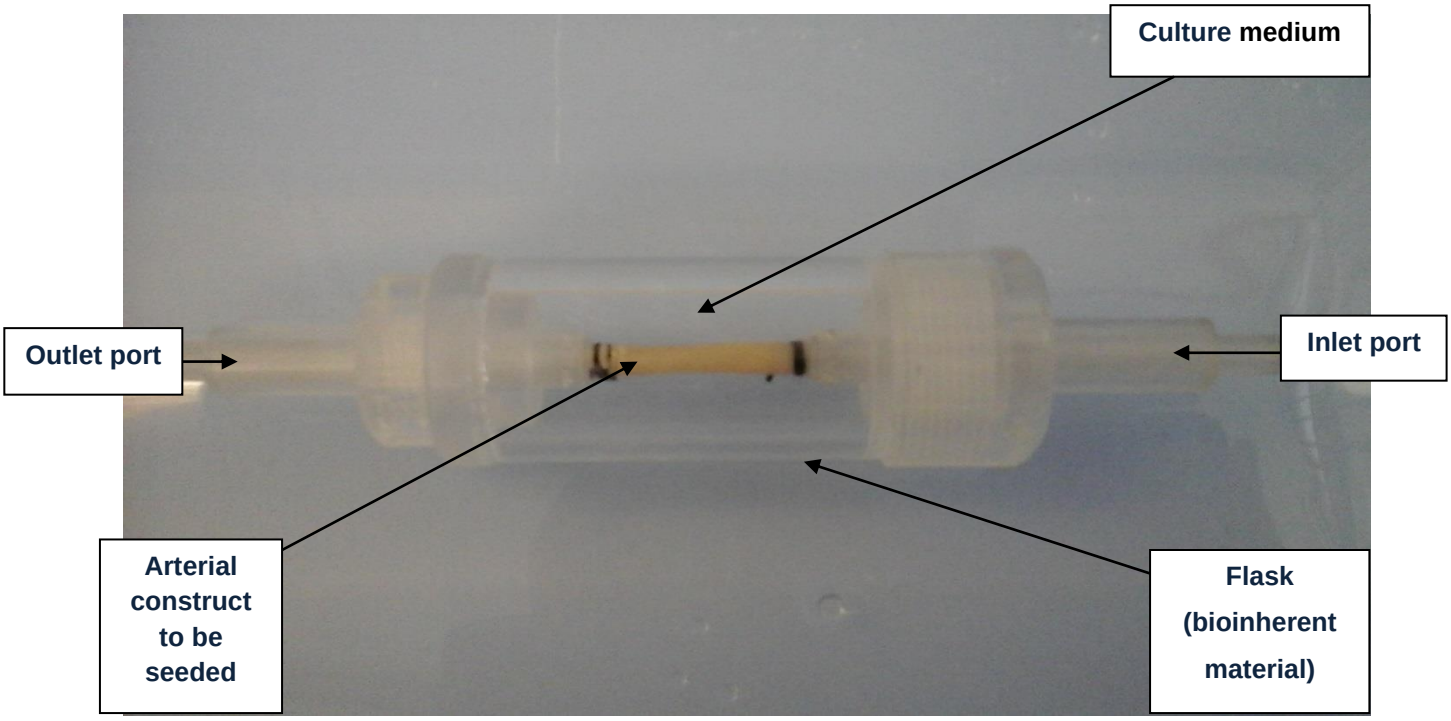

Figure 4-1: A static bioreactor with a blood vessel attached to both ends for seeding.

4.3.3.1. Cell seeding experiment

a. Surface coating

The decellularized artery was first cannulated on both ends to a stopcock. Then a 10µg/ml solution of fibronectin (Human plasma fibronectin, Gibco®, Grand Island, New York, USA) in 1 ml PBS solution was ejected into the vessel using a syringe. Enough solution was used to ensure that the entire surface lumen is covered. The fibronectin coating protein was used to facilitate EC attachment (endothelialisation) on the luminal surface of vascular grafts. The coating protein was diluted according to the manufacturer's instruction. After surface coating, the vascular graft was incubated at 37°C for 45 minutes to allow the fibronectin to absorb to the ECM. Thereafter, the unbound fibronectin was aspirated and the artery washed 2 times with sterile PBS. The coated arteries were allowed to dry and used immediately or incubated at 37°C for use at a later time.

b. Seeding of arteries

The arterial graft was sutured to the bioreactor on both ends, using 2 suture lines. Thereafter the bioreactor was filled with the endothelial growth medium (Clonestics™ EGM-2 Bullet kit Medium, Lonza Walkersville, Inc., Maryland, USA) and kept in a 5% CO_2 /air atmosphere incubator at 98% humidity at 37°C. Gas aeration to the static system was provided by surface aeration of the culture medium. The exact adjustment of the pH level was achieved by modulating the CO_2 supply. The static environment however provided a low shear stress environment that is crucial to the seeding process.

The artery construct was then washed 2x with PBS supplemented with an antibiotic solution (penicillin and streptomycin) to limit any contamination on the vascular grafts.

Cells were seeded at a density of 2.5 x 10^3 cells/cm^2 (recommended seeding density for HUVECs (Clonetics™, Lonza Walkersville, Inc., Maryland, USA)) on the freshly coated graft surfaces. The EC culture was suspended into the sutured graft within the bioreactor and the air removed. Endothelial cells (EC's) were precisely injected in the valve lumen through the specially designed cell seeding inlets. After injecting of EC's, the bioreactor was connected to a rotation device, the endostrabilisator, and placed in the CO_2 incubator at 37°C. The endostrabilisator was programmed to run for 180 minutes with 1800 rotations, 5 brakes and 120s pause after each rotation. The bioreactor was rotated to expose the entire luminal surface of the artery in order to achieve optimal attachment conditions. After 3 hours of seeding, the artery construct was rinsed with PBS to remove non-adherent cells. The arterial grafts were then maintained in fresh culture medium overnight at 37°C under 5% CO_2 incubator to allow ECs to grow onto the arteries in static culture medium. The arteries were incubated for additional 7 days, with the medium changed every 48 hours.

After the seeding procedure, day 1 and day 7, a small piece was cut from each seeded artery and sent for SEM and TEM to examine whether the endothelialisation of graft surfaces was successful. Cell viability of the cells seeded on the scaffold was also determined.

c. Cell viability on the scaffold

Strong cell attachment and proliferation on the scaffold demonstrate that seeded cells are viable. To verify this, we used the same MTT assay kit (MTT Cell Viability and Proliferation Assay Kit, ScienCell™, Corte Del Cedro, Carlsbad, CA) as with the cell culture. This kit verifies cell viability and proliferation by measuring the metabolic activity of cellular enzymes.

With MTT assay, the medium in the artery was first removed and replaced with fresh culture medium. Two small circular pieces (0.5 cm) were cut from each seeded artery using a tissue puncher. Thereafter cells were detached from the artery scaffold using trypsine/EDTA. Cells were then cultured into a 24-well plate for 24 hours. Endothelial cells from the cell culture was also included as a positive control. The MTT assay was then performed following the same procedure as in **4.3.2.1 (d)**.

4.3.4. Perfusion experiment

4.3.4.1. Blood sample collection

Blood samples (50 ml) were collected from 4 healthy baboons each using a 3.2% sodium citrate tube at the Animal Experimental Unit of the University of the Free State. Much less than 10% of the animal's blood volume (70 ml/kg) was collected from baboons that weighed between 10-15 kg. Blood samples were drawn by standard venipuncture of the femoral vein performed by head of the Animal Experimental Unit. Before drawing blood, the animals were anesthetized with Ketamine hydrochloride (10mg/kg).

Blood samples were then transported to the Department of Haematology and Cell Biology (Cell culture laboratory), and used within 4 hours for the perfusion experiment.

4.3.4.2. Perfusion experiment

The perfusion experiment was performed to investigate the possibility of thrombus formation on the control, decellularized+seeded and decellularized+not-seeded baboon arteries *in vitro* at room temperature

The *in vitro* flow chamber used for the perfusion experiment is a closed system connected to flexible plastic tubes and a palpitation pump which delivers a laminar flow to the arteries. Photos of the flow chamber are shown in **figure 4-2**.

The artery was sutured to the flow chamber on both ends using 2 suture lines. Care had to be taken to ensure that the artery wall did not get damaged in the process. A palpitation pump was then connected to the flow chamber. The sutured artery was first circulated with 50 ml of PBS supplemented with an antibiotic solution to wash the lumen (2 x 5 minutes). Then 50 ml of blood was circulated through the sutured baboon artery for 2 hours. A temperature of 37°C and a pressure of 120/60 mmHg was maintained throughout the experiment. The seeded baboon arteries were circulated with blood eight days after the onset of seeding. Thereafter, the artery was washed once with PBS for 5 minutes and removed from the flow chamber. Small pieces were cut out and sent for SEM analysis.

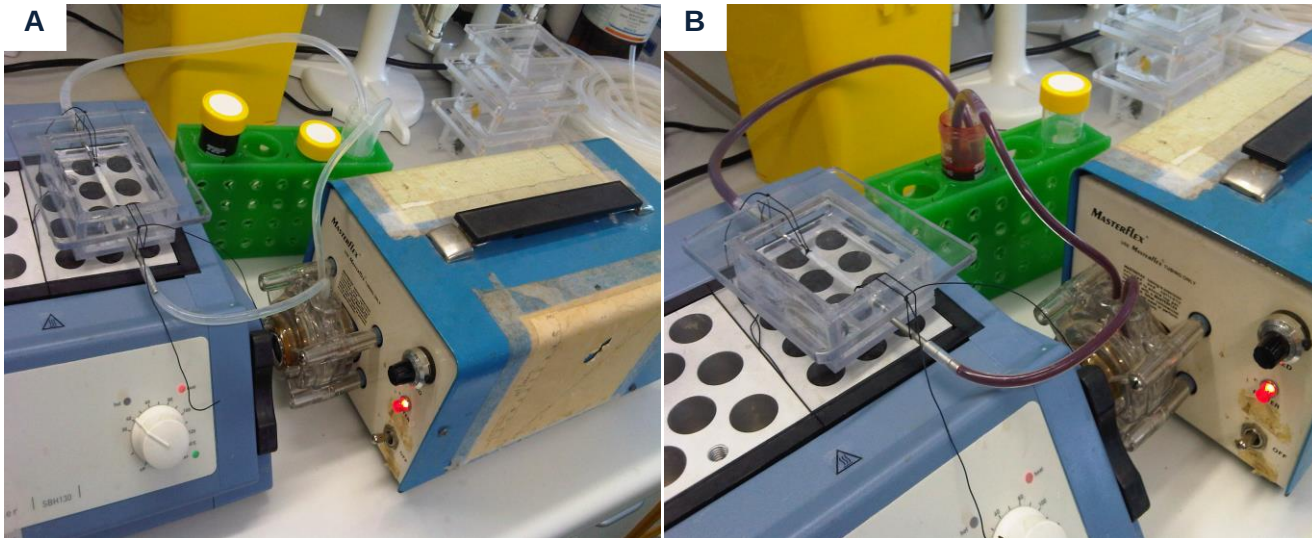

Figure 4-2: An in-vitro flow chamber connected to flexible plastic tubes and a palpitation pump which delivers a laminar flow to the sutured baboon artery. Two 50 ml containers filled with (A) PBS supplemented with antibiotic solution for washing the artery lumen and (B) baboon blood for perfusing the artery.

4.3.5. Morphological evaluation of decellularized and re-endothelialized baboon arteries

Histology, SEM and TEM studies were performed on the decellularized arteries to assess the morphology of the arteries before and after the seeding experiment. First, the general morphology of the native arteries was compared to the decellularized arteries using the histology, SEM and TEM. After the seeding experiment, SEM was applied to determine whether the luminal surfaces of the decellularized arteries were completely endothelialized. Arteries were also examined for any thrombosis and/or presence of activated platelets on the luminal surfaces after the perfusion experiment was performed using SEM. The SEM, TEM and histology methodologies are described in the next sections.

4.3.5.1. Scanning electron microscopy

In brief, samples for scanning electron microscopy (SEM) examination were fixed in a 0.1 M sodium phosphate-buffer (pH 7.0) with 3% glutardialdehyde for at least 3 hours. This was followed by 1 hour fixation in similarly buffered 1% osmium tetroxide. The samples were then dehydrated in a series of graded ethanol concentrations (50%, 70%, and 95%) for 20 minutes in each phase followed by two changes of 100% for 1 hour in each phase. The samples were dried using a critical-point dryer (Tousimis, Maryland, USA) in CO_2. After drying, the samples were mounted on stubs (Cambridge pin type 10 mm) by epoxy and gold coated (20 nm) with a sputter coater (Bio-Rad, United Kingdom). The samples were then examined with a Shimadzu SSX-550 scanning

electron microscope (Kyoto, Japan). Scanning electron microscopy examination was performed according to published methods of Bancroft and Gamble (2008).

4.3.5.2. Transmission electron microscopy

For TEM preparations, small sections that were cut from arteries were fixed in 3% glutaraldehyde for 3 hours at room temperature followed by further fixation in 1% osmium tetraxide for 2 hours. The vessels were then dehydrated in a series of graded alcohol concentrations and embedded in an epoxy. Ultrathin sections (1 µm) were cut and stained with uranyl acetate and lead citrate and scanned with TEM. The TEM procedure was performed according to the published methods compiled from Reynolds (1963), Spurr (1969) and Glauert (1974).

4.3.5.3. Histology

Histology methods were also performed according the published methods compiled from Reynolds (1963), Spurr (1969) and Glauert (1974).

Briefly, tissue samples were fixed with buttered formalin fixative to preserve the cells/tissue (both chemically and structurally) in a natural state. Then tissue processing was done to remove water from the biological tissue using a series of graded alcohol concentrations, followed by incubation in a non-polar solvent such as xylene, allowing extremely thin sections to be sliced. Tissue samples were then sectioned and processed into wax-blocks overnight. Once tissue processing was completed, the tissue was placed into a metal mold which was then filled with molten paraffin wax to allow the wax to harden and support the wax impregnated tissue which in turn allows the block to be cut. The tissue sample was orientated to make the lumen of the vessels visible when cut. The paraffin blocks were secured into a microtome clamp and slowly moved closer to the spinning blade for slices of the sample to be taken. The slices were then mounted on to glass slides and covered using cover-slips. Thereafter, mounted sections were stained with haematoxylin-eosin to highlight the nuclei and cellular materials in the artery tissue respectively. For the detection of collagen fibres in the arterial tissue, the paraffin embedded sections were stained with Masson trichrome (MT) stain. For identifying the elastic fibres in the artery tissue, the paraffin embedded sections were stained with Verhoeff-Van Gieson (VVG) stain. The stained sections were then analyzed by light microscopy.

4.3.6. Statistical analysis of data

Descriptive statistics were used. These include frequencies and percentages and where relevant, means with standard deviations. Because of small sample size, standard inferential tests were not used and only confidence levels for means were reported.

The histology of a normal and a decellularized baboon artery will be shown first, followed by TEM and SEM results of these arteries. The cell culture data will be shown next, thereafter SEM results of the seeded decellularized arteries. Lastly, SEM results of perfused arteries will be shown.

5.1.1. Histological analysis

a. Normal baboon artery

Histology of normal artery is shown in **figure 5-1** and that of a decellularized artery is shown in **figure 5-2**. A hemotaxylin and eosin (H&E) staining in **figure 5-1: A** and **B** revealed the nuclei of cells (hematoxylin) and the cytoplasmic and extracellular matrix proteins (eosin) of arteries. The nuclei of the endothelial cells (N-ECs), smooth muscle cells (N-SMCs) and fibroblasts (N-FBs) are clearly visible in a normal artery.

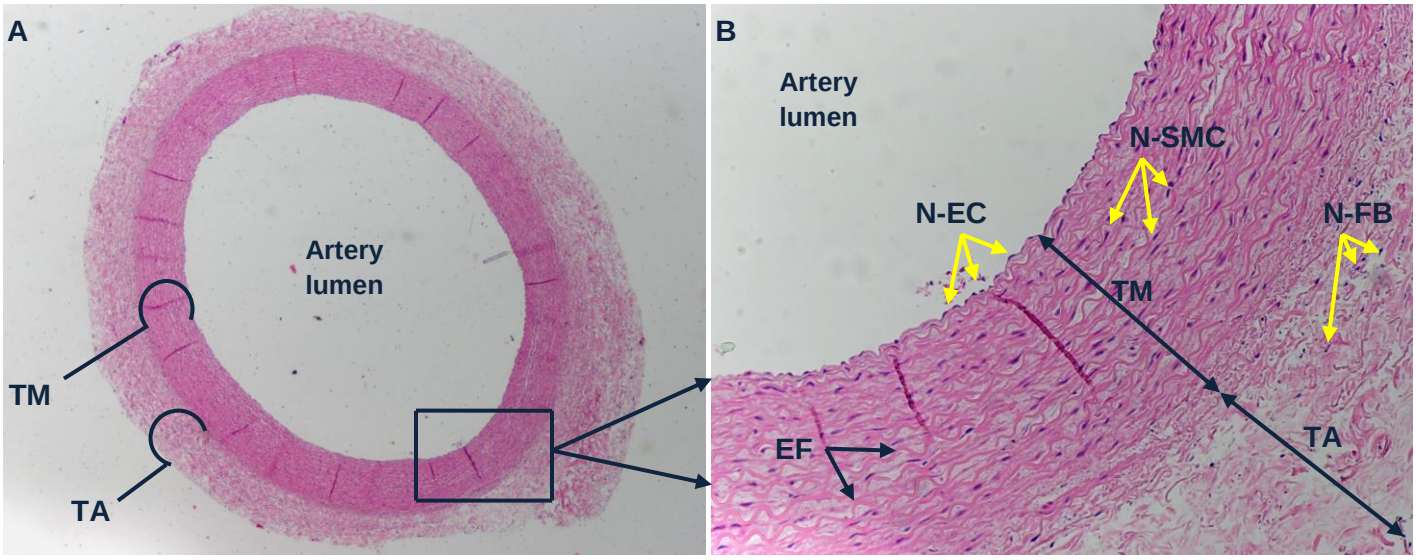

Figure 5-1: Histology of a normal medium-sized muscular artery (before decellularization) stained with H&E. Light microscopic image (A) shows the apparent tunica media (TM) and tunic adventitia (TA) at 2x magnification. Insert (B) is magnified 10x and shows the different layers of the artery. The nuclei of the endothelial cells (N-ECs), smooth muscle cells (N-SMCs) and fibroblasts (N-FBs) are stained dark violet indicated by the yellowe arrows. The nuclei of endothelial cells (N-ECs) present on the inner surface of the artery towards the lumen indicate the presence of the intact endothelium lining. The smooth muscle cells (indicated by the presence of nuclei (N-SMC)) and the many longitudinal wave-arranged elastin fibres (EF - stained deep pink) dominate the TM. The TA is underneath the TM and contains loose connective tissue that consists of elastic and collagen fibres.

The Masson's Trichrome (MT) stain was used to reveal the presence of the collagen fibres (stained blue) in the normal artery in **figure 5-2: A** and **B**. The bulk of tunica adventitia (TA) in a normal artery is made up with collagen fibres.

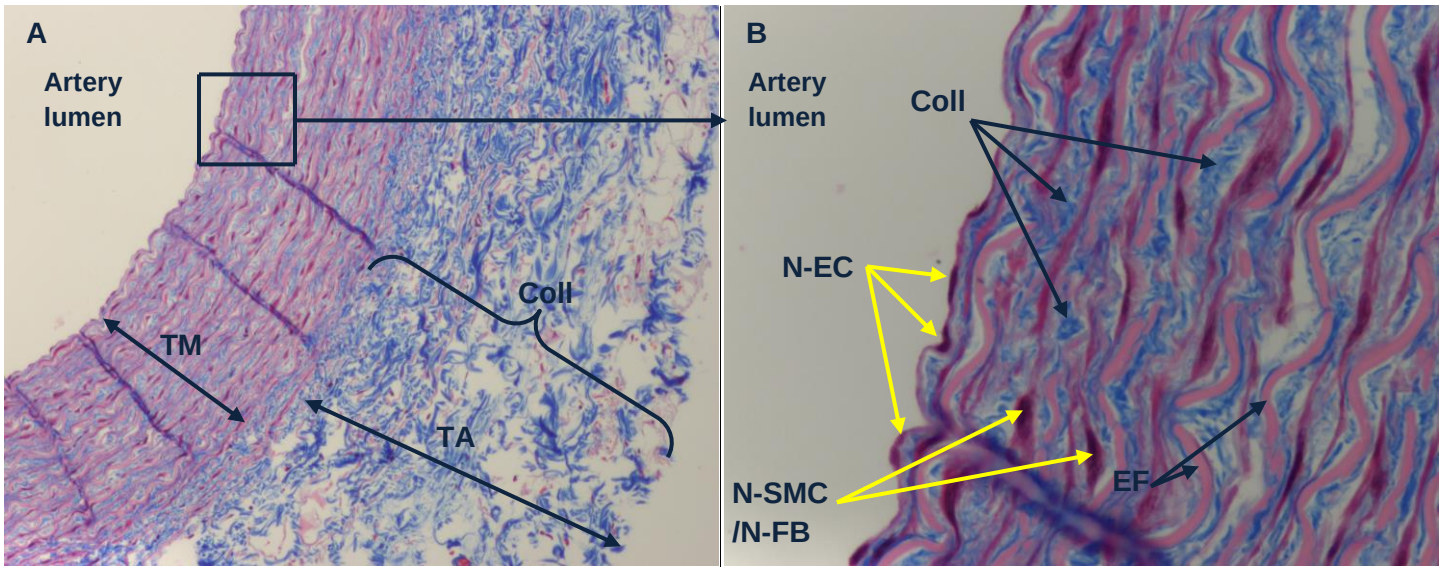

Figure 5-2: Histology of the collagen fibres in a normal medium-sized muscular artery (before decellularization) stained with Masson's Trichrome (MT) stain. The collagen fibres (Coll) are stained blue in image (A) at 10x magnification. Notice that the bulk of tunica adventitia (TA) of the artery consists of collagen fibres (indicated by the brace bracket in A). Insert (B) at 50x magnification, shows a close view of the present collagen fibres in the tunica media (TM) of the artery. The nuclei material of (N-ECs), (N-SMCs) and / or (N-FBs) are stained dark violet, and longitudinal wave-arranged elastic fibres (EF) are stained pink in the tissue (B).

A Verhoeff-Van Gieson (VVG) stain revealed the elastin fibres (EFs) and internal elastic lamina (IEL) separating the tunica intima (TI) and media in **figure 5-3: A** and **B**. The EFs and IEL are stained brown with the VVG stain. Again, the SMCs, elastin and collagen fibres are the major components of the tunica media in normal arteries.

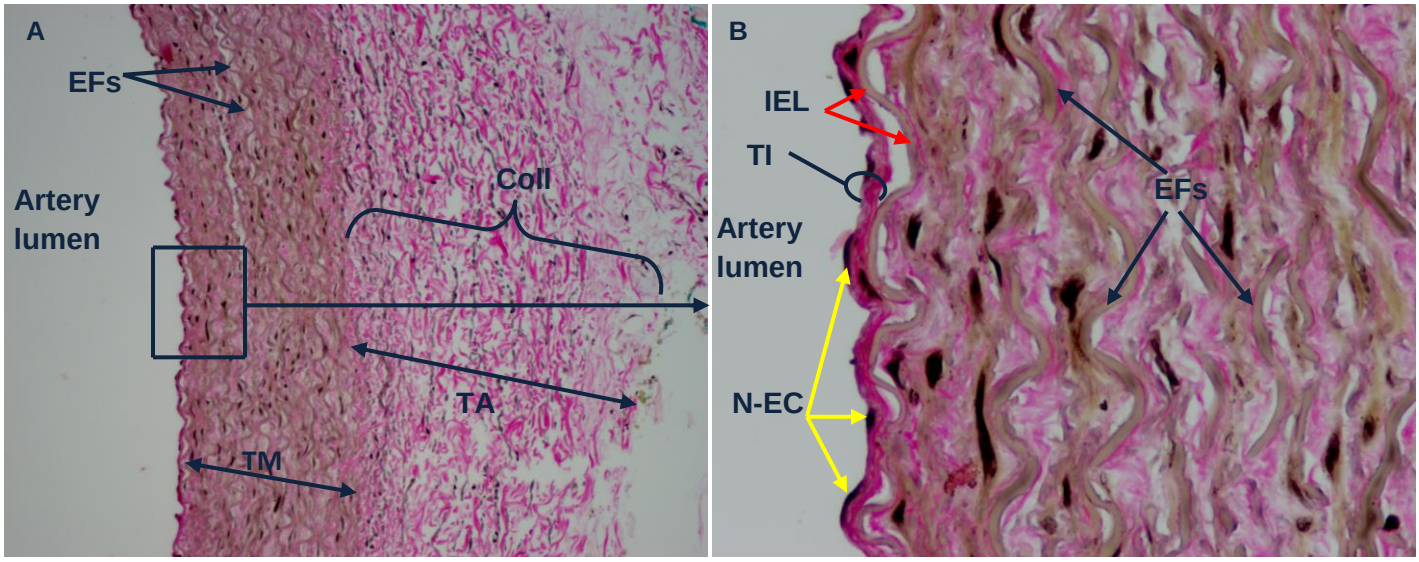

Figure 5-3: Histology of a normal medium-sized muscular artery (before decellularization) stained with Verhoef-Van Gieson (VVG) stain. The present elastic fibres (EFs) in (A) at 10x magnification are stained brown in the tissue. The tunica adventitia (TA) consists mostly of collagen fibres (Coll) stained pink. Insert (B) at 50x magnification, highlighted the EFs in tunica media (TM). The apparent internal elastic lamina (IEL) stained brown, marks the outer limit of the tunica intima (TI). This is indicated by the red arrows in (B). Few endothelial nuclei (N-EC stained dark) covering the luminal surface of the IEL can also be noticed in (B) indicated by the yellow arrows. The smooth muscle and collagen fibres (the major components of the tunica media) are stained pink in the background in (B).

b. Decellularized baboon artery

After decellularization, arteries were examined under a light microscope to verify the success of decellularization. The decellularized arteries were about 3-4 mm in diameter (**figure 5-4: A**). Histology examination showed that the histology architecture of the decellularized arteries was retained after the decellularization process with the medial layers still intact. The histology is shown in **figure 5-4: B**.

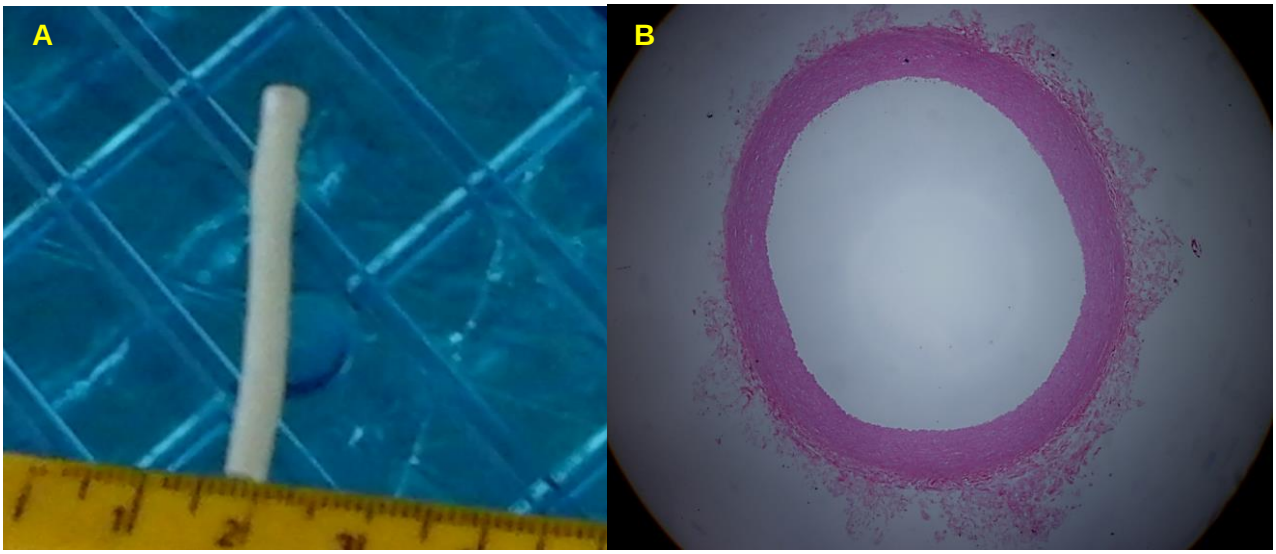

Figure 5-4: Image and histology of a medium-sized muscular artery obtained after decellularization. Image (A) is photo (JPEG image 2048x1152) of a decellularized artery with a diameter of 3 mm. Image (B) shows the histology of a decellularized baboon artery stained with H&E stain (at 2x magnification). Histology examination revealed the intact medial layers. Notice the minimized tunica adventitia (outer layer) of the artery done prior to decellularization.

Histology examination revealed that none of the 12 decellularized arteries were completely cell free when observed by light microscopy. These arteries showed multiple nuclear remnants (N) within the matrix of the artery after H&E staining (**figure 5-5**). Histology of carotid arteries are shown in **figure 5-5: A**, femoral arteries in **figure 5-5: B** and radial arteries in **figure 5-5: C**. Carotid and radial arteries showed less visible nuclear material than the femoral arteries.

The apparent internal elastic lamina separating the tunica intima with tunica media could be observed on all decellularized arteries after H&E staining. The endothelial layer on all the decellularized arteries was removed by the decellularization process (see **figure 5-5**). This was indicated by the absence of endothelial nuclei found on the luminal side of the artery. Thus all the arteries were not completely decellularized, rather only “de-endothelialized” (absent endothelial lining).

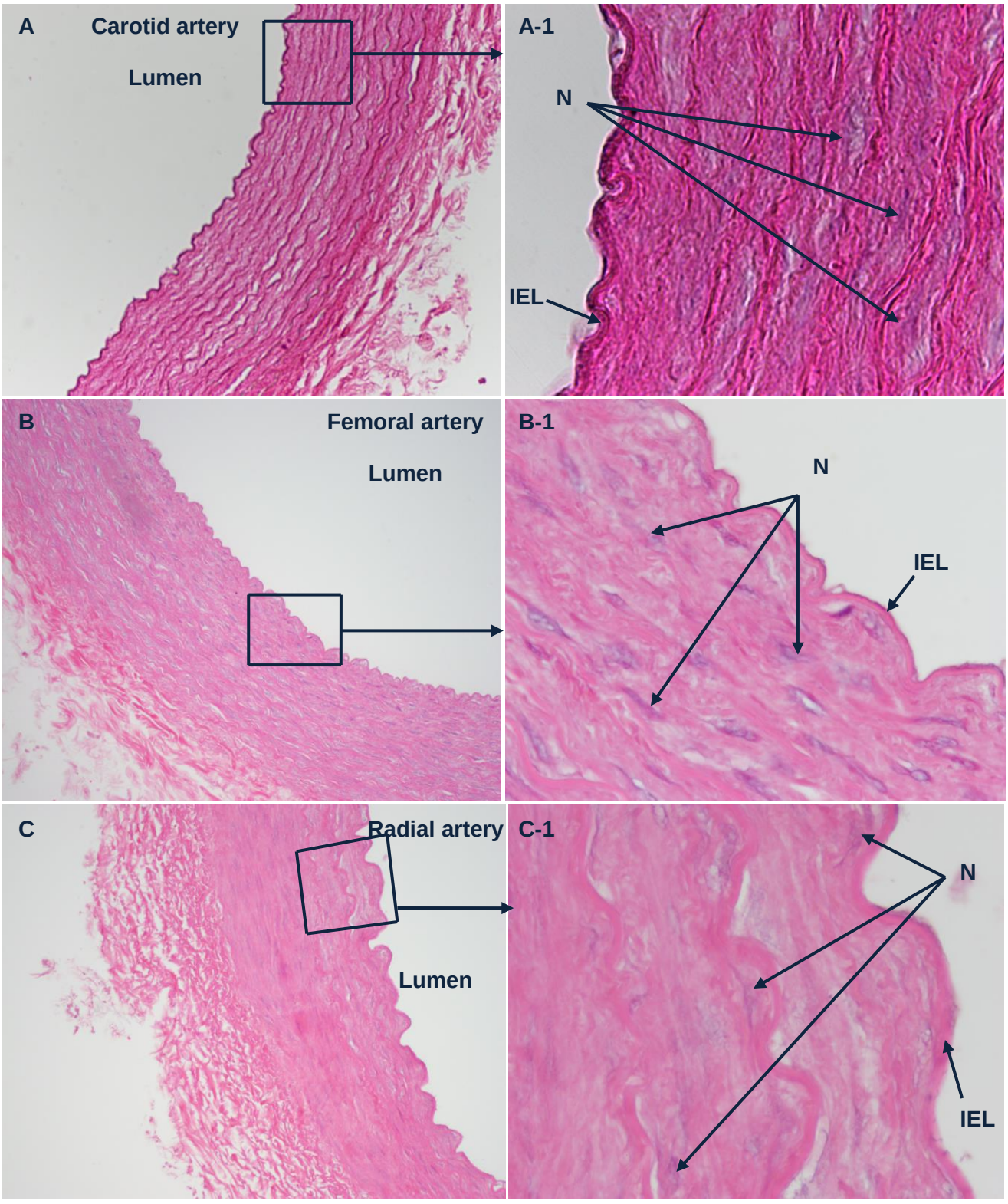

Figure 5-5: Histology of decellularized arteries stained with H&E stain. Images (A, B, C) at 10x magnification, and inserts (A-1, B-1 and C-1) at 50x magnification. The presence of nuclear (N) materials is indicated by a purple stain in all the images. A decellularized carotid in (A) and radial artery in (C) showed very little nuclear remnants present. Decellularized femoral arteries in (B), showed more nuclear material present within the ECM scaffold. All decellularized arteries (A, B and C) showed an apparent internal elastic lamina (IEL) that stained dark pink. The endothelial layer on the luminal side is missing.

The matrix of all the decellularized arteries was still preserved as indicated by the presence of collagen and elastic fibres stained by MT and VVG stains respectively in **figure 5-6**. Histological differences in tissue composition and density could be observed in the different decellularized carotid, femoral and radial arteries. The femoral artery (**figure 5-6: B**) showed a thicker tunica media, with dense elastic fibre distribution. The carotid artery (**figure 5-6: A**) showed a thinner media with many elastic fibres, but not as compact as the femoral artery. The radial artery on the other hand (**figure 5-6: C**) showed a tunica media with less elastic fibres that are loosely distributed. All arteries in **figure 5-6: D-F**, showed well preserved collagen fibres within the matrix after decellularization.

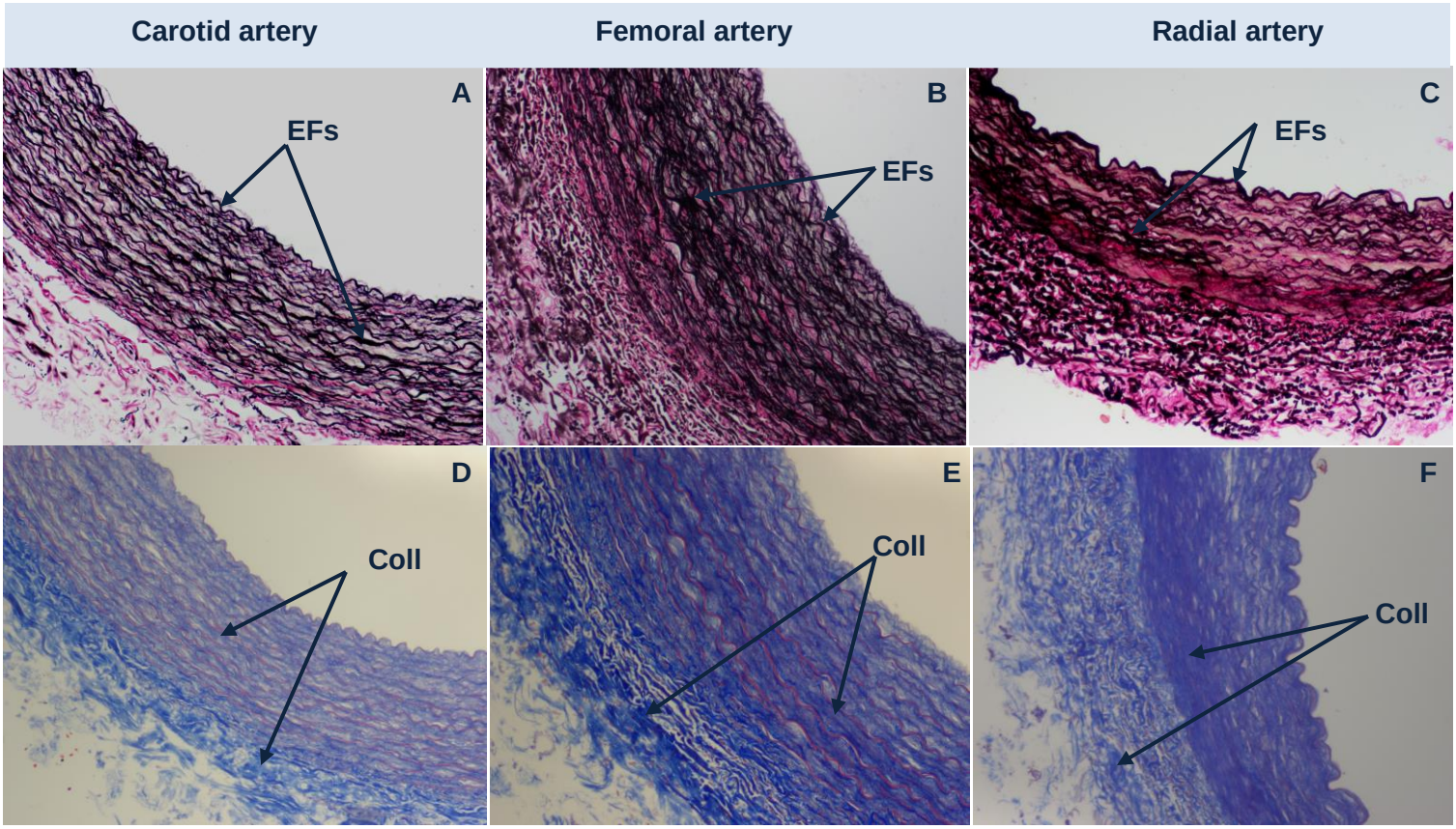

Figure 5-6: Histology of decellularized arteries after staining with VVG and MT stains respectively. Images A – C shows the presence of elastic fibres (EFs) stained black by VVG stain. Images D – F show the presence of collagen fibres (Coll) stained blue by MT stain. All images (A – F) were taken at 10x magnification.

5.1.2. Transmission Electron Microscopy (TEM) analysis

Transmission electron microscopic (TEM) examination of a normal baboon artery revealed the presence of the endothelial lining (red arrows in **figure 5-7: A**) with an intact basement membrane (blue arrows). In a decellularized artery (**figure 5-7: B**), the endothelial monolayer was clearly absent. The remaining cellular debris and cellular components of ECs and SMCs could be observed in a decellularized artery. This is indicated by the purple and orange arrows respectively in **figure 5-7: B**. TEM examination also showed an intact basement membrane on all the decellularized arteries as indicated by the blue arrows in **figure 5-7: B**.

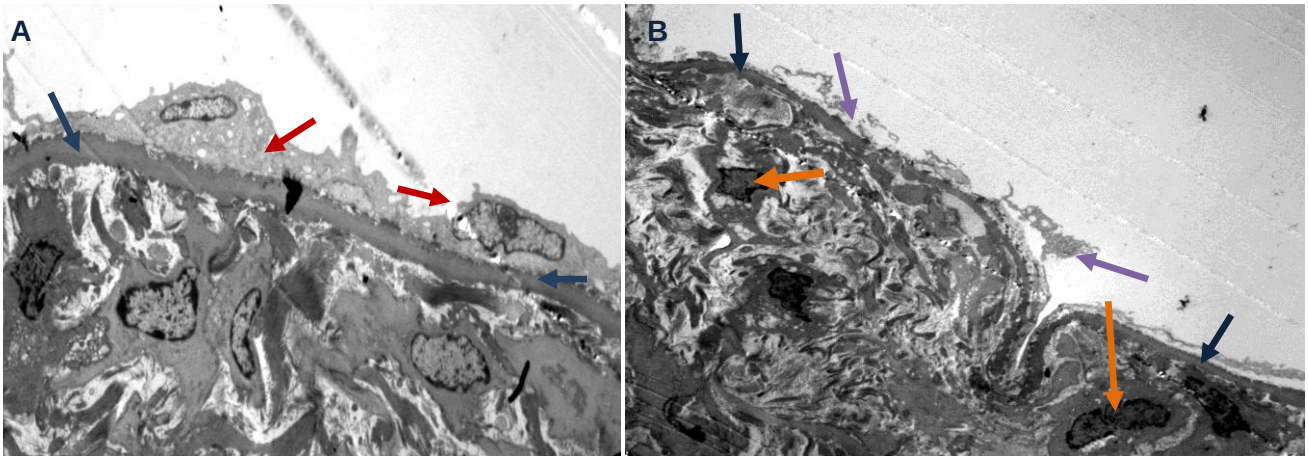

Figure 5-7: TEM images of a normal artery (A) and a decellularized artery (B). Both A and B were taken at 1,250x magnification. (A) shows an endothelial monolayer of a normal blood vessel indicated by the red arrows, with an intact basement membrane indicated by the blue arrows. (B) shows the missing endothelial layer of a decellularized artery. Notice the cellular debris observed on the intimal surface (indicated by the purple arrows) and ruptured remaining cellular material (indicated by the orange arrows) throughout the matrix scaffold. The intact basement membrane could also be observed as indicated by the blue arrows on the TEM of a decellularized artery.

5.1.3. Scanning Electron Microscopy (SEM) analysis

Morphological differences of an artery before and after decellularization were also detected by SEM images (**figure 5-8: A and B**). The normal artery showed a smooth surface, indicating the presence of the EC on the luminal surface. On the other hand, a decellularized artery had a rough surface showing the exposed collagen fibres. This proves the absence of an endothelial lining.

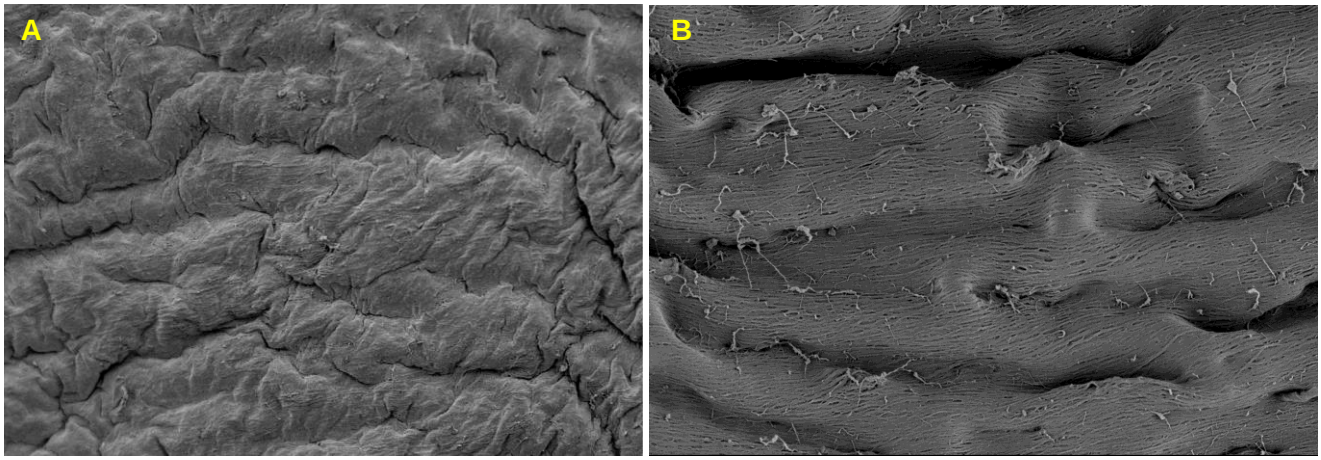

Figure 5-8: SEM images of a normal baboon artery (A) and a decellularized baboon artery (B). A normal artery shows a smooth luminal surface, indicating the presence of ECs, whereas a decellularized artery shows a rough surface indicating the missing endothelial lining. (Images at 1,000x magnification).

5.1.4. *Scaffold treatment and -sterilization*

Bacterial contamination was observed on the decellularized arterial grafts after SEM examination (**figure 5-9: A and B**). An aerobic gram negative bacterium (*stenotrophomonas maltophilia*) was identified by microbiology studies on small pieces (0.5 cm) of the graft. Bactrim antibacterial solution (Bactrim® IV Co-trimoxazole (480 mg/5 ml), Roche products (Pty) Ltd/Edms, Gauteng, South Africa) was indicated as a drug of choice for treating the contaminated arteries by the research laboratory in the Department of Medical Microbiology at the University of the Free State. The contaminated decellularized arteries were incubated for 3 days in 1.6 ml of Bactrim (480 mg/5 ml) solution diluted in sterile 5 ml PBS. A new bactrim solution was prepared each day. After the decontamination process, SEM examination revealed the absence of bacteria on the arterial segments (**figure 5-10: A** and **B**).

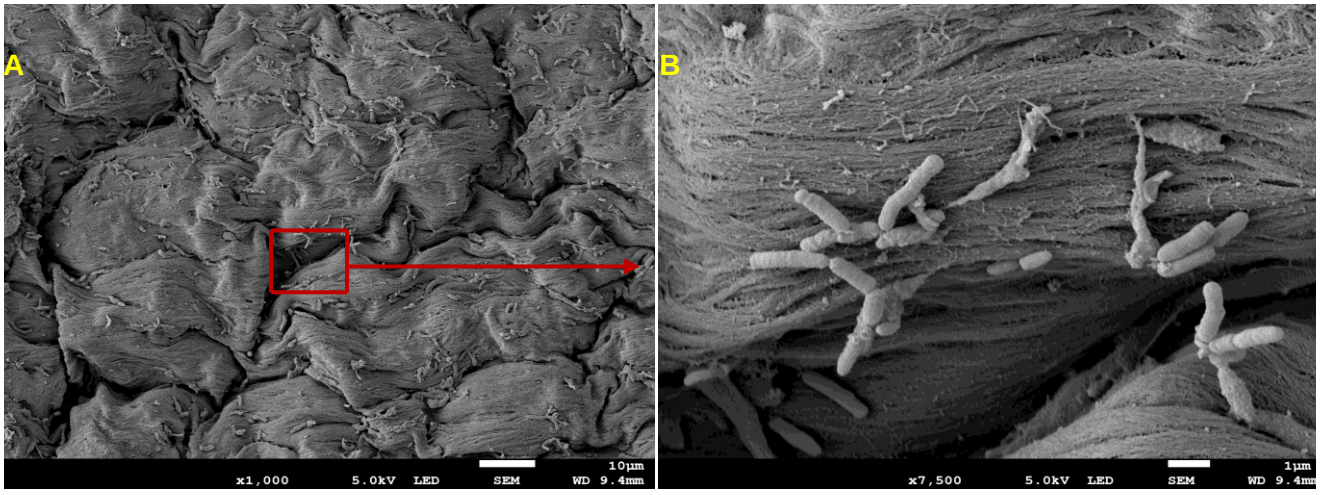

Figure 5-9: SEM images of a bacteria contaminated decellularized artery. SEM showed the presence of a contaminating bacillus bacteria (A at 1,000x magnification and B at x7,500 magnification).

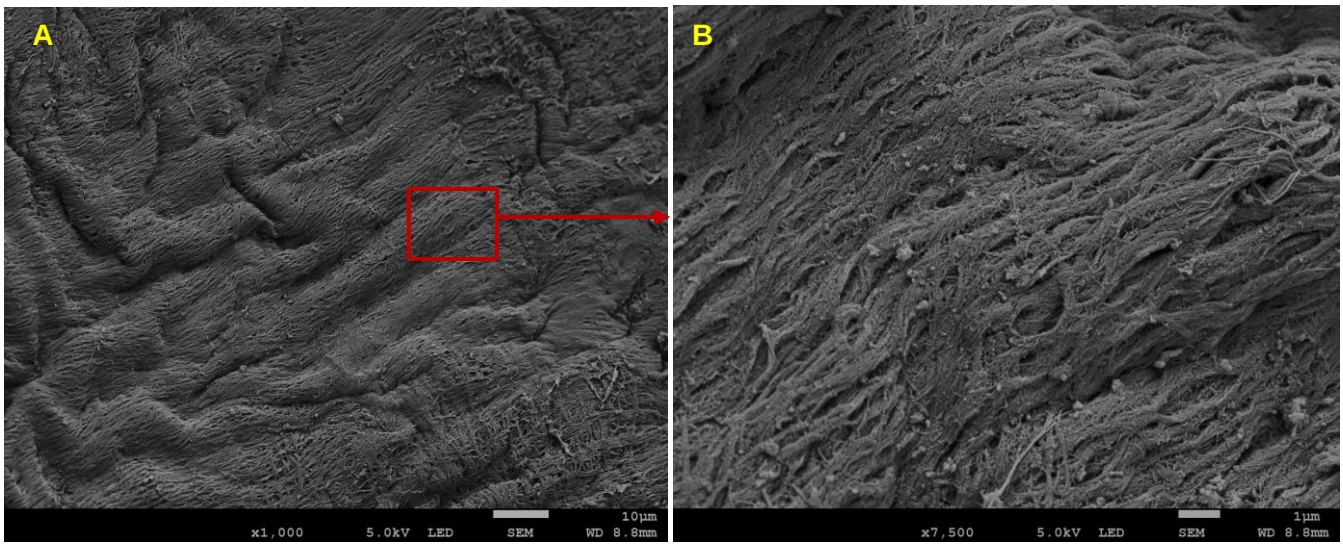

Figure 5-10: SEM images of a decellularized artery after decontamination with Bactrim antibiotic treatment. SEM showed the absence of bacteria on the luminal surface of the arterial segments (A at 1,000x magnification and B at 7,500x magnification).

5.2. HUVECs culture

5.2.1. Morphology and growth of ECs

Human umbilical vein endothelial cells (HUVECs) showed the typical cobblestone morphology of ECs in growing cultures (not shown).

Cells were passaged only 2 times and no contamination was observed during cell culture work. The ECs reached 70 – 80% confluency within 5 – 6 days, with a cell seeding density of 2, 5 x 10^3 cells/cm^2 after the second passage.

Figure 5-11 shows total cell count, live cell count and dead cell count from the primary culture before passage, after the first passage and after the second passage. The total and live cell count increased with each passage. **Figure 5-12** shows the percentage viability of the HUVEC cells after each passage. The percentage viability was high in the primary culture and still increased with each passage.

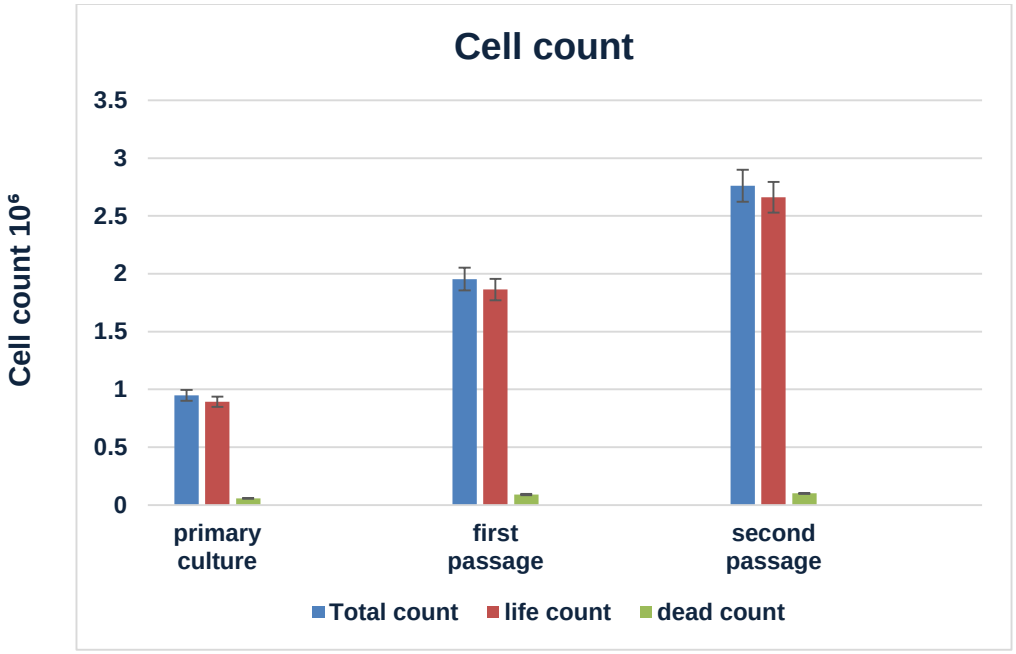

Figure 5-11: Total, live and dead cell counts of the primary culture, after the first passage and after the second passage. The total and life cell counts increased with every passage as determined using a TC10™ Automated Cell Counter. Values are expressed as mean ± 1 SD (n=2).

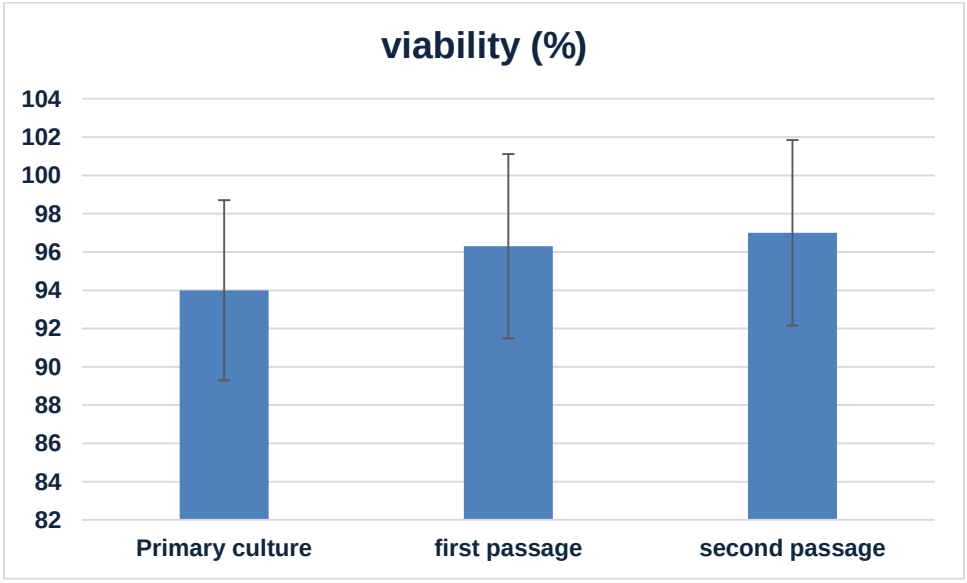

Figure 5-12: Viability counts of cells from primary culture, after first passage and second passage. As the number of cells increased, the viability of cells in culture also increased. Values are expressed as mean ± 1 SD (n=2).

5.2.2. Cell viability and proliferation rate

The cell viability increased after subculture as confirmed by the MTT viability and proliferation assay results in **figure 5-13**.

Viable cultured EC's with active metabolism converted MTT into a purple colored formazan product. Higher absorbance indicates increased cell proliferation. Longer incubation times (from 30, 45 and 60 minutes) also resulted in accumulation of color and increased sensitivity. Thus suggesting that the conversion of MTT to formazan by cells in culture is time dependent. **Figure 5-13** shows that the number of cells and the absorbance are linear related, thus as the number of cells increases, so does the absorbance.

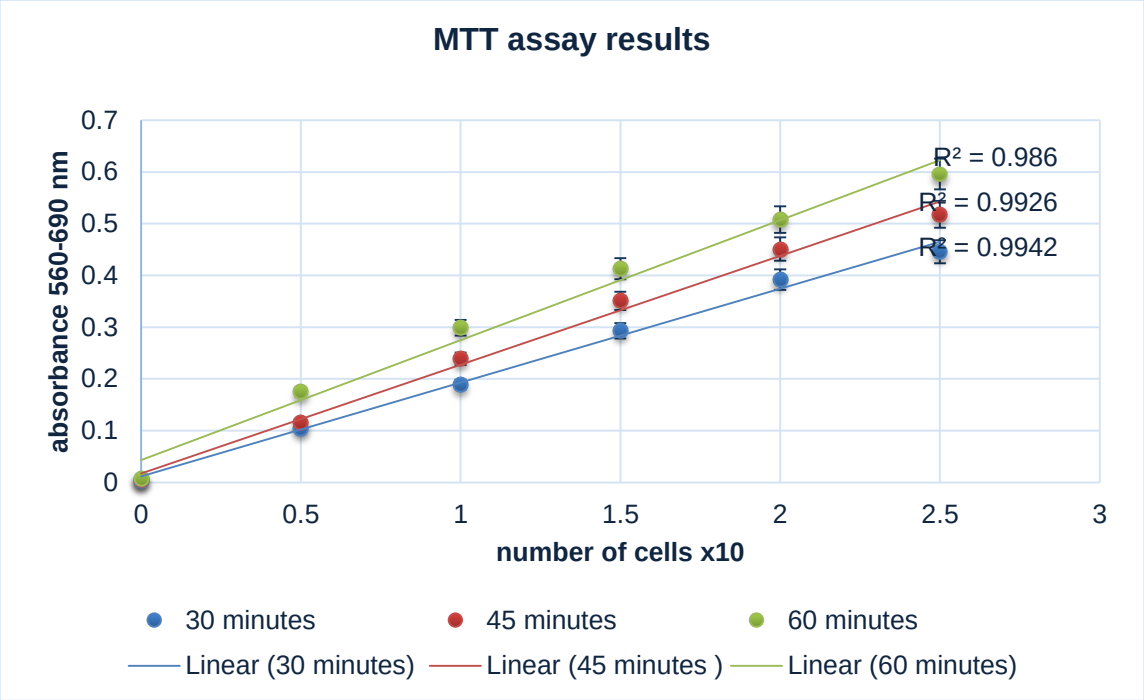

Figure 5-13: MTT assay of HUVECs in culture. The linear relationship between absorbance and the number of HUVEC showed a typical growth pattern in culture. Note the slight differences in absorbance after 30, 45 and 60 minutes.

5.3. Seeding of the decellularized baboon arteries

The decellularized arteries supported re-endothelialization as shown by scanning electron microscopy (SEM).

5.3.1. Scanning Electron Microscopy (SEM) analysis on seeded artery

Endothelial cells adhered to the decellularized artery after the seeding experiment (**figure 5-14: A**). **Figure 5-14: B** shows the proliferation and migration of the ECs on the decellularized artery after day 1 (indicated by the red arrows). The ECs had formed an almost confluent monolayer in the middle section of the arterial construct.

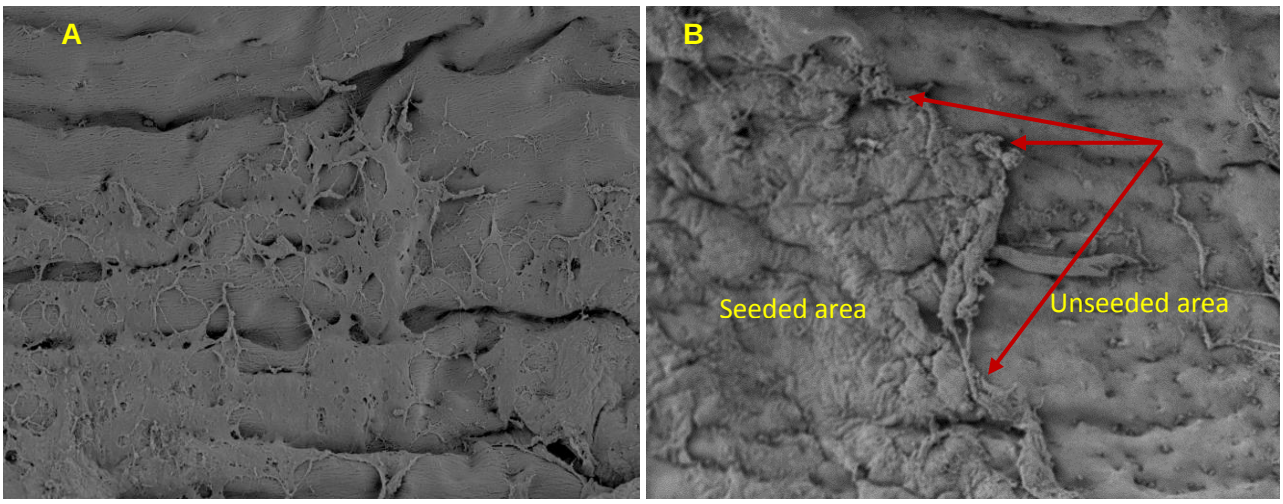

Figure 5-14: SEM images of a seeded decellularized artery. Attached ECs are visible on the scaffold after the seeding experiment as shown in image A at 1,000x magnification. After day 1, ECs started to form a confluent monolayer and appeared to be migrating towards the direction of the red arrows in image B at 450x magnification. Notice the smooth surface on the seeded area of the decellularized scaffold compared to the rough unseeded area of the artery in image B.

After 7 days of seeding, a confluent endothelial monolayer was observed on the arterial grafts (**figure 5-15: A and B**). The internal elastic lamina (IEL) separating the tunica intima and tunica media was observed through a cross sectional cut of the artery (**figure 5-15: C** and **D** as indicated by the red arrows). This clearly indicated the presence of the formed endothelial layer on the luminal surface of the arterial graft.

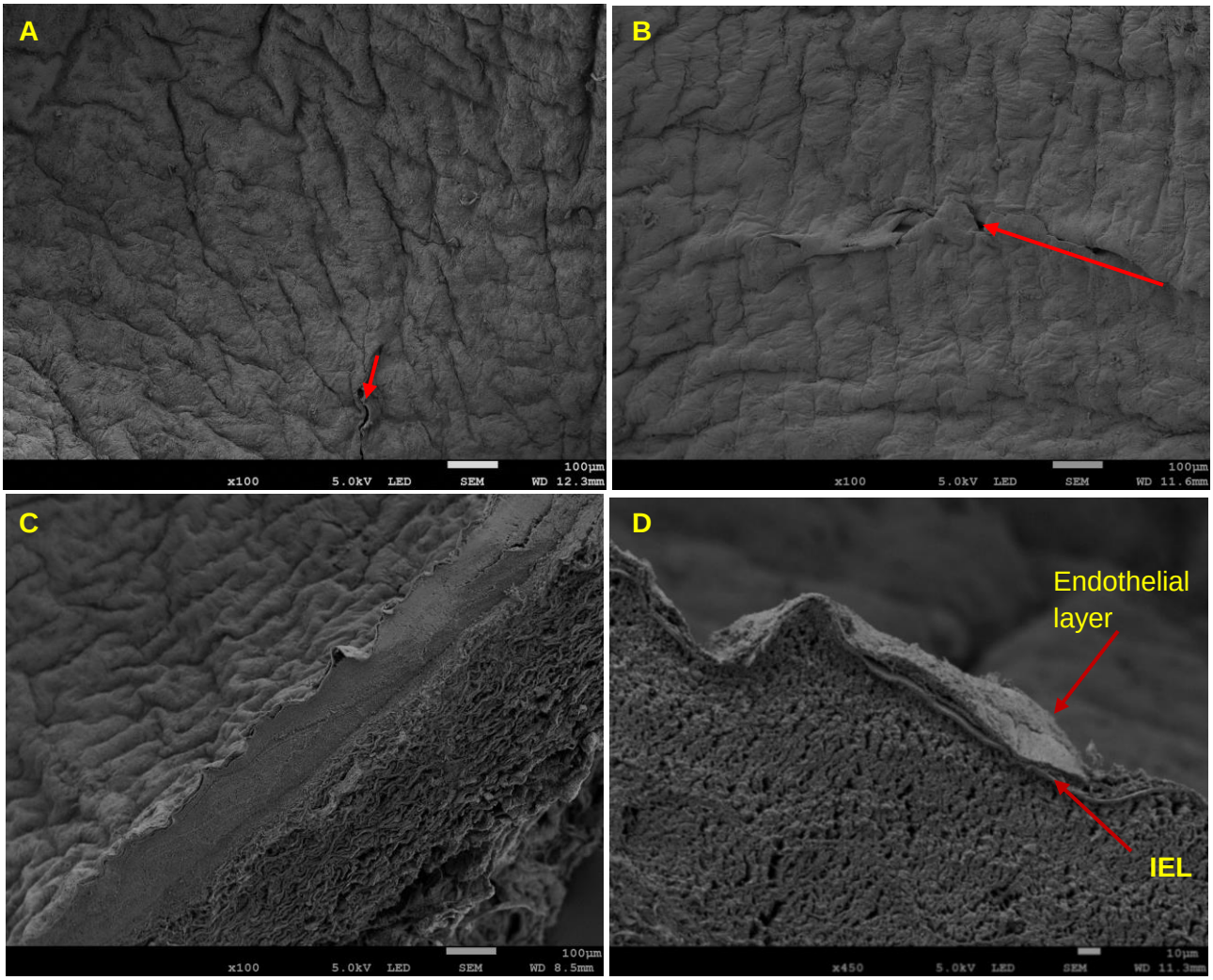

Figure 5-15: SEM of fully seeded arterial graft after 7 days of seeding. Images A and B shows that a complete endothelial layer was formed on the surface of the arterial sections (magnification 100x). Notice the smooth surface in (B) and a more rounded up rough surface in (A) of the endothelium on both images. A slight brakeage (indicated by the red arrows in A and B) of the endothelial layer occurred during sectioning. The internal elastic lamina (IEL) separating the tunica intima (TI) from the tunica media (TM) was observed from the cross sections of the arteries. This confirmed the presence of the endothelium.

Some of the arteries twisted and turned during the dissembling process of the bioreactor after seeding. This is because the bioreactor made it difficult to assemble and dissemble arteries before and after the seeding experiment. This led to the detachment of ECs at some places as shown in **figure 5-16** (indicated by the red arrows). Although parts of the vessel wall were still intact.

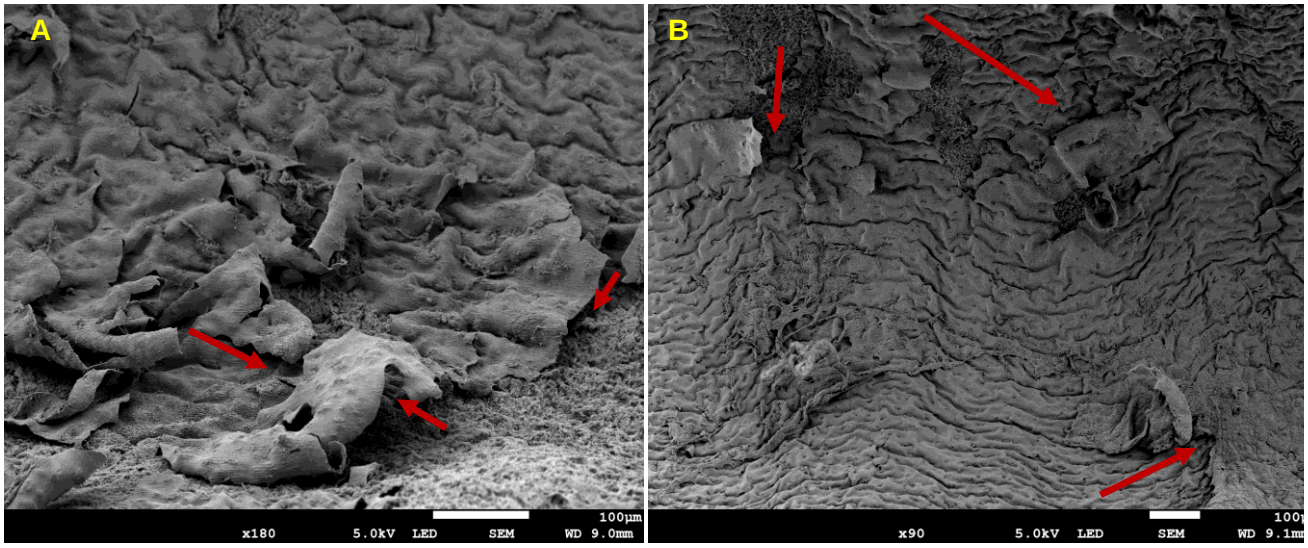

Figure 5-16: SEM revealed detached ECs from the decellularized artery. Image C taken at 180x magnification and D at 90x magnification. Both shows detachment of ECs on the on the lateral sides of the artery. This appeared due to twisting of the scaffold by accident.

Bacillus bacterial contamination was again observed on some arterial graft as indicated by the orange arrows in **figure 5-17**. This might have interrupted the attached cells from proliferating in certain surface areas on the scaffold.

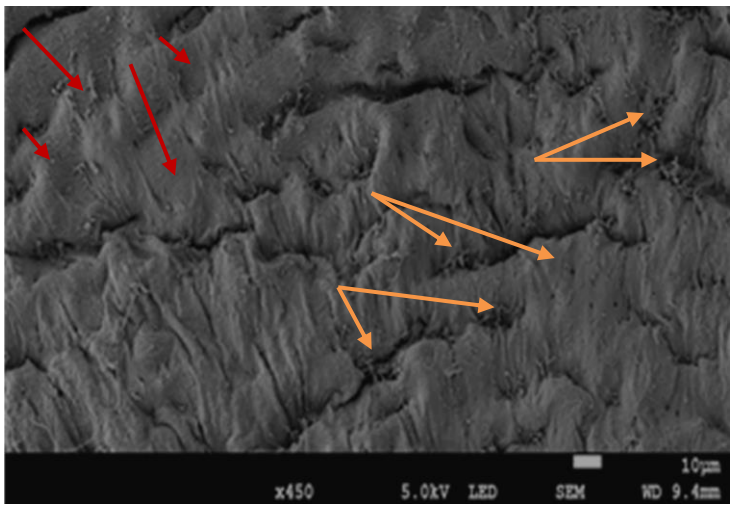

Figure 5-17: SEM image of seeded decellularized artery (at x450 magnification). Bacterial contamination was again observed on some arterial scaffolds, indicated by the orange arrows, interrupting endothelialization in those areas. Again notice the direction of the migrating ECs on the surface indicated by the red arrows.

5.3.2. MTT assay of seeded endothelial cells

Seeded HUVECs showed viability and proliferation after detachment of the arterial scaffold and 24 hours in culture as demonstrated by the MTT assay in **figure 5-18**. Again, longer incubation times (from 30, 45 and 60 seconds) also resulted in increased sensitivity. Furthermore, as a linear relationship between the number of cells increased, so did absorbance as in **figure 5-13**.

Slight differences in absorbance after 30, 45 and 60 minutes was also observed.

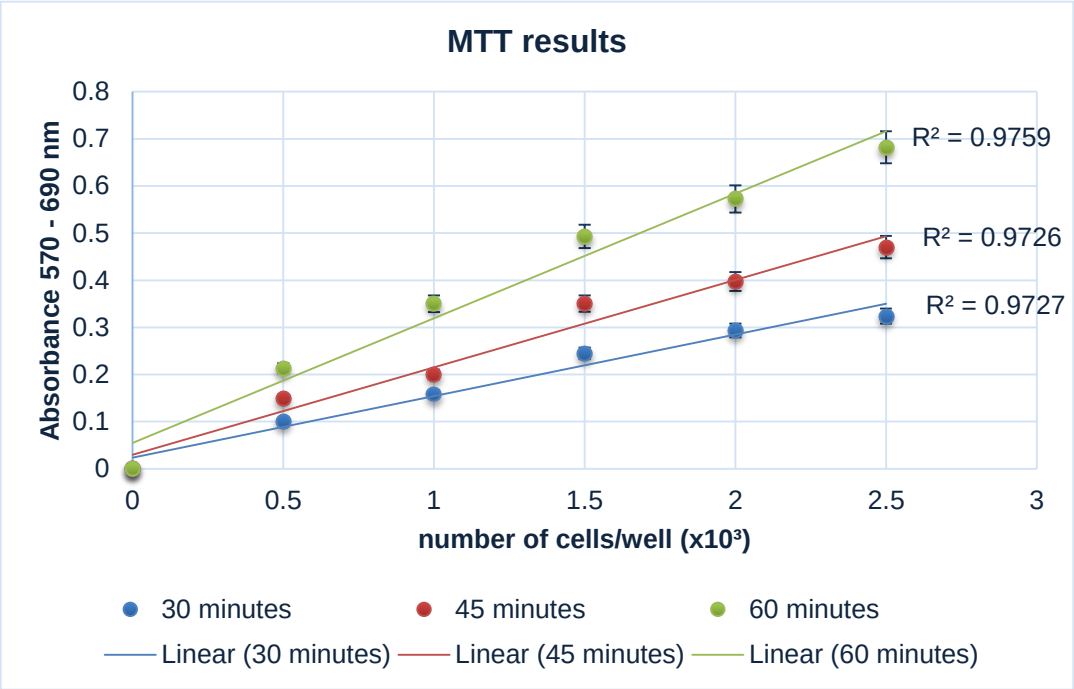

Figure 5-18: MTT assay of seeded endothelial cells. A linear relationship between the absorbance and the number of cells shows a typical ECs growth pattern after detachment from the arterial grafts. Note the slight differences in absorbance after 30, 45 and 60 minutes as expected in the MT assay.

5.4. Perfusion studies

Scanning electron microscopy

Figure 5-19 shows SEM of a decellularized artery (**A**), a normal artery (**B**), and a seeded decellularized artery (**C**). The normal artery and the seeded decellularized arteries showed no possible thrombus formation on their luminal surfaces. However, some areas of the seeded and normal arteries showed few isolated spots of platelets adhesion. This might be due to possible damage to the endothelial layer in those areas. The decellularized arteries on the other hand showed more platelet adhesion and activation on the surface of the ECM after perfusion with whole blood. Thus a decellularized scaffold does promote thrombosis.

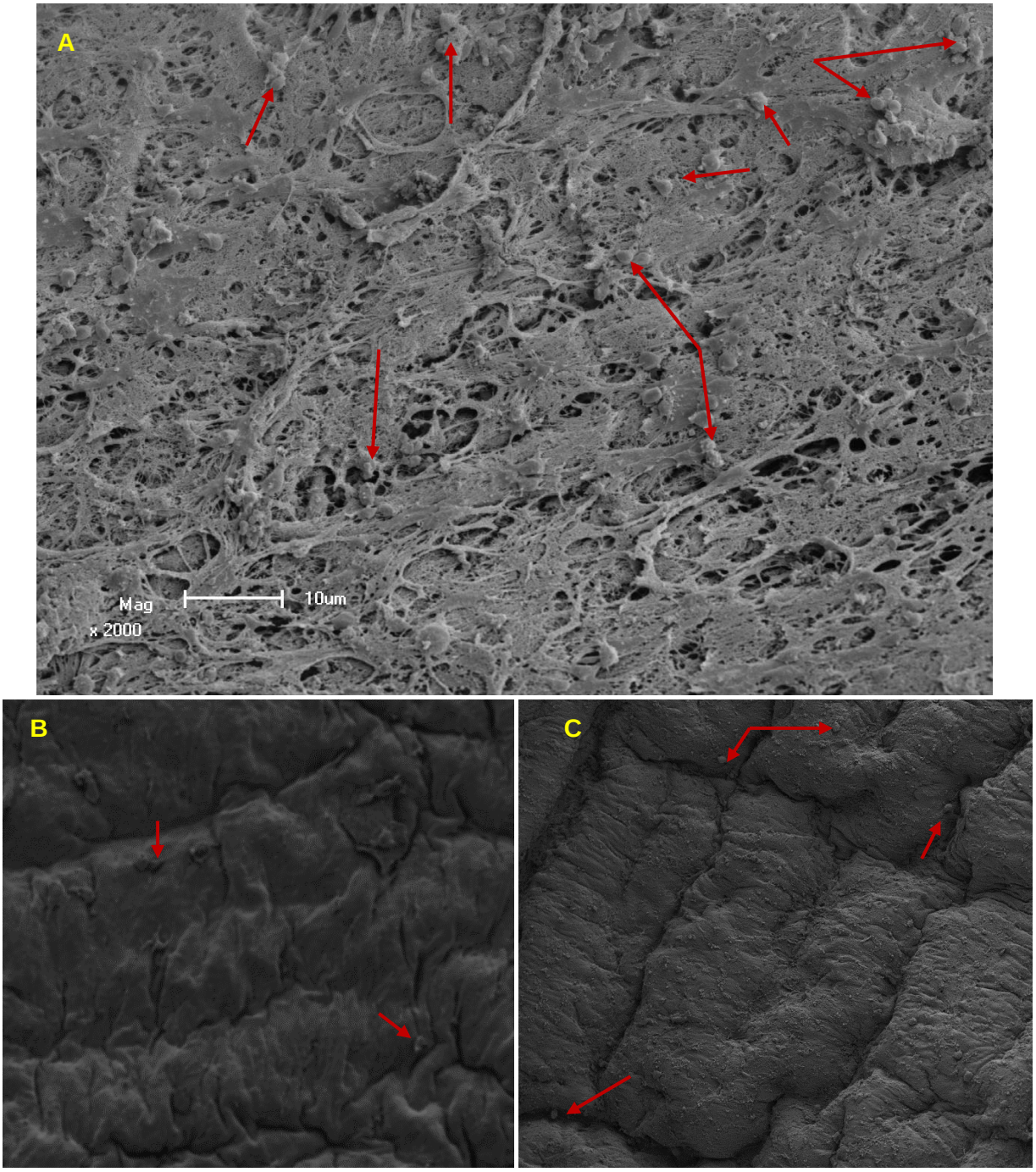

Figure 5-19: SEM results of decellularized, normal and seeded arteries after the perfusion experiments with whole blood. Image A (at 2,000x magnification) shows a decellularized with platelet adhesion and activation on ECM surface shown by the red arrows. Image B and C (at 450x magnification) shows a normal artery and seeded decellularized artery respectively. No thrombosis (platelet adhesion/activation) could be observed on the surfaces of the arteries, except for few areas with very little isolated adhered platelets (indicated by red arrows).

6. Discussion and Limitations

The use of decellularized biologic scaffolds for vascular graft construction has been the main focus of vascular engineering in the last few years. Decellularized arteries are histologically similar to native arteries in shape, size and strength. In addition, decellularized scaffolds might be used as conduits for small diameter grafting as they provide the suitable matrix for re-endothelialization (Moroni and Mirabella 2014; L'Heureux *et al.* 1998; Murase *et al*. 2006; Conklins *et al*. 2002).

In this study, 12 arteries from the carotid, radial and femoral origins were chosen for decellularization. These arteries are known to be strongly associated with atherosclerosis and thrombosis and thus are more prone to the development of vascular diseases (Rothewell 2001; Allan *et al*. 1997; Burke *et al*. 1995). The elastic, small diameter radial artery is often used as an arterial graft for coronary artery bypass grafting (Barner *et al*. 2012; Calligaro *et al.* 1997).

The first objective of this study was to investigate the histology of the extra-cellular matrix after decellurlarization. Histological examinations are normally used to determine the efficiency of decellularization (Almelkar *et al.* 2013; Gui *et al.* 2009; Quint *et al.* 2011; Kasmir *et al.* 2006; Kadner, 2004). Standard histological staining with H&E is often used to determine whether nuclear structures of cells are still present after decellularization (Gilbert *et al.* 2006). There are however various other methods available to determine the efficiency of cell removal from tissues. These include immuno-histochemical methods to examine specific proteins, such as actin and vimentin (Woods and Gratzer 2005); DAPI (4', 6-diamidino-2-phenylindole) staining (Kapuscinski and Skoczylas 1978) or Hoechst stains (Kakkar and Grover 2005) to inspect the presence of DNA; and DNA quantification studies using assays such as PicoGreen or propidium oxide (Kral *et al*. 2005). It was however not necessary for us to perform such assays, since the scaffolds were not going to be implanted and immunological issues related to DNA and RNA content in the scaffold were thus not relevant.

An important finding was that all decellularized arteries were found to be de-endothelialized as shown by electron microscopy in **figure 5-7**. The absence of the endothelial lining was further confirmed by SEM examination. According to Schaner *et al.* 2004, complete removal of the endothelial lining is not surprising when sodium dodecyl sulphate (SDS) is used even in low concentrations in the decellularization process. However the removal of smooth muscle cells requires higher concentration of SDS, which can cause damage to the remaining ECM. Thus smooth muscle cells and nuclear material were still retained **(figure 5-5)**. Furthermore, the carotid and radial arteries showed much less nuclear material remaining after decellularization compared to the femoral arteries (**figure 5-5**). This was to be expected, since the femoral arteries contain the

most muscle cells in the tunica media when compared to the radial and carotid arteries **(figures 5-5 and 5-6)**. Literature also indicates that the muscle content of the biological structure impairs the diffusion through the material and thus complicates the decellurization process (Montoya and McFetridge, 2009). Since the objective of this study was not to re-implant the arteries, the nuclear content was not relevant.

The decellularization process did not alter the morphology of the arteries, as the content of the collagen and elastic fibres remained unchanged. This finding is important as collagen fibres provide structural support and tensile strength, while elastic fibres provide elasticity and compliance to the graft (Humphrey *et al*. 1995). In addition, collagen type IV contains ligands important for firm EC attachment and retention during re-endothelialisation (Herbst *et al*. 1988).

It is important to provide the necessary mechanical properties when designing a scaffold for tissue engineered small diameter vascular graft (Murase *et al.* 2006; Conklin *et al*. 2002). Literature also shows the importance of preserving tensile strength and elasticity during the regeneration of small diameter vascular grafts (Abott *et al*. 1987; Hasson *et al*. 1985; Walden *et al.* 1980; Weston *et al*. 1996; Gilbert *et al*. 2006). In this study, no biomechanical evaluation and morphological studies were performed on the decellularized arteries in order to examine graft compliance and tensile strength. This is because the aim of the study was not to re-implant the arteries, but only to ensure seeding on the decellularized vessels. In this respect the decellularization process was successful.

Examination of the basement membrane using TEM showed that the decellularization processes was successfull. TEM showed the presence of an intact basement membrane on all the decellularized arteries shown in **figure 5-7**. An intact basement membrane is important to allow re-endothelialization of arteries after decellularization. The basement membrane is also involved in modulating cell-matrix interactions, by supporting cell adhesion, migration and proliferation during development and regeneration (Engvall 1995). Thus a preserved ECM contributes to maintaining a non-thrombotic environment in a graft material if used in bypass surgery (Abott *et al.* 1987; Walden *et al.* 1980; Hasson *et al.* 1985; Weston *et al.* 1996).

In order to obtain complete decellularized grafts with preserved ECM integrity and bioactivity, decellularization processes needs to be optimized for baboon arteries by making educated decisions regarding the reagents and techniques used during the process (Crapo *et al*. 2012). Different reagent concentrations and time exposure need to be adjusted until effective cell removal (efficiency of the decellularization) can be obtained. Gilbert *et al.* (2006) further suggests that a combination of physical, enzymatic and chemical treatment is required in order to obtain complete decellularization of tissues. Both studies agree that an effective decellularization process is

dependent on the tissue of interest. This highlights the complexity of decellularization protocols suitable for each specific tissue of interest in order to produce a cell free biological scaffold. Hence the Department of Cardiothoracic Surgery is still trying to develop an effective decellularization protocol that can efficiently be applied to different arteries.

Obtaining a cell free scaffold material after decellularization processes is an important component in TEVGs. The remaining matrix is considered a bioinert material that does not elicit immunogenic response when implanted (Conklins *et al.* 2002). In addition, complete cell removal eliminates antigenicity to limit any adverse biological reactions by the host. Although the experimental arteries still contained nuclear remnants, the remaining ECM was preserved with an intact basement membrane without an endothelial layer.

The primary objective of the study was to determine whether re-endothelialization of decellularized arteries protects the graft against thrombosis in an *in vitro* flow model, and not the immunological responses to implanted decellularized tissue containing nuclear debris. As the luminal surface of the arterial grafts was completely de-endothelialized and only nuclear debris remained in the arteries, re-endothelialization could go ahead, followed by flow studies.

The concept of repopulating biological scaffolds *in vivo* with endothelial and interstitial cells has been proposed by some workers (Da Costa 2004; Goldstein *et al.* 2000 and O'Brian *et al.* 1999). Although this concept has been successful in decellularized pulmonary and aorta homografts, smaller decellularized conduits developed thrombosis. Hence *in vitro* seeding techniques using cultured autologous cells are required to endothelialize smaller vascular constructs before implantation.

A two-stage seeding technique using HUVECs was performed to successfully re-endothelialize the de-endothelialized baboon arteries. Human Umbilical Vein Endothelial Cells (HUVECs) are used in literature to obtain ECs for seeding vascular grafts surfaces (Sgarioto *et al.* 2012; Dahan *et al.* 2011; Gui *et al.* 2009; Kasimir *et al.* 2006; Kasimir *et al.* 2005). There are other various sources from which ECs can be obtained from, such as to harvested endothelial progenitor cells obtained from peripheral blood (Boyer *et al*. 2000) and bone marrow (Cho *et al*. 2005; Kusuma and Gerecht 2010). Endothelial cells can also be isolated from umbilical cord vein by surgical procedure (Schmidt *et al*. 2005). However, HUVECs are easily accessible as they can be purchased from many available manufacturing companies. They can also be easily cultured under optimal conditions following the manufacturer's instructions. Validation and verification studies are also not necessary to perform as these studies are already performed on the cells. Another

advantage of using HUVECs is the growth potential of these cells when cultured under optimal conditions (Badylak *et al*. 2011).

The manufacturing company already passaged the purchased HUVECs once. These cells can only be passaged 3 times for the purpose of our study, because endothelial cells undergo a process of senescence (biological aging) and stop dividing when passaged more than 3 times due to contact inhibition (Almelkar *et al.* 2013). The growth rate, biological responsiveness and function of the cells also deteriorate with subsequent passages (Clonetics™, Lonza Walkersville, Inc., Maryland, USA). Thus these cells were only passage 2 times in our cell culture laboratory before seeding.

To optimize our cell culturing, the morphology of the cells was assessed for homogenous cobblestone morphology present throughout the culture. The EC cultures showed excellent viability and proliferation capabilities (**figure 5-12** and **figure 5-13**). Furthermore, the cell counts increase with every subculture indicated that more cells were growing than dying (**figure 5-11**). Thus we could obtain enough viable ECs to seed the luminal surfaces of three decellularized baboon arteries. The MTT assay also served as an important tool to optimize cell culture conditions, as it is an indicator of active metabolism. Although there are other viability assays available, the MTT assay can be used on different cell types including HUVECs (Riss *et al.* 2013).

An arterial construct can only be considered suitable for use as a biocompatible vascular graft if it is able to support endothelialization, since a confluent functional endothelial layer prevents thrombosis on the graft (Gui *et al.* 2009; Kasmir *et al.* 2005). We successfully seeded the decellularized arterial grafts with HUVECs in this study as shown by SEM. The seeded grafts exhibited endothelialization along their entire length from day 1 (**figure 5-14**), and a confluent monolayer of ECs was observed after 7 days of seeding (**figure 5-15**). Although a slight breakage of the endothelial layer occurred due to twisting during sectioning, a confluent monolayer could still be observed. This highlights the importance of precision during TEVGs.

The use of a bioreactor for cell seeding played a crucial part in re-endothelelialization of vascular constructs. According to Barron *et al*. (2003), a bioreactor provides the physiological environment with conditions necessary for functional tissue development. Bioreactors also promote the development of tissue-engineered constructs with better mechanical properties and morphological characteristics when compared to those created in static culture medium. There are different types of bioreactors currently in use for tissue engineering of vascular grafts (Barron *et al.* 2003). We used a static bioreactor system due to its availability. A static bioreactor allows efficient tissue growth by providing effective gas exchange and mass transfer at the surface of the tissue

construct. A continuous supply of oxygen, temperature, pH, humidity, nutrients and osmotic pressure can also be maintained. Furthermore, rotation of the bioreactor by an endostrabilisator enhanced homogenous cell distribution.

The last objective of this study was to determine if our re-endothelialized, decellularized biological scaffolds could reduce thrombogenecity when compared to the decellularized ones. It has been reported that decellularized constructs promote thrombosis formation due to the absence of an endothelial lining responsible for maintaining an antithrombotic surface. Our study supports this finding as unseeded decellularized arteries showed widespread platelet activation during the perfusion experiment with baboon blood (**figure 5-19**). The seeded arterial grafts and the normal artery on the other hand showed no thrombus formation. However, certain areas of the normal and seeded arteries showed few isolated platelets adhered to the surface. This might have been due to damaged ECs, exposing collagen fibres in those areas. This finding highlights the importance of maintaining an intact endothelial cell lining during tissue engineering. A study by Kasmir *et al.* in 2005 also showed the same results, with platelet adhesion on areas where the EC lining that was not intact. In addition, it is known that the exposed collagen in vascular injury leads to immediate activation of platelets through the activation of the coagulation pathway to seal the wound. It is therefore not surprising that the exposed fibrous structures of decellularized arteries would promote platelet activation.

Our data with the perfusion experiment supports the findings that endothelialization of decellularized vascular grafts provides a thrombus-free graft surface. Furthermore the perfusion did not damage the seeded endothelial cells. This is an important finding since endothelialization of vascular grafts is limited by the inability of endothelial cells to remain attached to the scaffold after exposure to flow (Sgarioto *et al*. 2012).

Bacterial contamination on biological substrates is a major concern in the development of TEVG's. To avoid potential bacterial contamination on the scaffold materials, the use of antibiotic cocktail solutions were included in every step of the experiment. However, bacterial contamination on the graft surfaces was constantly observed using SEM. **Figure 5-9** showed bacterial contamination already after decellularization. It is however not clear whether bacterial contamination occurred before, during or after the decellularization process. It has been reported that long processes of chemical decellularization methods can cause bacterial contamination on the remaining matrix (Gilbert *et al.* 2006). This might have been the case as chemicals were used to decellularize arteries for this study.

After we treated the scaffold matrix with antibacterial solution recommended by the Department of Microbiology, SEM confirmed the absence of bacteria. However, after the seeding experiment, SEM again revealed traces of the same bacterial contamination on the luminal surface of the graft as seen in **figure 5-17**. This resulted in an incomplete monolayer formation with ECs not being able to proliferate in certain areas. According to Mirzaie *et al.* (2007), even high concentrations of antibiotics cannot completely eradicate bacterial contamination. Thus the incidence of bacterial contamination increases with every exposure to any contaminating environment. Thus, this also highlighted the importance of appropriate facilities (aseptic working conditions and trained personnel) needed for this type of study as there was limited specialized laboratory space available.

The decision to use baboon arteries in our study was based on the increased need for use of animal models that can translate basic research into clinical therapy. Non-human primates are thought to be important for optimizing therapies for human clinical studies (Shi *et al*. 2012). Primate models also possess similar hemostatic characteristics to humans (Shepard *et al*. 1984; Shepard *et al*. 1986). Their coagulation system and platelet behavior closely resembles that of humans, where other animal species such as dogs, sheep and pigs do not. Their vascular EC growth characteristics are also thought to be similar to that of humans. They share about 98% homology to human genes, possess similar protein structures to humans and reflect the anatomical, physiological and behavioral makeup of humans (Shepard *et al*. 1984; Shepard *et al.* 1986). This is also the first study where human endothelial cells were seeded successfully on baboon arteries.

However primate models are not readily available for experimental studies due to ethical concerns surrounding them. An advantage for us was their availability as another study in our Department was already using baboons for other experimental studies. Thus we could easily access them and collect arteries of interest for our study after being euthanized. A limiting factor to our study was that we were not able to use more animals from which we could collect blood vessels.

This study was the first to make use of the bioengineering process of decellularized small calibre biologic grafts in our institution.

7. Conclusion

Tissue engineering of blood vessels is a promising concept to generate improved blood vessels to substitute diseased and damaged native blood vessels. In this study we showed that decellularization did not alter the morphology of the arteries, as the content of collagen and elastic fibres remained unchanged. The thickness of an artery (amount of smooth muscle cells) however plays an important role in the decellularization process, since the carotid and radial arteries showed much less nuclear material remaining compared to the thicker femoral arteries. The presence of an intact basement membrane on all the decellularized arteries, which is important to allow re-endothelialization of arteries after decellularization, was gratifying.

Although nuclear debris was still present after decellularization, this study showed that arteries, even when not completely decellularized, can still support re-endothelialization with human umbilical vein endothelial cells (HUVECs). This is the first study where human endothelial cells were seeded on baboon scaffold arteries.

Furthermore, we showed that endothelialization does prevent thrombus formation on all arterial graft surfaces after perfusion with whole blood at high shear rate. We also showed that decellularized arteries attract platelet adhesion and activation after perfusion.

An important finding is that the two-hour perfusion process did not damage the seeded endothelial cells. This is thus one of a few studies to show a successful seeding outcome.

This study was also the first to use make use of the bioengineering process of decellularized small calibre biologic grafts in our institution.

8. Future studies

The decellularization and sterilization process developed by the Department of Cardiothoracic Surgery, which is successful in other tissues and animal models, needs to be refined and adapted for the different arteries in the baboon model. An ideal process should also provide for a sterilization process that effectively prevents infection of the TEVG, the Achilles heel of decellularization processes.

Different animal species might be explored in order to obtain the different size arteries for use in humans. These grafts could be used as an option for patients without autologous tissues or those who are not candidates for synthetic grafts.

Harvesting vascular ECs from other sources, such as endothelial progenitor cells from peripheral blood of the recipient or stem cells could also be explored for potential use in endothelialization of vascular grafts. This would eliminate the problem of rejection by the patient.

Morphometric quantification of ECs can be included for post-seeding cultivation and for shearing in order to determine the continual proliferation of the seeded cells. The use of a better perfusion system that allow shearing of *in-vitro* lined endothelium at physiological forces could also be used as these systems have already been developed.

Tissue engineering work requires specialized equipment and trained personnel. Highly specialized laboratory settings with specific working stations are very important as this reduces the chances of cross contamination and bacterial infection.

9. Abstract

The use of decellularized biological scaffolds for the reconstruction of small-diameter (< 6 mm) vascular grafts has been the focus of tissue engineering studies. These biological materials constructed through decellularization processes are thought to be ideal graft materials for replacement of diseased vessels. However thrombogenecity is a major cause of obstruction in these vascular constructs and result in early graft occlusion. Seeding of the decellularized vascular constructs with endothelial cells (ECs) is an attractive proposition as the endothelial layer incorporates many of the anti-thrombogenic properties of blood vessels.

The aim of this study was to determine if we could successfully re-endothelialize decellularized baboon arteries, thereafter perfuse the newly engineered arteries with whole blood to investigate the possibility of thrombus formation.

First, the histology of the decellularized baboon arteries were compared to normal arteries in order to assess the efficiency of the decellularization process. Collagen and elastin fibres were also stained to determine whether the remaining extracellular matrix scaffold was preserved after decellularization. Human umbilical vein endothelial cells (HUVECs) were then cultured under optimal conditions. The viability and proliferation rate of the cultured ECs were assessed using the MTT cell viability and proliferation assay. The cultured endothelial cells were then used to seed the luminal surfaces of decellularized baboon arteries. The confluent endothelial monolayer of the seeded decellularized arteries were then assessed using scanning electron microscopy (SEM) after 7 days. The seeded cells were then detached from the graft surfaces of small sections and tested for viability (metabolic activity and proliferation) using the MTT assay again. Afterwards, normal, decellularized and seeded decellularized arteries were perfused for 2 hours with baboon whole blood collected in 3.2% sodium citrate tubes. Thereafter, small artery sections were examined with SEM to determine whether thrombi was formed on the luminal surfaces of all arteries.

Histology examinations showed that the decellularized arteries were not completely cell free. Nuclear and cellular remnants were still retained within the scaffold materials. Histology also revealed that the femoral arteries had retained more nuclear and cellular materials than the carotid and radial arteries which showed much less nuclear material remaining. However, the ECM of the decellularized arteries was preserved after the decllularization processes.

Enough viable ECs were obtained in culture to seed three decellularized baboon arteries. After 7 days post seeding, a confluent endothelial monolayer was observed on the luminal surfaces of the decellularized scaffolds using SEM.

The perfused normal artery and the seeded decellularized arteries showed no possible thrombus formation on their luminal surfaces. The decellularized arteries however showed wide-spread platelet adhesion and activation on the surface of the ECM.

In conclusion, decellularization of arteries was not successfully and proved to be dependent on the thickness of the tissues used. However, the decellularization process produced morphologically preserved extracellular matrix. The endothelialization process was successful since the endothelialization of decellularized vascular grafts does prevent thrombus formation on artery surfaces after perfusion with whole blood, while a decellularized scaffold does promote thrombus formation.

Key words: Decellularization, tissue engineering, extracellular matrix, re-endothelialisation, seeding, thrombogenecity.

10. Abstrak

Die gebruik van gedeselluleerde biologiese stutte om klein-deursnee (<6mm) vaskulêre oorplantings op te bou, is die hooffokus van weefsel-ingenieurswese. Hierdie biologiese materiale wat deur desellulering opgebou word, word as die ideale oorplantingsmateriale beskou om beskadigde weefsels te vervang. Maar trombusvorming is ‘n groot oorsaak van die verstopping van hierdie vaskulêre stutte. Deur die gedeselluleerde vaskulêre stut met endoteelselle te besaai, is ‘n aantreklike opsie omdat die endoteellaag teen-trombogeniese eienskappe van bloedvate bevat.

Die doel van hierdie studie was om vas te stel of ons gedeselluleerde bobbejaanarteries weer kan her-endoteliseer. Daarna het ons die nuwe opgeboude arteries met heelbloed geperfuseer om the bepaal of trombi daarop vorm.

Eers het ons die histologie van die gedeselluleerde bobbejaanarteries vergelyk met dié van normale arteries om vas te stel of die deselluleringsproses suksesvol vas. Ons het kollageen- en elastienvesels ook gekleur om te bepaal of die ekstrasellulêre matriks stut behoue gebly het na die deselluleringsproses. Menslike naelstring endoteelselle (HUVEC) is onder optimale kondisies gekultuur. Die oorlewings- en vermeerderingstempo van die selle in kutuur is bepaal met die MTT-seloorlewingstoets. Die kultuurselle is dan gebruik om die luminale oppervlaktes van die gedeselluleerde bobbejaanarteries te besaai. Die aaneenlopende endoteel enkellaag wat op die besaaide gedeselluleerde arteries gevorm het, was dan bestudeer deur skandeerelektronmikroskopie (SEM) na 7 dae. Die besaaide selle is dan van die stut los gemaak en getoets vir oorlewing en vermeerdering deur weereens die MTT-toets te gebruik. Daarna is ‘n normale, gedeselluleerde en ‘n besaaide arterie met bobbejaan-heelbloed in 3.2% natriumsitraat vir 2 ure lank geperfuseer. Klein deeltjies van die arterie is dan d.m.v. SEM ondersoek om te bepaal of trombi op die luminale oppervlaktes gevorm het.

Histologiese ondersoeke het aangetoon dat die gedeselluleerde arteries nie heeltemal van selle bevry was nie. Kerne en seloorblyfles was nog in die stutte sigbaar. Hierdie ondersoeke het ook aangedui dat die femorale arteries heelwat meer kerne en seloorblyfsels bevat. Nogtans het die ekstrasellulêre matriks (ECM) van die gedeselluleerde arteries behoue gebly na die deselluleringsproses.

Genoegsaam oorlewende endoteelselle kon gekultuur word om die gedeselluleerde bobbejaanarteries te besaai. Na 7 dae van saaiing kon ons ‘n aaneenlopende monolaag van endoteelselle op die luminale oppervlakte van die gedeselluleerde stut deur middel van SEM sien.

Die geperfuseerde normale en besaaide arteries het geen trombusvorming op hulle luminale oppervlaktes getoon nie. Die gedeselluleerde arteries het egter wydverspreide plaatjie klewing en aktivering op die oppervlak van die ECM getoon.

Om af te sluit, die desellulering van arteries was nie suksesvol nie en blyk afhanklik van die dikte van weefsel te wees. Nogtans het die deselluleringsproses ‘n ECM geproduseer waarvan die morfologie behoue gebly het. Die endoteliseringsproses van die vaskulêre oorplantings het wel trombusforming op die arteriële oppervlakte verhoed na perfusie met heelbloed waar ‘n gedeselluleerde stut trombusforming aangehelp het.

Sleutelwoorde: Desellulering, weefselopbouing, ekstrasellulêre matriks, her-endotelisering, seeding, trombusvorming

11. References

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