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Role of Endothelial and Vascular Smooth Muscle Cells in the Origin, Progression and Therapy of Vascular Diseases

Subtitle: Tissue Engineering of Vascular Grafts

PhD Thesis

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ABSTRAKT

Úvod: Cévní chirurgie čelí nedostatku vhodných materiálů k bypassu. Syntetické cévní protézy zahrnují polyetylén tereftalát (PET) a expandovaný polytetrafluoroetylén (ePTFE). Jako cévní náhrady o malém průsvitu (< 6 mm) však podléhají trombóze díky absenci endotelové výstelky. Cílem práce bylo modifikovat PET cévních protéz adhezními povrchy tkáňového inženýrství a zlepšit jejich biokompatibilitu pro cévní hladké svalové buňky (HSB) a endotelové buňky (EB).

Metodika: Použili jsme protein krevní srážlivosti fibrin (Fb) a proteiny extracelulární matrix kolagen (Co), laminin (LM) a fibronectin (FN). Připravili jsme povrchy: Co, Co/LM, Co/FN, Co/Fb, Co/Fb/FN a provedli experimenty s buněčnými kulturami: 1. planární statické, 2. planární dynamické, simulující krevní proud, 3. tubulární dynamické a 4. implantace laboratornímu praseti.

Výsledky: Růst EB a HSB na komerčních protézách (ePTFE, PET a PET/Co) byl omezený. Růst obou typů buněk byl nižší na PET/Co než na PET. Po modifikaci proteiny dosáhly EB nejvyšších počtů na PET/Co a PET/Co +Co/Fb. Nebyl rozdíl v hustotě HSB na různých površích. Finální počet HSB byl vyšší než EB, ale nikoli významně na PET/Co a PET/Co +Co/Fb. Impregnace PET vstřebatelnými kopolymery snížila růst EB, následné pokrytí proteiny jej zvýšilo, zejména na Fb vzorcích. Při průtoku média exprimovaly buňky na Co/FN adhezní a metabolické geny více než na Co/LM. V tubulárních cévních protézách měly nejvyšší odolnost vůči 2 h průtoku buňky na PET/Co +Fb a +Fb/FN. Laboratorními zvířaty byl nejlépe tolerován aorto-ilický interní bypass s ligací zevních pánevních tepen. Z pěti cévních náhrad osazených vlastními EB došlo k trombóze 2× spontánně, 2× pro pooperační komplikace a jedna zůstala průchodná se zachovaným endotelem.

Závěr: Modifikace PET pomocí Fb povrchů a/nebo adhezních proteinových povrchů podporuje růst buněk. Fibrin je pro růst EB unikátní. Konstruovali jsme tri-komponentní planární vzorky složené z cévních protéz PET, vstřebatelných kopolymerů a proteinových povrchů, které podpořily tvorbu endotelové vrstvy. Kombinované matice Co/LM a Co/FN jsou výhodnější pro nasazení lidských EB a jejich odolnost k průtoku než samotný Co. Matrix Co/FN navíc indukuje příznivější buněčný fenotyp než Co/LM. Potažení tubulárních PET/Co cévních protéz povrchem Fb nebo Fb/FN zlepšuje retenci lidských EB během průtoku. Na modelu mini-prasete může nasazení autologních EB na modifikovanou cévní protézu zlepšit průchodnost implantovaných bypassů.

Klíčová slova: endotelové buňky, cévní hladké svalové buňky, tkáňové inženýrství, fibrin, extracelulární matrix, kolagen, laminin, fibronectin, ateroskleróza, cévní chirurgie, cévní protéza, polyetylén tereftalát, polytetrafluoroetylén

ABSTRACT

Introduction: Vascular surgery for atherosclerosis is confronted by the lack of a suitable bypass material. Synthetic vascular prostheses include polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE). However, these materials become thrombosed in small-caliber applications (<6 mm) because of the lack of an endothelium. The objectives of this study were to make modifications to clinically-used PET vascular prostheses with tissue-engineered surfaces to improve their bio-compatibility towards vascular smooth muscle cells (VSMC) and endothelial cells (EC).

Methods: Blood coagulation protein fibrin (Fb) and extracellular matrix proteins collagen (Co), laminin (LM) and fibronectin (FN) were used. Cell adhesive assemblies were prepared: Co, Co/LM, Co/FN, Co/Fb, Co/Fb/FN. Cell culture experiments were performed: (1) planar static, (2) planar dynamic with simulation of blood flow, (3) tubular dynamic, and (4) animal porcine implantation.

Results: The growth of EC and VSMC on commercial prostheses (ePTFE, PET and PET/Co) was low. The growth of both cell types was lower on PET/Co than on PET. After modification with protein assemblies, the highest numbers of EC were reached on PET/Co and on PET/Co +Co/Fb. There was no difference in the densities of VSMC among various assemblies. The final populations of VSMC were higher than those of EC. However, this difference was not significant on PET/Co and on PET/Co +Co/Fb. Impregnation of PET with degradable copolymers made it less adhesive for EC. However, additional coating with protein assemblies enhanced the cell growth, especially on Fb-containing surfaces. Under flow, the cells plated on Co/FN up-regulated adhesion and metabolic genes more than the cells on Co/LM. Cells on PET/Co +Fb and +Fb/FN tubular prostheses showed excellent retention during 2h of flow. In animals, the best tolerated anatomical setting was the aorto-internal iliac bypass with ligation of the external iliac arteries. From the 5 grafts seeded with own EC, two occluded spontaneously, 2 occluded due to complications, and one remained patent with a preserved endothelium.

Conclusion: Modification of PET with Fb assemblies or with adhesive protein assemblies promotes cell growth. Fibrin-containing surfaces are unique for the growth of EC. Tri-component planar grafts composed of PET, copolymers and adhesive assemblies were fabricated to promote the endothelial lining. Combined Co/LM and Co/FN matrices are more suitable for human EC seeding and retention under flow than a Co surface alone. Moreover, a Co/FN matrix promotes slightly more favorable cellular phenotype than Co/LM. Pre-coating the tubular PET/Co prosthesis with Fb or Fb/FN improves the retention of human EC under flow. In a minipig model, seeding autologous EC onto a modified vascular prosthesis may enhance the patency of the bypass graft.

Key words: endothelial cells, vascular smooth muscle cells, tissue engineering, fibrin, extracellular matrix, collagen, laminin, fibronectin, atherosclerosis, vascular surgery, blood vessel prosthesis, polyethylene terephthalate, polytetrafluoroethylene

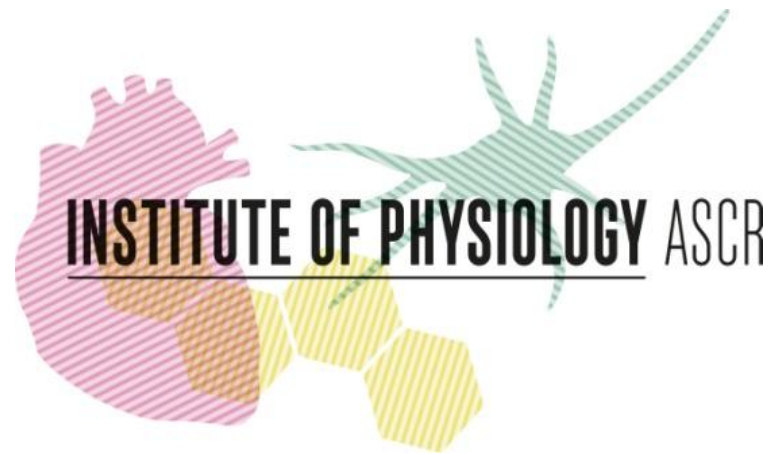
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2. Abbreviations

2D	two-dimensional	HCAEC	human coronary artery endothelial cells
3D	three-dimensional	HKC	human keratinocytes
a.	arteria	HMC	human mesothelial cells
AAA	abdominal aortic aneurysm	Hr	hirudin
AFM	atomic force microscopy	HSVEC	human saphenous vein endothelial cells
AT III	antithrombin III	HUVEC	human umbilical vein endothelial cells
AVF	arterio-venous fistula	ICAM-1	intercellular adhesion molecule 1
BAEC	bovine aortic endothelial cells	IH	intimal hyperplasia
b-FGF	basic fibroblast growth factor	KLF	Kruppel-like factor
BM	basal membrane	LM	laminin
CAD	coronary artery disease	MCP-1	monocyte chemotactic protein 1
Co	collagen	MEM	Minimum Essential Eagle Medium
Coll	commercial collagen impregnation	NIH	neo-intimal hyperplasia
CPAE	bovine pulmonary artery endothelial cells of the CPAE line	NO	nitric oxide
CVD	cerebral vascular disease	ns.	non-significant
DMEM	Dulbecco-modified Minimal Essential Eagle Medium	PAD	peripheral artery disease
EC	endothelial cell(s)	PAF	platelet activating factor
ECM	extracellular matrix	PAI-1	plasminogen activator inhibitor
ELISA	enzyme-linked immunosorbent assay	PCL	poly(ϵ -caprolacton)
EPC	endothelial progenitor cells	PDEC	human umbilical cord progenitor-derived endothelial cells
ePTFE	expanded polytetrafluoroethylene	PDGF	platelet-derived growth factor
ET-1	endothelin-1	PECAM-1, CD-31	platelet-endothelial cell adhesion molecule 1
Fb	fibrin	PET	polyethylene terephthalate
FB	fibroblast(s)	PET/Coll	polyethylene terephthalate with commercial collagen impregnation
Fbg	fibrinogen	PGA	poly(glycolid acid)
FBGC	foreign-body giant cell(s)	PGI ₂	prostacyclin
FBS	fetal bovine serum	PGS	poly(glycerol sebacate)
FDA	Food and Drug Administration		
FN	fibronectin		
h	hour		

PLA	poly(L-lactic acid)	vWF	von Willebrand factor
PLDLA	poly(D,L-lactic acid)	WHO	World Health Organization
PLG	poly(L-lactide-co-glycolide)		
PLGC	poly(L-lactide-co-glycolide-co- ϵ -caprolactone)		
PLLACL	poly(L-lactide-co- ϵ -caprolactone)		
PPACK	D-phenylalanyl-L-prolyl-L-arginine chlormethyl ketone		
PS	polystyrene		
PTA	percutaneous balloon angioplasty		
PTFE	polytetrafluoroethylene		
PU	polyurethane		
Res.	result		
S	serum		
SEM	standard error of mean / scanning electron microscopy		
SMC	smooth muscle cell(s)		
SPR	surface plasmon resonance		
SREBP-1	sterol regulatory element binding protein 1		
SVG	saphenous vein graft		
TCPS	tissue culture polystyrene		
TEBV	totally-engineered blood vessel		
TEM	transmission electron microscopy		
TF	tissue factor, thromboplastin		
TFPI	tissue factor pathway inhibitor		
TGF- β	transforming growth factor β		
Thr	thrombin		
TM	thrombomodulin		
tPA	tissue plasminogen inhibitor		
VCAM-1	vascular cell adhesion molecule 1		
VEGF	vascular endothelial growth factor		
vs.	versus		
VSM	<i>vena saphena magna</i>		
VSMC	vascular smooth muscle cell(s)		

3. Introduction

Cardiovascular disease has become a major cause of morbidity and mortality in the Western world, claiming 17 million lives worldwide each year. It accounts for almost half of all deaths from non-infectious diseases and nearly one third of all deaths globally. This trend is also observed in developing countries, and it is becoming a major global health and economic burden (WHO 2010).

Cardiovascular disease in children comes mainly in the form of inborn defects in the development of the heart or of vessels. In adults, it comes in the form of **atherosclerosis** of arteries in most cases, and in the form of other degenerative or inflammatory diseases in a minority of cases. Atherosclerosis can be clinically silent for decades, but in its advanced forms it causes narrowing (stenosis) and occlusions of arteries, and decreased blood supply to the organs results in symptomatic diseases, such as coronary heart disease (CAD) (*e.g.* myocardial infarction, angina pectoris and heart failure), cerebrovascular disease (CVD) (stroke, brain infarction), peripheral arterial disease (PAD) of the extremities, which can lead to amputation of the limb (*e.g.* diabetic foot syndrome) and, last but not least, visceral ischemia – impaired blood supply to inner organs such as kidneys or bowels. All these disorders can occur in an acute form or in a chronic form. In the acute form, a blood clot (thrombosis) usually develops on top of a chronic atherosclerotic lesion, and this situation is usually referred to as athero-thrombosis. A blood clot can also travel through the vascular system from the site of its origin (*e.g.* heart with arrhythmia) and get stuck in any distant vascular region without an underlying atherosclerotic disease – an arterial embolism, resulting in acute ischemic events (Norgren, L. *et al.* 2007). Another vascular disease entity is aneurysm formation (dilation), which endangers the patient with thrombosis, embolism or even rupture and serious bleeding.

There has been a huge advance in non-invasive therapy options (pharmacology) and in minimally-invasive procedures in the treatment of cardiovascular disease, such as endovascular angioplasty and stent placement. Nevertheless, surgical repair is often necessary for an advanced and multiple vascular lesion. The most frequently performed cardiovascular surgical procedures include **bypass grafting** (“bridging”), substitution (replacement) and patch angioplasty (broadening the lumen). Cardiac surgery usually deals with diseases of the heart and diseases of the thoracic aorta. **Vascular surgery** has developed into a separate discipline, and it deals with all other types of lesions throughout the body. Most procedures are performed to restore blood flow to the lower extremities and to the brain, to repair peripheral aneurysms (*e.g.* abdominal aortic aneurysm, AAA) and to create a vascular access – arterio-venous fistula (AVF) for hemodialysis in patients with end-stage renal disease.

Current treatment modalities for bypassing or replacing diseased vascular regions include the use of autografts (autologous – patient’s own artery or vein), allografts (human

donor vessels), xeno-grafts (heart valves or vessels from animals) and artificial synthetic prostheses. However, each of these methods has its limitations, which include shortage of own or donor tissue, pathogen transfer, anticoagulation therapy, infection risk and limited durability. More importantly, they do not allow full regeneration and functional recovery and hence reduce the patient's life expectancy or increase the probability of complications and redo surgery (Bouten, C.V. *et al.* 2011).

For more than 50 years, synthetic vascular grafts (**vascular prostheses**) have been made from one of two materials: polyethylene terephthalate (PET, Dacron[®]) and expanded polytetrafluoroethylene (ePTFE, Teflon[®], Gore-Tex[®]). These types of commercial implants are ready for use off-the-shelf, and they are steady and safe. However, their healing characteristics and their hemocompatibility are very limited in humans (Pasquinelli, G. *et al.* 1990). They perform well as substitutes for large-caliber arteries, such as the aorta and the iliac arteries, but they would fail as a bypass for small-caliber arteries (< 4-6 mm), such as the coronary or leg arteries. There is currently a vast clinical need for a better functioning vascular graft for small-diameter applications (Chlupac, J. *et al.* 2009).

Tissue engineering involves the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions (Langer, R. and Vacanti, J.P. 1993). The main types of cells in the vascular wall are endothelial cells (EC), which form the lining of blood vessels, vascular smooth muscle cells (VSMC, SMC), which are responsible for the strength and contractility of the vessel wall, and finally fibroblasts, which form the outer layer. The behavior (**phenotype**) of both EC and VSMC can be healthy (quiescent, physiological) or pathological (Muto, A. *et al.* 2007). Therapeutic strategies in cardiovascular disease are mainly aimed at these cell types, and they also include efforts to influence their phenotype. By analogy with this, tissue engineering of vascular grafts makes an effort to introduce living cells into the design of vascular implants in order to make vascular grafts more biocompatible. Concurrently, these cells are expected to express a healthy phenotype (Bacakova, L. *et al.* 2007; Bacakova, L. *et al.* 2011).

This work deals with the interaction of vascular smooth muscle and mainly endothelial cells with prosthetic grafts, with the aim to modify clinically-used vascular prostheses and improve their blood-compatibility for the purposes of vascular tissue engineering and surgical treatment of atherosclerotic disease.

3.1 Vascular physiology

The vascular system exists to facilitate continuous nutrient delivery and waste removal to sustain tissue viability in organisms larger than 200µm in size due to diffusion limitations (Stegemann, J.P. *et al.* 2007). In the structure of the vascular wall, three different layers can be

distinguished: *tunica intima* (inner layer), *tunica media* (middle layer) and *tunica adventitia* (outer layer). The detailed structure of the vascular wall is presented in Figure 1.

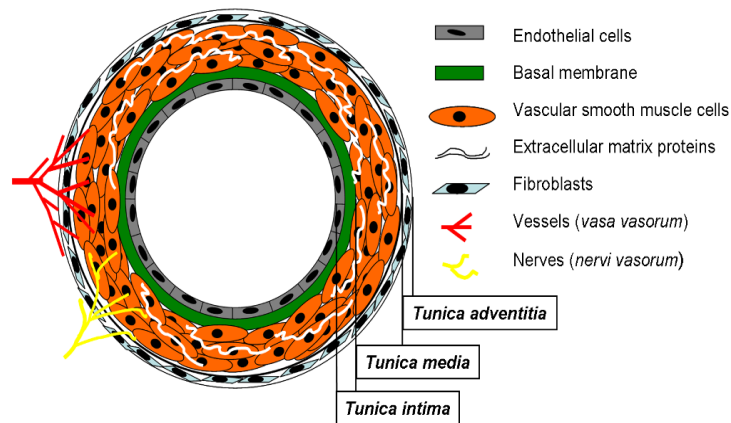


Figure 1. Structure of the vascular wall on a cross-section of an artery.

Tunica intima consists of a monolayer of endothelial cells which line the vessel lumen and form the blood-contacting surface. The ECs function as a dynamic interface which actively maintains blood circulation. They prevent blood clotting, inflammation and infection, and ensure further signaling from the lumen towards the VSMC in the *media*. Vascular cells are embedded in an **extracellular matrix** (ECM) composed largely of proteins, glycoproteins, glycosaminoglycans and proteoglycans. The protein components are often fibrillar and provide strength in tension, whereas the proteoglycans sequester water and ions and provide compressive resistance. Different ECM composition in tissues results in different specialized functions. The EC are supported by a thin sub-intimal layer of ECM called endothelial basal membrane (BM). BM is rich in collagen (Co) type IV and laminin (LM), and it contains a fenestrated acellular layer called internal elastic lamina (*lamina elastica interna*).

Tunica media is the thickest layer and it contains contractile vascular smooth muscle cells, which are responsible for the vascular tone. VSMCs have a circumferential or spiral pattern, they are innervated and they receive signals from luminal EC and from the blood. Vascular smooth muscle cells are the most abundant cell type in the vessel wall and comprise the muscular layer of vessels (mainly arteries). They are critical for maintaining blood pressure and continuous blood flow. To achieve this, there is interplay between the cells and the ECM in the vessel wall. Collagen type I and III and elastin are the most abundant and major structural proteins in the *media*, endowing the vessels with excellent tensile strength and elasticity, respectively. Whereas collagen maintains the structural integrity of a vessel, elastin acts to recoil the vessel wall after stretch, preventing excessive vessel dilation and contributing to the compliance of the vessel (Tschoeke, B. *et al.* 2009). Physiological blood pressure is 120/80 mmHg. The strength of human arteries is characterized by the so-called “burst pressure”, which amounts to almost 2000 mmHg (Bouten, C.V. *et al.* 2011). There is also a lesser amount of

other components – fibronectin (FN), laminin (LM), vitronectin and other ECM proteins. A wavy layer of compacted elastin is located in the outer part of the *media* and is called the external elastic lamina (*lamina elastica externa*) (Stegemann, J.P. *et al.* 2007).

In the *tunica adventitia* there are fibroblasts and a loose connective tissue matrix. The *adventitia* provides further structural support. The tiny vessels supplying the vascular wall from the outside run there (*vasa vasorum*) (Kachlik, D. *et al.* 2007).

3.2 Vascular pathophysiology

3.2.1 Endothelial dysfunction

When tissue damage occurs, EC drive local thrombosis and inflammation and enable efficient wound repair. Thrombosis involves three interconnected regulatory systems: the coagulation cascade, the complement cascade, and the cellular components of the blood, such as leucocytes and platelets. Moreover, the fibrinolytic system and regulation of the vascular tone influence blood clot lysis and blood flow (McGuigan, A.P. and Sefton, M.V. 2007). However, there are a number of pathological states in which chronic or repeated endothelial injury exceeds a certain limit and provokes an undesired response.

Two main phenotypes of EC are distinguished: **quiescent EC** and **activated EC**. Quiescent and activated EC differ in the expression of molecules involved in hemostasis (coagulation, cell adhesion, complement, fibrinolysis, vasoreactivity) and in atherogenesis (inflammation, lipid metabolism, cell permeability, cell turnover, cell cycle regulation, ECM remodeling and cytoskeleton organization). Normal, healthy, unactivated confluent EC support normal blood flow and inhibit thrombosis. The process of thrombosis has significant redundancy and complexity, with a steady state resulting from the dynamic balance of many molecules involved in coagulation and fibrinolysis cascades. A healthy endothelial phenotype is characterized by up-regulated expression of molecules with anti-atherogenic, anti-coagulant, anti-inflammatory, anti-apoptotic and anti-proliferative properties. Moreover, a quiescent endothelial phenotype is consistent with elongated cells, low cell turnover, low permeability of an EC monolayer and the production of molecules inhibiting thrombosis and supporting vasodilation. For example, in unactivated EC the expression of the tissue factor (TF) is low or absent and thrombomodulin (TM) expression is high. Additionally, there is low secretion of nitric oxide (NO), prostacyclin (PGI₂), von Willebrand factor (vWF) and adhesion molecules: intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD-31) and P-selectin. Some of the molecules are listed in Table 1.

Activation of the EC reverses this profile. Stimulated (pro-coagulant, pro-atherogenic) phenotype of EC is characterized by an increased expression of NO, PGI₂, vWF, surface

adhesion molecules, diapedesis of leucocytes, increased permeability and lipid insudation, migration of VSMC into the sub-endothelial layer and ECM remodeling and promotion of thrombosis and atherosclerosis (Brooks, A.R. *et al.* 2004). In experimental settings, the activation of EC usually occurs by exposing them to lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF- α) (McGuigan and Sefton 2007).

Table 1. Molecules expressed by endothelial cells for control of coagulation (TM, TF, TFPI, AT III, vWF), adhesion of blood cells (ICAM-1, VCAM-1, PECAM-1, P-selectin, E-selectin), control of vascular tone (NO, PGI₂, PAF, ET-1), complement cascade (C1 inhibitor), fibrinolysis (tPA, PAI-1), sensing of biomechanical stimuli (KLF-1), inflammation (MCP-1) and lipid metabolism (SREBP-1). Abbreviations: Low-density lipoprotein receptor (LDL-R), Hydroxy-methyl-glutaryl-coenzyme A synthase (HMG CoA synthase). Adapted from (Brooks, A.R. *et al.* 2004; Chien, S. 2007; McGuigan, A.P. and Sefton, M.V. 2007).

Molecule	Function	Quiescent Endothelium	Activated Endothelium
Thrombomodulin (TM)	Inactivates thrombin, activates protein C and protein S	Constitutively expressed	Downregulated
Tissue factor, thromboplastin (TF)	Receptor for factor VII, initiates coagulation	Low expression	Expressed in response to an agonist
Tissue factor pathway inhibitor (TFPI)	Inhibits TF activity	Constitutively expressed	
Antithrombin III (AT III)	Inactivates free thrombin	Constitutively expressed	
Von Willebrand factor (vWF)	Platelet adhesion to exposed ECM	Stored in Weibel-Palade bodies	Expressed in response to an agonist
Intercellular adhesion molecule 1 (ICAM-1)	Adhesion and diapedesis of leucocytes		
Vascular cell adhesion molecule 1 (VCAM-1)			Expressed in response to an agonist
Platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD-31)			
P-selectin, E-selectin			
Nitric oxide (NO)	Inhibits platelet adhesion and aggregation, vasodilator		Expressed in response to an agonist
Prostacyclin (PGI ₂)	Inhibits platelet adhesion, vasodilator		
Platelet activating factor (PAF)	Stimulates platelets and leucocytes		Secreted in response to an agonist or increased shear
Endothelin-1 (ET-1)	Vasoconstrictor		
C1 inhibitor	Complement inhibitor	Constitutively expressed	
Tissue plasminogen inhibitor (tPA)	Plasmin generation and thrombus fibrinolysis	Constitutively low secretion	Rapid secretion in response to an agonist
Plasminogen activator inhibitor 1 (PAI-1)	Inhibits action of tPA	Constitutively low secretion (but higher than tPA)	
Kruppel-like factor (KLF)	Flow-responsive transcription factor	Central switch between quiescent and activated cells	
Monocyte chemotactic protein 1 (MCP-1)	Proinflammatory molecule	Decreased expression	
Sterol regulatory element binding protein 1 (SREBP-1)	Increased expression of LDL-R, HMG-CoA synthase, fatty acid synthase	Low expression	Sustained activation under disturbed flow

3.2.2 Vascular smooth muscle cells dysfunction

Under healthy, homeostatic conditions, the VSMC in the *media* of the vascular wall have a spindle-shaped morphology and exhibit a differentiated, **contractile** phenotype with a low turn-over rate, and only baseline levels of both proliferation and apoptosis. This quiescent phenotype is induced by heparin or by transforming growth factor- β (TGF- β). Vascular injury is associated with a phenotypic switch towards a proliferative or **synthetic** phenotype. The VSMC then dedifferentiate and migrate from the *media* into the neo-intima, increase the rate of proliferation and apoptosis and extensively synthesize ECM (Bacakova, L. *et al.* 2007). This activated phenotype with a rhomboid morphology is induced by basic fibroblast growth factor (b-FGF) and platelet-derived growth factor (PDGF). There are three potential sources of these VSMC that contribute to vascular remodeling: *in situ* expansion, migration of the cells from *adventitia* or accumulation from distant sources (blood-borne), e.g. homing of stem cells from the bone marrow. These processes are thought to be involved in the pathogenesis of atherosclerosis, hypertension, transplant-arteriopathy, re-stenosis after an endovascular intervention (balloon angioplasty and/or stent deployment) or surgical bypass grafting (Muto, A. *et al.* 2007).

3.2.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arteries that is driven by elevated plasmatic levels of lipids. It is a normal part of the wear of human vessels, starting approximately in the second decade of human life. However, pathological atherosclerosis originates as a **response to vascular injury** (Ross, R. 1999), which can be triggered by some of the well-known risk factors for atherosclerosis: hypertension, hyperlipidemia, smoking, diabetes mellitus, unhealthy diet, chronic stress and a sedentary lifestyle. Some of the atherosclerotic events are depicted in Figure 2.

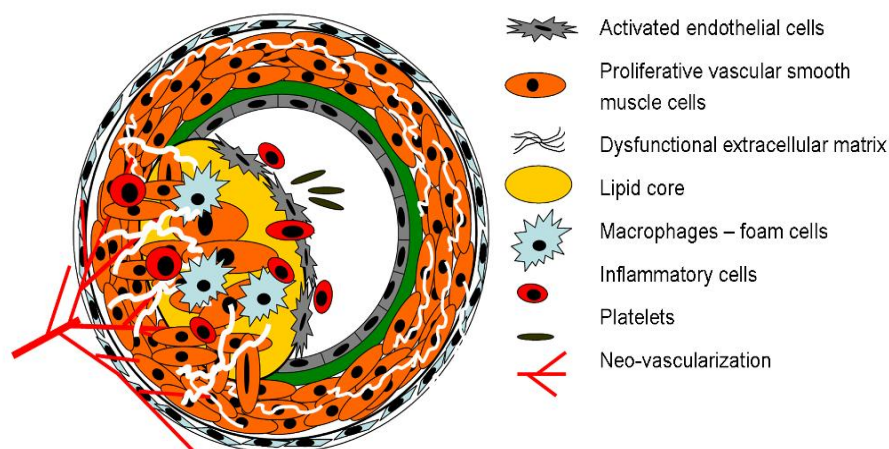


Figure 2. Atherosclerotic plaque, which has caused stenosis of an artery.

Atherosclerosis starts as **fatty streaks** of the *intima*, characterized by intracellular lipid accumulation. The streaks can gradually progress into **atheroma** – a lipid core composed of extracellular lipids and macrophages that phagocytose oxidized low-density lipoproteins (LDL) containing cholesterol and triglycerides, thus forming large foam cells. Activated endothelium becomes more adherent for circulating monocytes and more permeable for plasma lipoproteins; adhering platelets (thrombocytes) produce proliferative signals and the VSMC migrate from the *media* into the sub-endothelial space of the *intima*, acquire a proliferative phenotype and synthesize the ECM (Poledne, R. *et al.* 2009). Neo-vascularization can cause bleeding into the atherosclerotic plaque. All these events lead to progressive **stenosis** or **occlusion** and sometimes to calcification of the vessel. So-called vulnerable plaques are rich in foam cells, and have a very thin fibrous cap. They can rupture, expose the underlying ECM and cause **thrombosis** of the artery or a distant embolism.

When the atherosclerotic lesion stenoses the artery gradually, **collateral vessels** usually develop that allow for at least baseline blood supply to the affected organs (Figure 3A). In cases when the lesion has to be surgically treated by a vascular graft, there are two ways of implanting the graft. First, the affected region is removed and the graft is placed in end-to-end fashion as a vascular **substitution**. This method gives a better fit of the hemodynamic force, but the collateral vessels are not preserved (Figure 3B). This way is used only in replacing short arterial segments or in surgery for aneurysms. A second option is to bridge long and often multilevel lesions with a **bypass** graft in end-to-side fashion. The collateral vessels are then preserved (at least temporarily) for the case of the occlusion of the graft (Figure 3C). Most bypass surgery on the heart and on the lower extremities is performed in this fashion, and the bypass graft can be as long as 60-80 cm (Schouten, O. *et al.* 2005).

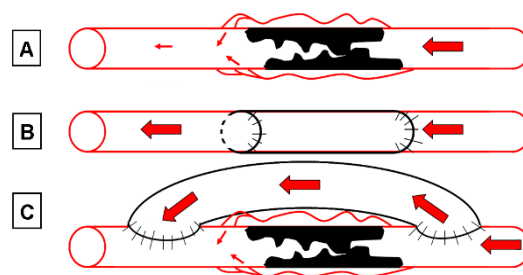


Figure 3. **A.** Longitudinal section of an atherosclerotic vascular lesion with developing collateral vessels. **B.** An atherosclerotic lesion has been resected and substituted by a vascular graft in end-to-end fashion. The collaterals have been damaged. **C.** The lesion has been bypassed by a vascular graft in end-to-side fashion. The collaterals are preserved.

3.2.4 Intimal hyperplasia

Intimal hyperplasia (IH) or neo-intimal hyperplasia (NIH) is a reaction to a non-natural (iatrogenic) injury, such as surgical vascular bypass grafting or endovascular stenting. It is characterized by denudation of the EC, proliferation and migration of the VSMC, excessive synthesis of ECM and progressive narrowing or even loss of the vascular lumen. Inflammation plays a major role and IH has many similarities with the pathogenesis of atherosclerosis. After placing an arterial or arterio-venous **prosthetic bypass graft** in end-to-side fashion, the predilection site for the development of IH is the site of the connection between the graft and the artery – the **anastomosis** (Rotmans, J.I. *et al.* 2004). IH in the distal anastomosis is encountered more often than IH in the proximal anastomosis. Typical locations are the toe, the heel and the arterial floor of the anastomosis (Haruguchi, H. and Teraoka, S. 2003) (Figure 4).

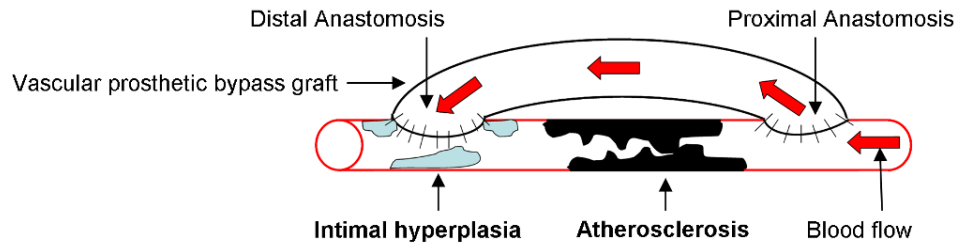


Figure 4. Atherosclerosis of an artery that has been bypassed by a vascular prosthetic graft sutured in end-to-side fashion. The development of intimal hyperplasia in the site of the distal anastomosis can be seen at the toe, at the heel and at the arterial floor of the anastomosis (blue regions).

After placing a biological **saphenous vein graft** (SVG) in the arterial circulation, IH can develop throughout the length of the vein graft (Human, P. *et al.* 2009). A diameter mismatch between the graft and the artery contributes to the development of IH, and this is an issue in both prosthetic and vein grafts. The caliber of the target artery or the “run-off” or “outflow” vessel is usually smaller than the caliber of the vascular graft, resulting in a cross-sectional quotient ($Q_c = \text{runoff diameter/graft diameter}$) across the distal anastomosis $Q_c < 1$, sometimes even reaching values around $Q_c = 0.2$. After being surgically hydro-dilated and sutured into the arterial high-pressure system, SVG undergoes significant dilation and remodeling. Decreased shear stress (tangential friction force) to the endothelium and increased wall tension bring the SVG wall to a response of **diffuse intimal hyperplasia**. Moreover, physiological luminal irregularities (intimal “cushions”, valves, ligated side branches) and fluctuations in diameter across the length of the vein graft lead to eddy flows, resulting in **focal intimal hyperplasia**, which ultimately contributes to vein graft occlusion (Human, P. *et al.* 2009).

It has been experimentally confirmed in a clinically relevant non-human primate model that external constriction of the inner diameter of the vein graft by ca 25% with a Nitinol (nickel and titanium alloy) mesh successfully suppresses IH in a majority of SVGs. This corresponds to a shear stress threshold of 5 dyn/cm², which has been proposed for mitigating IH. In this model, reversed superficial femoral vein was implanted as an interposition graft of the superficial femoral artery in senescent baboons. In addition, further graft constriction of ca 50% (corresponding to shear stress of 20 dyn/cm²) of the diameter resulted in preservation of endothelial integrity (Zilla, P. *et al.* 2009).

Interestingly, although coronary bypass surgery and lower leg bypass surgery both use saphenous veins as conduits, they undergo significantly **different remodeling** processes (including IH) in these two anatomic positions (Zilla, P. *et al.* 2012). It has been proved experimentally that IH can be mitigated by external constriction in both vascular regions (Moodley, L. *et al.* 2013). However, therapeutic vein constriction to decrease IH has not yet been adopted in clinical practice.

3.3 Vascular grafts

3.3.1 Introduction

Although advanced pharmacological and minimally-invasive techniques offer a growing therapy option (Met, R. *et al.* 2008), a surgical bypass of blood vessels on the heart or on a lower extremity remains the procedure of choice for a number of patients with **advanced multilevel disease** (Guyton, R.A. 2006). The gold standard material in bypass surgery for blood vessels remains the patient's own artery or vein. However, this material may be unavailable, or may suffer vein graft disease.

A synthetic tube or a vascular prosthesis has to be implanted when the patient's own artery or vein is not available. After more than half a century of development work, the results achieved with currently available synthetic materials are not optimal in terms of healing and tissue regeneration. Long-term patency rates of prosthetic grafts are satisfactory in large-caliber arteries (> 8 mm), where thrombogenicity (*i.e.*, disposition to blood coagulation) may be overcome by massive blood flow, and the 5-year patency of aorto-iliac substitutes is 90% (Brewster, D.C. 1997) (Figure 5A-C). However, in small-caliber vessels (< 6 mm), such as coronary arteries (heart), infrainguinal arteries (below the inguinal ligament), and particularly in low-flow infrageniculate arteries (below the knee joint), the outcomes of vascular prostheses are rather disappointing. Five-year primary patency of prosthetic (ePTFE) above-the-knee femoropopliteal bypass grafts is as low as 39%, whereas autovenous bypasses (*i.e.*, performed with own vein) have a rate of 74% (Klinkert, P. *et al.* 2004) (Figure 5E).

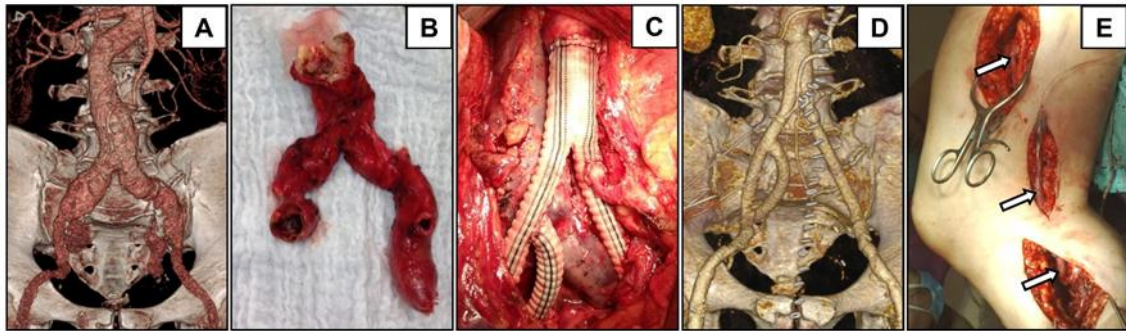


Figure 5. Vascular substitutes for large-caliber arteries (synthetic vascular prosthesis) or small-caliber arteries (autologous vein graft) in clinical use. **A, B:** Multiple aneurysms of the abdominal aorta and iliac arteries that threaten the patient with rupture or thrombo-embolism. **C, D:** Replacement with polyethylene terephthalate vascular prosthesis (large-caliber replacement). **E:** Femoral-popliteal distal bypass (below the knee joint) for ischemia (trophic ulcers) of the lower limb performed with the patient's own saphenous vein graft (*vena sahena magna*, arrows). Computed tomography angiograms (**A** and **D**), courtesy of the Dept. of Radiology, IKEM, Prague.

Currently available vascular prostheses fail due to thrombogenicity of the artificial surface and intimal hyperplasia. Unlike in animal models, most of the prosthetic blood-contacting surfaces remain uncovered by tissue in humans (Berger, K. *et al.* 1972). The etiology of vascular trauma that leads to IH is multi-factorial. It includes a compliance mismatch between a relatively rigid prosthesis and the more elastic native artery (Sarkar, S. *et al.* 2006), graft/artery diameter mismatch, lack of endothelial cells (EC), surgical trauma and flow disturbances in the anastomosis resulting in adaptive changes in the sub-endothelial tissue, characterized by proliferation and migration of the VSMC, synthesis of ECM proteins, restenosis and stagnation thrombosis (Haruguchi, H. and Teraoka, S. 2003) (Figure 6).

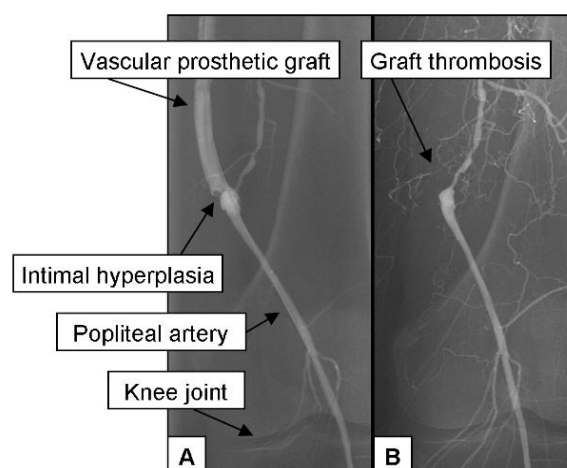


Figure 6. Angiogram of distal anastomosis of a lower leg femoro-popliteal prosthetic bypass graft. Intimal hyperplasia causing stenosis (**A**) and graft thrombosis (**B**). Courtesy of the Dept. of Radiology, IKEM, Prague.

An **ideal vascular graft** would possess the following characteristics: mechanical strength and compliance to withstand long-term hemodynamic stresses; non-toxicity; non-immunogenicity; biocompatibility; “off-the-shelf” availability in various sizes for emergency care; operative suturability and simplicity of surgical handling; resistance to *in vivo* thrombosis; ability to withstand infection; complete incorporation into the host tissue with satisfactory graft healing and ability to grow when placed in children (Kakisis, J.D. *et al.* 2005), and, last but not least, reasonable manufacturing costs. The task is so demanding and the potential rewards are so great that research in the field of small-caliber vascular substitutes has been compared with the search for the Holy Grail (Conte, M.S. 1998).

3.3.2 Biological vascular grafts

The gold standard for a vascular replacement remains an **autologous** (patient’s own), native vessel, which possesses the most physiological properties. In coronary artery bypass grafting (CABG), the internal mammary artery (*a.thoracica interna*) and the radial artery are superior to a great saphenous vein graft, which is also often used (Beghi, C. *et al.* 2002). In the case of lower limb or peripheral bypass surgery, the material of choice is also great saphenous vein (*vena saphena magna*) (VSM). However, it should be noted that, despite good clinical performance, SVG is also liable to atherosclerosis and intimal hyperplasia occurring throughout the length (Sarjeant, J.M. and Rabinovitch, M. 2002). In addition, almost 30-40% of patients lack an appropriate saphenous vein (Farries, P.L. *et al.* 2000) due to previous phlebitis, vessel removal, varicosities, hypoplasia or anatomical unsuitability. In addition, any surgical vessel harvesting is associated with inevitable donor site morbidity (Svenne, C.L. *et al.* 2006).

Although **allografts** (vessels from cadaveric or live human donors) are not routinely used (except in limb-threatening situations, in redo surgery or in an infected field), some studies have suggested improved patency of these grafts, *e.g.*, in the case of preserved externally-supported human umbilical vein (harvested from newborns) in comparison to ePTFE (Johnson, W.C. and Lee, K.K. 2000).

The application of heterografts (**xenografts**, *i.e.*, tissue from different species) (Schmidt, C.E. and Baier, J.M. 2000) has been investigated in studies on alternative hemodialysis vascular access. Importantly, decellularized natural tissue of allogenic (human) or xenogenic (animal) origin serves as a scaffold for cell seeding within the scope of tissue engineering of vascular grafts (Dahl, S.L. *et al.* 2003). A summary of biological vascular conduits is presented in Table 2.

Table 2. Biological vascular grafts in clinical use.

	Biological vascular grafts				
	Autografts		Allografts (homografts)		Xenografts (heterografts)
	Arterial	Venous	Arterial	Venous	
Advantages	Closest approximation, less diameter mismatch, IMA anatomically nearby, excellent function	Durable and versatile, good results, infection resistance, relative availability	Off the shelf availability, better resistance to infection, transplant-recipient patients		
Disadvantages	Availability, vasospasm (radial artery), donor site morbidity	Availability, harvest injury, vein graft disease	Antigenicity, graft deterioration, early occlusions, chronic rejection, intake of drugs, infection risk		
Healing	Intimal thickening, myointimal hyperplasia (radial artery)	Endothelial desquamation, vein dilation, wall thickening, arterialization, re-endothelialization	Endothelial denudation, immune response, fibrotization		
First use	(Jaboulay, M. and Briau, E. 1896)	(Goyannes, J. 1906)	(Gross, R.E. <i>et al.</i> 1948)	(Linton, R.R. 1955)	
Review e.g.	(Nezic, D.G. <i>et al.</i> 2006)	(Cooper, G.J. <i>et al.</i> 1996)	(Fahner, P.J. <i>et al.</i> 2006)	(Dardik, H. <i>et al.</i> 2002)	(Schmidt, C.E. and Baier, J.M. 2000)

3.3.3 Synthetic vascular grafts

The first vascular prosthesis made of Vinyon “N” was implanted in humans in 1954 as a substitute for AAA (Blakemore, A.H. and Voorhees, A.B., Jr. 1954). For more than 50 years, two non-degradable polymers have been used for synthetic vascular prostheses: polyethylene terephthalate (PET, Terylene®, Dacron®), and polytetrafluoroethylene (PTFE, Teflon®, Gore-Tex®) (Figure 7). Both of these molecules are highly crystalline and hydrophobic.

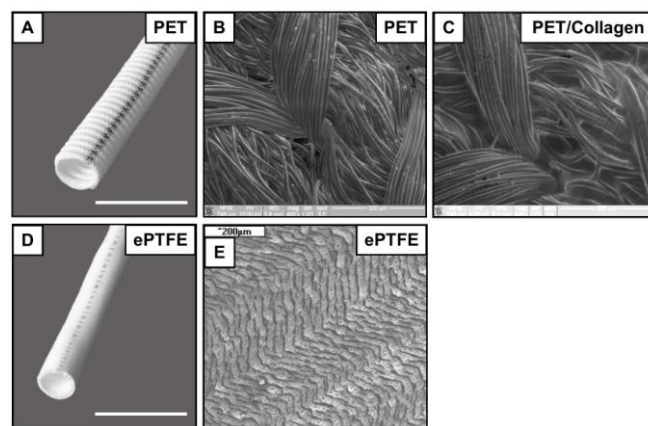


Figure 7. Synthetic vascular prostheses in clinical use: polyethylene terephthalate (PET) (A) and expanded polytetrafluoroethylene (ePTFE) (D), bar = 1 cm. Ultrastructure of the textile knitted PET (B), knitted PET with commercial bovine type I collagen impregnation (C) (VÚP Joint-Stock Comp., Brno, Czech Republic) and non-textile ePTFE (E). Scanning electron

microscopy (NOVA nanoSEM 200, FEI, USA), magnification 400× and bar = 300 μm (**B, C**) and bar = 200 μm (**E**).

PET (polyethyleneterephthalate, [-O-C=O-C₆H₄-O-C=O-CH₂-CH₂-]) was introduced in England in 1939, and was further developed and patented as Dacron by DuPont in 1950. It is a thermoplastic polymer resin of the polyester family and is used in synthetic fibers of round cross-section. These fibres are bundled into multifilament yarns, which can be woven (over-and-under pattern) or knitted (looped fashion) into textile vascular graft fabrics and tubes.

A crimping technique (an undulating surface) is sometimes used to increase distensibility and kink-resistance. The porosity of a textile Dacron graft is defined by its water permeability, which is greater for knitted Dacron. Knitted Dacron is impregnated with albumin, collagen or gelatin to make it more impervious, to decrease the porosity/permeability, and to avoid the need for blood preclotting prior to implantation. Depending on the cross-linking agent (formaldehyde or glutaraldehyde), the albumin impregnation is degraded 2 to 8 weeks after implantation (Marois, Y. *et al.* 1996). Although collagen impregnation led to increased platelet deposition and delayed the healing process in a dog experiment (Guidoin, R. *et al.* 1996), a promising randomized trial with aorto-iliac prostheses indicated that collagen impregnation does not stimulate the coagulation cascade more than conventional Dacron (De Mol Van Otterloo, J.C. *et al.* 1991). There are no differences in clinical graft patency between woven and knitted Dacron, when used as an aorto-iliac bypass graft (Quarmby, J.W. *et al.* 1998).

Host reactions to the vascular prosthesis start immediately after restoration of blood circulation. The tissue-prosthesis and blood-prosthesis interfaces are complex microenvironments, and the physico-chemical properties of the surface of the prosthesis, e.g. charge, energy, wettability and roughness, may be responsible for the graft patency. The first step is the plasma protein adsorption/desorption process typical for any blood/material interface (Vroman, L. and Adams, A.L. 1969), followed by platelet deposition, white blood cell and erythrocyte adhesion, and then endothelial and smooth muscle cell migration. Fibrin deposits, which contain platelets and blood cells, build up during the first few hours to days after implantation. They are stabilized over a period of up to 18 months and form an inner compacted fibrin layer. Fibrin also fills the interstices within the graft wall. Generally, this fibrin/platelet pseudointima remains acellular; however, after 5 months, capillaries and fibroblasts can grow into the tight interstitial spaces even in humans (Stewart, G.J. *et al.* 1975) and reach the inner fibrin layer. Only a few sparse small islands of endothelialization appeared in areas remote from the anastomosis region on woven excised Dacron grafts (Wu, M.H. *et al.* 1995). The external fibrin matrix surrounding the graft is gradually invaded by macrophages, and granulation tissue and foreign-body giant cells (FBGC) are usually seen under the external surrounding connective tissue capsule (Rahlf, G. *et al.* 1986). In the case of knitted Dacron, endothelial islands can occasionally be observed on human explants during redo surgery or autopsy 1-11 years after

implantation (Shi, Q. *et al.* 1997). Mature endothelium and a well-developed smooth muscle layer, described only in animal studies, is attributable to extended trans-anastomotic ingrowth rather than a midgraft healing process (Clowes, A.W. *et al.* 1987; Zacharias, R.K. *et al.* 1987).

The structure of knitted Dacron and, to a lesser extent, also of woven Dacron allows a certain degree of transmural tissue ingrowth. However, the compacted inner fibrin layer forms a barrier and, even if the barrier is overcome by undifferentiated connective tissue, the capillaries remain unconnected to the poorly endothelialized blood surface (Herring, M.B. *et al.* 1979) (Xue, L. and Greisler, H.P. 2003).

PTFE (polytetrafluoroethylene, [-CF₂-CF₂-]) was patented by DuPont as Teflon in 1937, and ePTFE was patented by Gore as Gore-Tex in 1969. ePTFE is an expanded polymer which is manufactured by a heating, stretching and extruding process, resulting in a non-textile porous tube composed of irregular-shaped solid membranes (“nodes”). The molecule is relatively biostable, *i.e.* less prone to deterioration in biological environments than PET (Guidoin, R. *et al.* 1993a) (Guidoin, R. *et al.* 1993b), and the graft surface is electronegative, which minimizes its reaction with blood components. It is characterized by a node-fibril structure, and its average porosity is described by the internodal distance (IND), which is usually 30 to 90 µm. However, the actual available ingrowth spaces between fibrils are much smaller than IND (Nishibe, T. *et al.* 2007).

Optimal IND of 60 µm (high porosity) was experimentally proposed for tissue ingrowth and endothelialization of 4mm ePTFE grafts in a baboon model (Golden, M.A. *et al.* 1990). Though a human trial with high-porosity ePTFE showed capillary ingrowth, it did not extend more than half the distance from the outside, and did not produce an endothelial lining (Kohler, T.R. *et al.* 1992). Human host responses to standard low-porosity ePTFE (IND ≤ 30 µm) material are similar to the responses to Dacron grafts: a thin fibrin coagulum or amorphous platelet-rich material develops over time, and a lack of luminal surface endothelial cellular coverage is found after human implants. Collagenous external encapsulation develops within 1 to 6 months, penetrating the material structure with minimal cellular infiltration (Guidoin, R. *et al.* 1993a). Systematic evaluation and meta-analysis of randomized controlled trials comparing Dacron and ePTFE showed no evidence of an advantage of one material over the other (Roll, S. *et al.* 2008).

Polyurethanes (PU) comprise a large family of elastic polymers containing a urethane [-NH-(CO)-O-] group. Polyurethanes were originally developed in Germany in the 1930s. They were commercialized by DuPont in 1962, and have been available for biomedical applications since the 1960s (Boretos, J.W. and Pierce, W.S. 1967). Generally, they are copolymers consisting of three different monomers: crystalline (hard) and amorphous (soft) segments, the former providing rigidity and the latter providing flexibility, which can be varied by the manufacturer. The third monomer serves as a chain extender (Zdrahala, R.J. 1996). The

disadvantage of first-generation polyester-based PU was hydrolytic biodegradation, which resulted in one of the clinical trials being aborted (Zhang, Z. *et al.* 1997). The next generation of polyether-based PU, which is hydrolysis-resistant but more susceptible to oxidation, underwent successful clinical assessment as a haemodialysis access graft (Glickman, M.H. *et al.* 2001), and received Food and Drug Administration (FDA) approval in 2000.

The latest generation of polycarbonate-based PU is hydrolytically and oxidatively stable, and promoted faster luminal endothelialization and less neointimal formation as small-calibre vascular prostheses in a rat experiment than ePTFE (Jeschke, M.G. *et al.* 1999). After 36-month implantation in aorto-iliac position in dogs, a histological analysis of poly(carbonate-urea) PU grafts showed well-developed neointima only in distal anastomosis, only minor hydrolysis of the amorphous segments and, in addition, the grafts retained their compliance with time – a feature not observed in Dacron or ePTFE. These promising results led to a phase I clinical trial (Seifalian, A.M. *et al.* 2002)..

According to their microstructure, polyurethanes can be divided into fibrillar or foamy. Both structures tend to lack communicating spaces for potential capillary ingrowth. Upon implantation, the blood surface of the fibrillar PU is covered by a fibrin layer that is thinner than on knitted Dacron, and the outer surface is encapsulated by scar formation containing FBGC. In microporous foamy PU with 15 μm pore size, relatively little capillary ingrowth can be accomplished, whereas with increasing pore size up to 157 μm , it was possible to diminish the undesired inflammatory FBGC reaction and capillary sprouting was allowed from the outside in a Chacma baboon model. However, before its completion, transmural ingrowth slowed down when reaching the dense inner fibrin layer that was “squeezed” into the pores from the inside (Zilla, P. *et al.* 2007).

Although PU grafts possess many interesting features, *e.g.* the presence of EC under poor hemodynamic conditions, better compliance, excellent healing, good surgical handling and low suture bleeding, further investigations are required before more recommendations can be made, because there is a lack of evidence for their use in human peripheral bypass surgery (Dereume, J.P. *et al.* 1993; Tiwari, A. *et al.* 2002). An outline of synthetic vascular prostheses is summarized in Table 3.

Table 3. Synthetic vascular prostheses in clinical use. Expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (PET), inter-nodal distance (IND).

Synthetic vascular grafts						
	PET (Dacron, Terylene)		ePTFE (Teflon, Gore-Tex)		Polyurethane	
	Woven	Knitted	Low-porosity (< 30 µm 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 haemo- and biocompatibility, less thrombogenicity	
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?	
Healing	Inner fibrinous capsule, outer collagenous capsule, scarce endothelial islands	Fibrin luminal coverage, very sporadic endothelium, trans-anastomotic endothelialization in animals	Luminal fibrin and platelet carpet, connective tissue capsule with foreign body giant cells, no transmural tissue ingrowth	Macrophages and polymorpho-nuclear invasion, capillary sprouting, fibroblast migration, certain angiogenesis, thicker neointima, endothelialization in animals	Thin inner fibrin layer, outside foreign body cells, limited ingrowth	Better ingrowth with bigger pores
First use	(Ku <i>et al.</i> 1957)		(Norton, L. and Eiseman, B. 1975)		(Boretos, J.W. and Pierce, W.S. 1967)	
Review e.g.	(Xue, L. and Greisler, H.P. 2003)		(Nishibe, T. <i>et al.</i> 2007)		(Tiwari, A. <i>et al.</i> 2002)	

3.3.4 Composite vascular grafts

Attempts at improving disadvantageous anastomotic hemodynamics include alternative surgical techniques and interposition of an autologous vein segment. When the patient's own vein graft is of insufficient length, feasible options that increase the patency rates may be: a *composite bypass* - prosthesis and vein are spliced together with an additional anastomosis (Bastounis, E. *et al.* 1999), a *sequential bypass*, which is formed as a distal venous extension graft to the preceding proximal prosthetic bypass (Mahmood, A. *et al.* 2002), and a *bridge graft*, which connects two patent distal arteries with a short vein segment (Deutsch, M. *et al.* 2001).

Own venous tissue can be placed in the form of a vein patch or cuff between an ePTFE graft and the artery to reduce the anastomotic compliance mismatch and to improve the prosthesis patency. Procedures implemented during the 1980s and 1990s include the Miller cuff (Figure 8), the Linton patch, the Taylor patch and the St. Mary boot (Moawad, J. and Gagne, P. 2003).

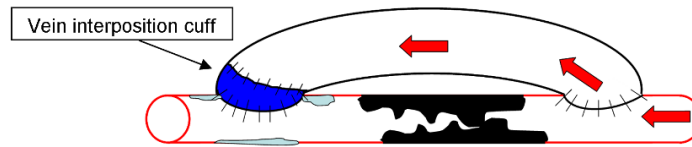


Figure 8. Vein interposition cuff between the prosthetic vascular graft and the native artery that may decrease the intimal hyperplastic response.

Vein interposition techniques are generally useful for distal anastomosis located below the knee (Mamode, N. and Scott, R.N. 2000), but are unimportant in above-the-knee position (Twine, C.P. and McLain, A.D. 2010; Khalil, A.A. *et al.* 2012). The underlying mechanism consists in reducing the compliance mismatch and suppressing IH (Cabrera Fischer, E.I. *et al.* 2005).

3.4 Tissue engineering

3.4.1 Introduction

Tissue engineering is the discipline that strives to reestablish not only the mechanical function but mainly the essential biological functions of body implants. A viable layer of endothelial cells is the best anti-thrombogenic surface and, moreover, properly functioning EC may induce a quiescent phenotype of the underlying VSMC (Stegemann, J.P. *et al.* 2005). There are three possible sources of spontaneous **graft lumen endothelialization** *in vivo* post implantation (Figure 9): trans-anastomotic ingrowth from the native artery, trans-mural tissue and capillary ingrowth through the prosthesis wall, and a “fall-out” or blood-borne source from circulating progenitor cells. Unlike animals, **humans** seldom achieve spontaneous endothelialization more than 1-2 cm from an anastomosis, and trans-mural ingrowth is hampered by the structure of currently-used prostheses (Pennel, T. *et al.* 2013). Blood-borne endothelialization of vascular prostheses from bone marrow stem cells is also sporadic. The blood-contacting surface therefore remains **unhealed** and is covered with a layer of compacted plasma proteins only (Pasquinelli, G. *et al.* 1990). Synthetic vascular grafts therefore fail in part due to thrombosis resulting from lack of endothelium (Zilla, P. *et al.* 2007).

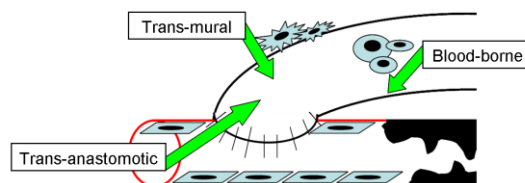


Figure 9. Three possible sources of a spontaneous endothelial lining of vascular prosthetic grafts. None of them proved to be of much significance in humans.

To overcome the fundamental inconveniencies of vascular prostheses, novel biomaterials research (Shin, H. *et al.* 2003) and particularly tissue engineering modalities are increasingly being adopted (Isenberg, B.C. *et al.* 2006). According to the original definition, **tissue engineering** is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions (Langer, R. and Vacanti, J.P. 1993). The main types of cells in the vascular wall are EC and VSMC. By analogy with this, tissue engineering of vascular grafts makes an effort to introduce these living cells into the design of vascular implants in order to make the vascular grafts more hemocompatible or more biocompatible and finally to improve their clinical performance (Figure 10). Promising and clinically proven outcomes have already been achieved with tissue-engineered vascular grafts (Meinhart, J.G. *et al.* 2001).

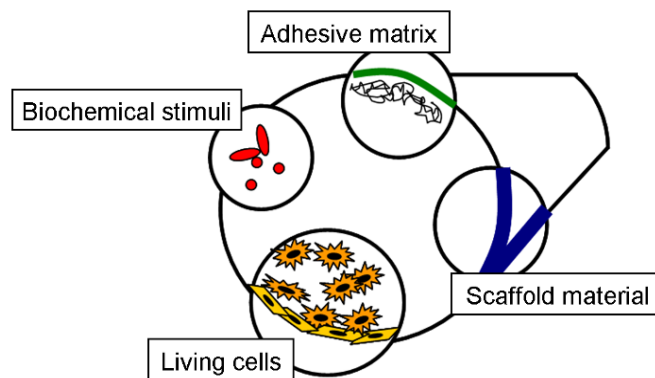


Figure 10. Scheme of the tissue engineering of a vascular graft.

3.4.2 Biosynthetic vascular grafts

The concept of seeding endothelial cells onto the graft lumen before implantation was experimentally implemented in the late 1970s (Herring, M. *et al.* 1978), and consequently this autologous EC “transplantation” managed to improve the patency of human Dacron prostheses (Herring, M. *et al.* 1984) in a non-smoker population. Endothelial cells from a subcutaneous vein segment (*e.g.* saphenous, cephalic or jugular vein) are harvested and immediately seeded onto the graft lumen within the timeframe of one operation. The results of the first clinical trials performed during the 1980s were controversial and disappointing, mainly due to insufficient cell density (Bordenave, L. *et al.* 1999). However, sophisticated EC extraction and retention techniques (Tiwari, A. *et al.* 2001) and the search for more abundant cell sources, such as microvascular endothelium from fat tissue or an omentum biopsy, improved the outcome of this **single-stage cell seeding** method (Alobaid, N. *et al.* 2005).

On the other hand, the **double-stage** approach involves a prolonged incubation and cell multiplication period (2-4 weeks) between cell harvesting and implantation (Bordenave, L. *et al.* 2005). Nevertheless, *in vitro* flow pretreatment or shear stress preconditioning of the cell-material constructs before exposing them to arterial conditions enhances cell retention by

inducing structural changes and adaptation (Rademacher, A. *et al.* 2001). This means that an endothelial cell monolayer is physiologically exposed to certain mechanical forces, namely hydrostatic pressure resulting from blood pressure, shear stress resulting from tangential friction of blood flow against the vessel wall, and finally longitudinal and circumferential cyclic stretch resulting from repeated blood vessel distension due to the cardiac cycle (Lehoux, S. *et al.* 2006). Chronic laminar versus turbulent **shear stress** seems to be of utmost importance for endothelial and vascular smooth muscle cell function, regulating signal transduction in these cells, their alignment, molecule secretion, cytoskeleton reorganisation, gene expression, cell migration, proliferation and survival (Chien, S. 2007). These processes ultimately influence thrombogenesis and atherogenesis, and similar cellular events and phenotypes can be observed when cells are seeded on biomaterials (Fernandez, P. *et al.* 2007).

Early results of clinical *in vitro* double-stage endothelialization were promising (Figure 11) and were described as closing the gap between prosthetic and vein grafts (Meinhart, J. *et al.* 1997). A relatively recently published paper reported overall 5-year and 10-year patency of endothelialized fibrin glue-precoated ePTFE femoro-popliteal bypass grafts of 69% and 61%, respectively (Deutsch, M. *et al.* 2009). Interestingly, the feasibility of endothelium-seeded vascular prostheses was also confirmed in coronary artery bypass grafting, though it is not widely used (Laube, H.R. *et al.* 2000).

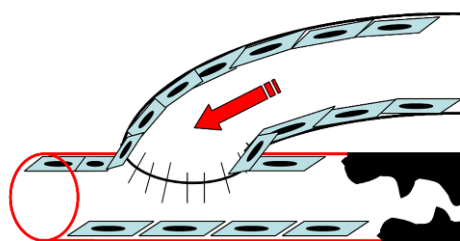


Figure 11. Biosynthetic graft: a vascular prosthesis was lined with endothelial cells *in vitro* prior to implantation in order to achieve less thrombogenicity.

In an attempt to achieve better clinical success, additional cell sources and cell retention technologies are being investigated. Vascular cells are **anchorage-dependent**, and integrin receptor-mediated adhesion occurs via ECM proteins that are adsorbed from the cell culture media *in vitro* or from the blood *in vivo* (Bacakova, L. *et al.* 2004). Thus, to regulate cell attachment, biomaterials are coated with ECM proteins such as collagen, laminin and fibronectin (Vara, D.S. *et al.* 2005), or are modified by covalent bonding of short adhesive peptides which may be cell specific, such as the Arg-Gly-Asp (RGD) sequence (Bacakova, L. *et al.* 2007). Fibrin, as a natural scaffold for cell migration and healing, plays a pivotal role in tissue engineering of vascular grafts (Filova, E. *et al.* 2009).

3.4.3 Living vascular grafts

The concept of completely biological living grafts (totally-engineered blood vessel, TEBV) implies the ability to remodel, grow, self-repair and respond to the immediate environment. Similarly to the native artery, the graft would consist of a functional endothelial cell layer resting on metabolically active smooth muscle cells which are in a contractile phenotype. The graft would also contain enough collagen and elastin proteins to display desirable viscoelastic properties, and would lack any synthetic foreign material that would initiate chronic inflammatory responses or be susceptible to infection. If a synthetic material is used, it should be non-immunogenic, non-thrombogenic and of appropriate compliance. In the ideal case, it should be degradable, providing a temporary scaffold for vascular tissue regeneration, and should be gradually removed and replaced by the newly-forming tissue.

In the construction of tissue-engineered vascular grafts, three major components must be addressed: a scaffold to provide the initial graft shape and strength, an adhesive matrix, and living vascular cells (Baguneid, M.S. *et al.* 2006). For the scaffold, four major approaches can be identified: permanent synthetic support, natural acellular tissues, a biodegradable scaffold, and non-scaffold technology (Campbell, J.H. *et al.* 1999).

The first seminal attempt involved seeding bovine EC, VSMC and fibroblasts onto collagen gel tubes (Weinberg, C.B. and Bell, E. 1986). An external Dacron mesh reinforcement had to be added due to poor mechanical strength. An example of the use of a natural scaffold involves seeding human cells on decellularized porcine aorta (Bader, A. *et al.* 2000). An example of the use of a biodegradable scaffold is a landmark experiment with polyglycolic acid (PGA). The VSMC followed by EC were seeded onto PGA, and the resulting vascular grafts were implanted in pigs, with excellent results (Niklason, L.E. *et al.* 1999). Seeding bone-marrow cells (BMC) onto a biodegradable scaffold enabled the establishment of a totally-engineered autograft implanted into the inferior vena cava of a dog, and explant analysis showed that BMCs were able to differentiate in both endothelial and smooth muscle cells (Matsumura, G. *et al.* 2003).

The first case of a tissue-engineered pulmonary artery transplant composed of biodegradable synthetic polymer (polycaprolactone-poly-lactic acid copolymer reinforced with woven PGA) seeded with autologous cells obtained from peripheral vein specimen was performed in Tokyo (Shin'oka, T. *et al.* 2001). Twenty-five grafts seeded with autologous bone marrow-derived cells were implanted as cavo-pulmonary conduits (low-pressure circulation) mainly in pediatric patients with congenital heart defects, and clinical safety, feasibility and excellent patency were reported with maximum follow-up of nine years. One graft developed non-significant mural thrombosis and four (16%) of the grafts developed stenosis, which was successfully treated with percutaneous balloon angioplasty (PTA) (Hibino, N. *et al.* 2010).

The totally-engineered blood vessel for high-pressure application was introduced as early as in 1998 in an animal model (L'Heureux, N. *et al.* 1998), and was clinically implanted into human patients in 2004. The non-scaffold or “self-assembly” approach produced a graft composed exclusively of human cells, which were harvested from a small skin and vein biopsy. A vascular access graft for hemodialysis (high-pressure circulation) was fabricated by a cell sheet-based technique without using any synthetic material by wrapping and culturing fibroblast and VSMC “cellular sheets” on a PTFE mandril, which was then removed and ECs were seeded in the lumen. This was the first completely biological TEBV to display burst strength comparable to that of human vessels (2 000 mmHg). Ten patients received the conduit in the Cytograft clinical trial in centers in Poland and Argentina between 2004 and 2007 (L'Heureux, N. *et al.* 2006; L'Heureux, N. *et al.* 2007b). Three grafts failed, one patient was withdrawn from the study, another patient died of unrelated causes, and five patients had grafts functioning for hemodialysis 6-20 months after implantation. Primary 1-month patency was 78% and primary 6-month patency was 60%, meeting the approved criteria for a high-risk patient cohort (McAllister, T.N. *et al.* 2009). A recent paper by this group reports 13 implanted patients and an adverse event rate of 0.7 events/patient-year, which is within the range observed for synthetic grafts and native fistulas (AVF) in the general hemodialysis patient population (Peck, M. *et al.* 2012). Another interesting approach is the use of an acellularised deceased donor iliac vein graft re-seeded with autologous EC and VSMC differentiated from bone marrow stem cells. This graft was transplanted in a 10-year-old girl with extrahepatic portal vein obstruction as a mesenterico-portal shunt to alleviate portal hypertension and gastrointestinal bleeding (Olausson, M. *et al.* 2012).

A relatively novel approach using *in situ* or *in vivo* **tissue engineering** has recently been adopted in order to avoid supplemental procedures such as cell harvesting and cell seeding (Bouten, C.V. *et al.* 2011). For example, fast degrading elastomeric vascular grafts have been prepared with a **cell-free technology**: synthetic grafts were composed of poly(glycerol sebacate) (PGS) and polycaprolactone fibers soaked in heparin and implanted in rat aorta. The appropriate pore size enabled host remodeling and cell in-growth during degradation of the polymers, thus forming a neo-artery with distinct functional cellular layers in 3 months, and this neo-artery was pulsating synchronously with the host aorta (Wu, W. *et al.* 2012). In another study, PCL scaffolds were implanted into rat abdominal aorta for up to 18 months. Despite endothelialization and excellent structural integrity, regression of cellularity and calcifications were observed between 12-18 months after implantation (de Valence, S. *et al.* 2012). An outline of recent experimental and clinical success in tissue engineering of vascular grafts is presented in Figure 12.

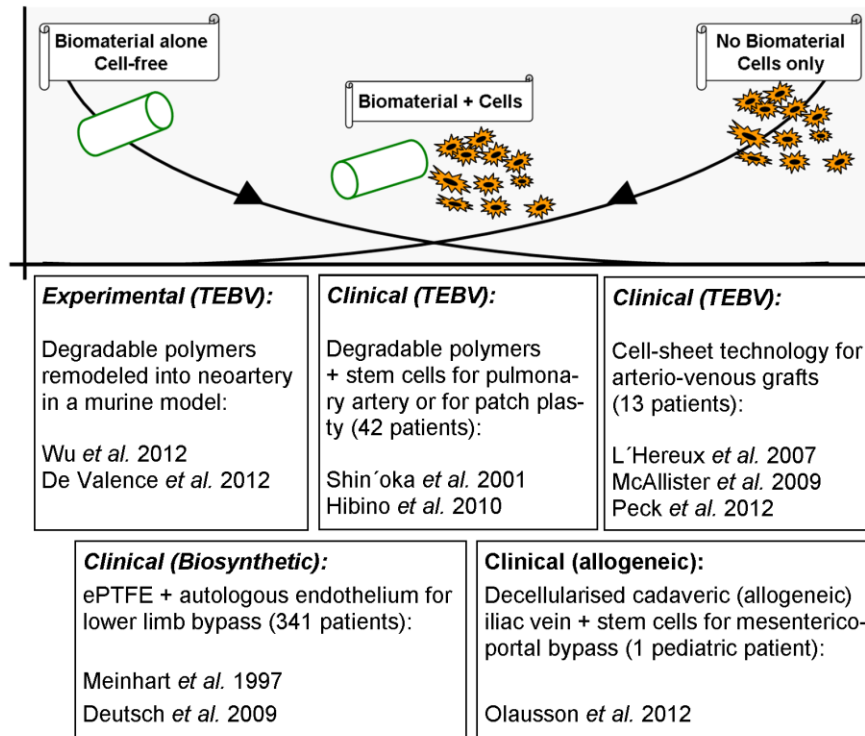


Figure 12. Outline of recent clinical and experimental success in tissue engineering of vascular grafts. Totally-engineered blood vessel (TEHV), expanded polytetrafluoroethylene (ePTFE).

A summary of composite, biosynthetic and totally-engineered vascular grafts is outlined in Table 4. For a more concise review of vascular replacements and tissue engineering of vascular grafts, see Appendix 4 (Chlupac, J. *et al.* 2009).

Table 3. Composite, biosynthetic and totally engineered grafts in experimental and clinical use.

	Composite grafts	Biosynthetic grafts		Totally engineered blood vessels	
	Clinically	Experimentally	Clinically	Experimentally	Clinically
Advantages	Reduced compliance mismatch and intimal hyperplasia, improved patency in femoro-distal bypasses, easier redo surgery	Antithrombogenic, better patency		Responsiveness, non-thrombogenicity, self-repair, growth, metabolically active, potentially cost-effective	
Disadvantages	Technically demanding, prolonged surgery time	No emergency use, cell amplification problems, cell culture contamination risk		Demanding fabrication, time-consuming and expensive, bioreactor cell laboratory, specialized centers only	
Healing	Less intimal hyperplasia	Self-renewing functional endothelium		Complete integration	
First use	(Siegman <i>et al.</i> 1979)	(Herring, M. <i>et al.</i> 1978)	(Herring, M. <i>et al.</i> 1984)	(Weinberg, C.B. and Bell, E. 1986)	(Shin'oka, T. <i>et al.</i> 2001)
Review e.g.	(Moawad, J. and Gagne, P. 2003)	(Seifalian, A.M. <i>et al.</i> 2002)	(Bordenave, L. <i>et al.</i> 2008)	(Isenberg, B.C. <i>et al.</i> 2006)	(L'Heureux, N. <i>et al.</i> 2007a)

4. Objectives of the work

The fundamental issues in vascular tissue engineering and the logical sequence of actions are: choice of the scaffold material, fabrication of the adhesive substrate, cell sourcing, adhesion and proliferation of the cells in a static culture, cell retention in a dynamic culture under flow, maintenance of a functional phenotype, testing in two-dimensional (2D) and three-dimensional (3D) systems, *in vivo* animal study and possible human implant.

The ultimate objectives of the work were to modify **clinically-used PET** vascular prostheses in order to improve their **biocompatibility towards endothelial cells** for the purposes of vascular tissue engineering. To achieve this, the following steps were performed with increasing experimental complexity:

1. Planar 2D static endothelial cell (EC) culture experiments *in vitro*:

- 1.1. EC on fibrin assemblies (**published**)
- 1.2. EC on fibrin assemblies with ECM proteins (**published**)
- 1.3. EC on PET vascular prostheses with fibrin assemblies and VEGF
- 1.4. EC and VSMC on PET, PET/Collagen and ePTFE vascular prostheses
- 1.5. EC and VSMC on PET/Collagen vascular prostheses with adhesive protein assemblies
- 1.6. EC on PET with degradable copolymers and adhesive protein assemblies (**published**)

2. Planar 2D dynamic cell culture experiments *in vitro*:

- 2.1. Phenotype of human primary EC on ECM protein assemblies under flow (**published**)

3. Tubular 3D dynamic cell culture experiments *in vitro*:

- 3.1. Human primary EC on PET/Coll with protein assemblies under flow (**published**)

4. Tubular 3D animal (porcine) implantation experiments *in vivo*:

- 4.1. Exploring the feasibility of various surgical anatomy
- 4.2. Double-stage seeding of autologous EC on PET/Coll with adhesive protein assemblies
- 4.3. Comparison of the clinical performance of seeded and unseeded grafts

5. Materials and methods

5.1 Planar 2D static cell culture experiments *in vitro*

5.1.1 EC on fibrin assemblies

A detailed description of materials and methods is given in Appendix 2 (Filova, E. *et al.* 2009). Briefly, polystyrene (PS) wells in 24-well plates were coated with 10 types (a-j) of 2D and 3D fibrin gels. The PS (k) served as a control surface. **2D Fb structures** (a-b) were prepared by repeated adsorption of fibrinogen (Fbg), incubating the surface with thrombin (Thr) solution, and inhibiting surface-attached Thr (by hirudin (Hr) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, PPACK). **Ultrathin 3D Fb gels** (c-f) were obtained if Thr inhibitors AT III and heparin were added into an Fbg solution. **Thick 3D Fb networks** (g, h) and **Fb bulks** (i, j) were formed by catalytic action of surface-attached Thr on an ambient Fbg solution. A summary of the samples was as follows:

- a. 2D Fb layer, low-density, inhibitors used: PPACK and Hr
- b. 2D Fb layer, high-density, inhibitors used: PPACK and Hr

- c. 3D thin surface Fb gel, low concentration, inhibitors used: AT III + Hep
- d. 3D thin surface Fb gel, high concentration, inhibitors used: AT III + Hep
- e. 3D crosslinked thin surface Fb gel, low concentration, inhibitors used: AT III + Hep
- f. 3D surface Fb gel from heparinized blood plasma

- g. 3D thick surface Fb gel, low concentration
- h. 3D thick surface Fb gel, high concentration

- i. 3D bulk Fb gel
- j. 3D bulk gel from human blood plasma

The formation of the surface Fb structures was observed using surface plasmon resonance (SPR), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). A polylactide fibrous scaffold was modified with a surface Fb film without filling the inner pores with a bulk gel. The growth of bovine pulmonary artery EC of the CPAE line (17 000 cells/cm²) seeded on PS surfaces coated with the Fb films was evaluated by the number and the morphology of the adhering EC and the concentration of β -actin, vinculin, α_v -integrin, and von Willebrand factor.

For better orientation, all the Fb assemblies used in all of the experiments are listed in Table 4. Similar Fb samples used in different experiments are highlighted with the same color.

Table 4. List of experiments with endothelial cells on fibrin assemblies. Similar Fb samples used in different experiments are highlighted with the same script and color. Thr inhibitor is a substance that inhibits thrombin during sample preparation. Abbreviations: antithrombin III (AT III), bovine pulmonary artery endothelial cells of the CPAE line (CPAE), collagen (Co), concentration (conc.), cross-linked (X-linked), density (dens.), D-phenylalanyl-L-prolyl-L-arginine chlormethyl ketone (PPACK), endothelial cells (EC), extracellular matrix (ECM), fibrin (Fb), fibrinogen (Fbg), fibronectin (FN), heparin (Hep), hirudin (Hr), human saphenous vein endothelial cells (HSVEC), laminin (LM), poly(L-lactide-co-glycolide) (PLG), poly(L-lactide-co-glycolide-co- ϵ -caprolactone) (PLGC), polyethylene terephthalate (PET), polyethylene terephthalate with commercial collagen impregnation (PET/Coll), polystyrene (PS), three-dimensional 3D, thrombin (Thr), two-dimensional (2D), von Willebrand factor (vWf).

Experiments with Endothelial Cells on Fibrin Assemblies

Fb assemblies (CPAE)		Thr inhibitor	Cell response
2D thin Fb gels	a 2D Fb layer, low-density	PPACK + Hr	Best spreading, the highest population
	b 2D Fb layer, high-density	PPACK + Hr	
3D ultrathin Fb gels	c 3D thin surface Fb gel, low concentration	AT III + Hep	Best spreading
	d 3D thin surface Fb gel, high concentration	AT III + Hep	
	e 3D X-linked thin Fb gel, low conc.	AT III + Hep	
3D thick Fb networks	f 3D Fb gel from heparinized blood plasma		Best cell differentiation (vWF), the highest population.
	g 3D thick surface Fb gel, low conc.		
3D Fb bulk Fb	h 3D thick surface Fb gel, high conc.		
	i 3D bulk Fb gel		
Control	j 3D bulk gel from human blood plasma		
	k PS		

Fb assemblies + ECM proteins (CPAE)

Fbg monolayer	a Fbg monolayer, low-density		
2D Fb films	b Double-layer film Fb, low-density	PPACK + Hr	
	c Triple-layer film Fb, low-density	PPACK + Hr	
	d Triple-layer film Fb, high-density	PPACK + Hr	
	e Double layer film + LM, low-density	PPACK + Hr	
	f 3D surface Fb network (low-density)	AT III + Hep	
3D thin Fb networks	g 3D Fb network, X-linked, low density	AT III + Hep	Best initial attachment
	h 3D surface Fb network + LM, low-dens.	AT III + Hep	Increased numbers of longer and thicker focal adhesion plaques of vinculin
3D Fb networks + ECM proteins	i 3D surface Fb network + FN, low-density	AT III + Hep	
	j 3D surface Fb network + CO, low-density	AT III + Hep	
Bulk Fb gel	k Bulk Fb gel		Low initial attachment
Control	l PS		

PET + Fb assemblies (CPAE)

3D thick Fb networks	1 3D thick Fb network		The highest initial and final cell density Higher cell density than on PET
	2 3D thick Fb network + incorporated VEGF		
3D bulk Fb gels	3 3D bulk Fb gel		
	4 3D bulk Fb gel + incorporated VEGF		
	5 3D bulk Fb gel + surface-attached VEGF		
Control	6 PET		

PET/Coll + adhesive protein assemblies (CPAE)

PET/Coll +Co/Fb	5 3D thin ("thicker") surface Fb gel, "medium" concentration		High final density
PET/Coll +Co/Fb/FN	6 3D thin ("thicker") surface Fb gel, "medium" concentration + attached FN		Low growth

PET + degradable copolymers + adhesive protein assemblies (CPAE)

PET or PET-PLG or PET-PLGC +Co/Fb	5 3D thin ("thicker") surface Fb gel, "medium" concentration	AT III	Enhanced cell growth
PET or PET-PLG or PET-PLGC +Co/Fb/FN	6 3D thin ("thicker") surface Fb gel, "medium" concentration + attached FN	AT III	

Tubular PET/Coll + adhesive protein assemblies under flow (HSVEC)

PET/Coll +Fb	3 3D thin ("thicker") surface Fb gel, "medium" concentration	AT III	Increased flow resistance
PET/Coll +Fb/FN	4 3D thin ("thicker") surface Fb gel, "medium" concentration + attached FN	AT III	

Porcine experiment (autologous EC)

PET/Coll +Fb/FN	3D thin ("thicker") surface Fb gel, "medium" concentration + attached FN	AT III	Possibly increased bypass patency
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5.1.2 EC on fibrin assemblies with ECM proteins

A detailed description of the materials and methods is given in Appendix 3 (Filova, E. *et al.* 2014). Briefly, 11 types (a-k) of structures were prepared on the bottoms of PS wells in 24-well plates: a **fibrinogen** layer (a), **2D Fb assemblies** (b-e) and **thin 3D Fb assemblies** (f-j) using various Thr inhibitors and Fbg concentrations and **bulk Fb gel** (k). Some of the assemblies were subsequently coated with **LM** (e, h), type I **Co** (j) or **FN** (i). Sample g was cross-linked. The samples were summarized as follows:

a. Fbg (low-density) monolayer

b. Double-layer film Fb (low-density), inhibitors used: PPACK and Hr

c. Triple-layer film Fb (low-density), inhibitors used: PPACK and Hr

d. Triple-layer film Fb (high-density), inhibitors used: PPACK and Hr

e. Double layer film with attached laminin, (low-density), inhibitors used: PPACK and Hr

f. 3D surface Fb network (low-density), inhibitors used: AT III + Hep

(- sample (c) from previous experiment)

g. 3D surface Fb network, crosslinked, (low-density), inhibitors used: AT III + Hep

(- sample (e) from previous experiment)

h. 3D surface Fb network with attached laminin (low-density), inhibitors used: AT III + Hep

i. 3D surface Fb network with attached fibronectin (low-density), inhibitors used: AT III + Hep

j. 3D surface Fb network with attached collagen (low-density), inhibitors used: AT III + Hep

k. Bulk Fb gel

l. PS

The samples were observed by SPR and atomic force microscopy (AFM). The attachment, spreading, morphology, growth and maturation of EC (CPAE line, 17 000 cells/cm²) seeded on top of the Fb layers were evaluated. Immunofluorescence staining and an enzyme-linked immunosorbent assay (ELISA) of vinculin, talin (proteins of focal adhesions), vascular endothelial cadherin (VE-cadherin) (protein of cell-cell junctions), PECAM-1 (CD-31) and vWF was performed. In addition, ELISA of ICAM-1 and VCAM-1 was performed.

5.1.3 EC on PET vascular prostheses with fibrin assemblies and VEGF

The materials and methods are similar to those given in Appendix 2 (Filova, E. *et al.* 2009). Briefly, planar patches (1×1 cm) of clinically-used vascular prostheses made of PET

(VÚP, Joint-Stock Comp., Brno, CZ) were put in 24-well plates and modified with 5 different Fb assemblies:

1. Fb, a thick 3D Fb network: fibrin 50 µg/ml 60 min, Tris, Thr 2.5 U/ml 10 min, Tris, Fbg 1.5 mg/ml.

- This sample was similar to sample (h): a thick 3D Fb gel (high concentration) from the experiment with EC on Fb assemblies.

2. Fb+VEGF (vascular endothelial growth factor, human recombinant VEGF 165, BioVision), a thick 3D Fb network with incorporated VEGF: Fb 50 µg/ml 60 min, Tris, Thr 2.5 U/ml 10 min, Tris, Fbg 1.5 mg/ml + VEGF 100 ng/ml.

3. Fb bulk, Fb 3D gel: Fbg 1.5 mg/ml and Thr 2.5 U/ml. .

- This sample was similar to sample (i): 3D bulk Fb gel from the experiment with EC on Fb assemblies.

4. Fb bulk+VEGF, 3D Fb gel with incorporated VEGF: Fbg 1.5 mg/ml and Thr 2.5 U/ml and VEGF 100 ng/ml.

5. Fb bulk/VEGF, 3D Fb gel with surface-attached VEGF: Fbg 1.5 mg/ml and Thr 2.5 U/ml, after network formation VEGF 100 ng/ml for 120 min.

6. PET.

The modified grafts were seeded with bovine pulmonary artery EC cells of the CAPE line (5.0×10^4 cells/cm²). The EC were grown in Minimum Essential Eagle Medium (MEM) (Sigma, USA) supplemented with 20% of BFS (Sebak, Germany). Cell densities were assessed on days 1, 4 and 7 using an automatic cell counter (Vi-CELL XR 2.03, Beckman Coulter, Inc., Miami, FL, USA). Pure PET was used as positive control surfaces. Some of the samples were fixed with 70% ethanol, the EC were immunostained for β -actin (mouse monoclonal anti- β -actin primary antibody, clone AC-15, Sigma, Cat. No. A5441; goat anti-mouse IgG (H+L) secondary antibody, F(ab')₂ fragment, conjugated with Alexa Fluor 488 (2 mg/ml, Invitrogen, Cat. No. A11017), the cell nuclei were counterstained with propidium iodide (5 µg/ml, Sigma, Cat. No. P4170) and the cells were observed using an epifluorescence microscope (Olympus IX51) equipped with a digital camera (Olympus DP70, Japan).

5.1.4 EC and VSMC on PET, PET/Collagen and ePTFE vascular prostheses

Planar patches (1×1 cm) of clinically-used vascular prostheses made of ePTFE (Gore and Assoc., Flagstaff, AZ, USA), PET and PET with collagen (PET/Coll) (VÚP, Joint-Stock Comp., Brno, CZ) and were put in 24-well plates (TPP, Switzerland) and seeded with bovine pulmonary artery EC cells of the CAPE line (8 100 cells/cm² on ePTFE and 45 300 cells/cm² on both PET and PET/Coll) and primary rat aortic VSMC (15 700 cells/cm² on ePTFE and 45 300 cells/cm² on both PET and PET/Coll). The ECs were seeded and grown in Minimum Essential Eagle Medium (MEM) (Sigma, USA) supplemented with 20% of BFS (Sebak, Germany) and

the VSMC were grown in Dulbecco's Modified Minimum Essential Medium (DMEM) (Sigma, USA) with 10% BFS. Cell densities were assessed on days 2, 3 and 7 on ePTFE and on days 1, 5 and 7 on PET and PET/Coll, using an automatic cell counter (Vi-CELL XR 2.03, Beckman Coulter, Inc., Miami, FL, USA). Tissue culture polystyrene (TCPS) and a standard microscopy cover glass slide were used as positive control surfaces. Some of the samples were fixed with 70% ethanol, the cell nuclei were stained with propidium iodide (5 µg/ml, Sigma, Cat. No. P4170) and were observed using an epifluorescence microscope (Olympus IX51) equipped with a digital camera (Olympus DP70, Japan).

5.1.5 EC and VSMC on PET/Collagen vascular prostheses with protein assemblies

The materials and methods were similar to those described in detail in Appendix 5 (Chlupac, J. *et al.* 2014a). Briefly, planar patches 1.6 cm in diameter were punched from clinically-used non-crimped PET vascular prostheses with bovine type I collagen (PET/Coll) (VÚP, Joint–Stock Comp., Brno, Czech Republic) and inserted into tissue culture 24-well plates. The luminal surface of the grafts was coated with collagen type I (Co), to which ECM proteins LM, FN, or surface Fb gel were attached. Five types of protein assemblies (+Co, +Co/LM, +Co/FN, +Co/Fb, +Co/Fb/FN) were fabricated. Pure PET/Coll served as a control surface.

The constructs were seeded with EC of passage 20-22, derived from bovine pulmonary artery (line CPAE ATCC CCL-209, Rockville, MA, USA) or with primary rat aortic VSMC of passage 7-10 at a density of 5.0×10^4 cells/cm². The VSMC were harvested using the explantation method (Bacakova, L. *et al.* 2007). The VSMC were cultured in DMEM essential medium supplemented with 10% FBS and the EC were cultured in MEM essential medium with 20% FBS. Cell densities were counted on culture days 1, 4 and 7. The EC were immunostained for β -actin (mouse monoclonal anti- β -actin primary antibody, clone AC-15, Sigma, Cat. No. A5441; goat anti-mouse IgG (H+L) secondary antibody, F(ab')₂ fragment, conjugated with Alexa Fluor 488 (2 mg/ml, Invitrogen, Cat. No. A11017). The VSMC were stained for α -actin (anti α -smooth muscle actin monoclonal antibody, clone 1A4, Sigma and FITC-anti-mouse secondary antibody, Sigma) and the cell nuclei were counterstained with propidium iodide (5 µg/ml) (Sigma, Cat. No. P4170). The cells were observed in a Leica TCS SP2 AOBs confocal microscope at magnification 10× and 40×.

5.1.6 EC on PET with degradable copolymers and adhesive protein assemblies

The materials and methods are described in detail in Appendix 5 (Chlupac, J. *et al.* 2014a). Briefly, knitted PET vascular graft patches (VÚP, Joint–Stock Comp., Brno, Czech Republic) were impregnated with the degradable copolymer poly(L-lactide-co-glycolide) (PLG) or with the terpolymer poly(L-lactide-co-glycolide-co- ϵ -caprolactone) (PLGC). The luminal

surface of the grafts was coated with collagen type I (Co), to which ECM proteins laminin (LM), fibronectin (FN), or surface fibrin gel (Fb) were attached. Three types of prostheses (PET, PET-PLG and PET-PLGC) and 5 types of protein assemblies (+Co, +Co/LM, +Co/FN, +Co/Fb, +Co/Fb/FN) were fabricated. The surface topography and the wettability were evaluated by scanning electron microscopy and by measurements of the water contact angles. Colonization with bovine pulmonary artery endothelial cells of the CPAE line (5.0×10^4 cells/cm²) was studied in a static culture system for 1 week.

5.2 Planar 2D dynamic cell culture experiments *in vitro*

5.2.1 Phenotype of human primary EC on ECM protein assemblies under flow

The materials and methods are given in detail in Appendix 6 (Chlupac, J. *et al.* 2014b). Briefly, cell-adhesive protein assemblies were fabricated on glass slides as combinations of collagen type I (Co), laminin (LM) and fibronectin (FN), resulting in three samples: Co, Co/LM and Co/FN. The surface topography, roughness and wettability were determined. Human saphenous vein endothelial cells were harvested from cardiac patients, cultured on the assemblies for 48h and then submitted to laminar shear stress of 12dyn/cm² in a flow chamber for 40, 80 and 120min (Figure 13). Cell retention was assessed post shear stress and total mRNA was extracted to perform qRT-PCR. The expression profile was determined of some adhesion genes (vascular endothelial cadherin (VE-cadherin), vinculin, kinase domain insert receptor (KDR) or vascular endothelial growth factor receptor-2 (VEGFR-2), a cluster of differentiation molecule-31 (CD-31) or platelet-endothelial cell adhesion molecule-1 (PECAM-1), β_1 -integrin chain) and metabolic genes (tissue plasminogen activator (t-PA), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), endothelial nitric oxide synthase (eNOS) and matrix metalloproteinase-1 (MMP-1)). Quantitative immunofluorescence of VE-cadherin, vinculin, KDR and von Willebrand factor was performed after 2h and 6h of flow. The static control sample was excluded from shearing.

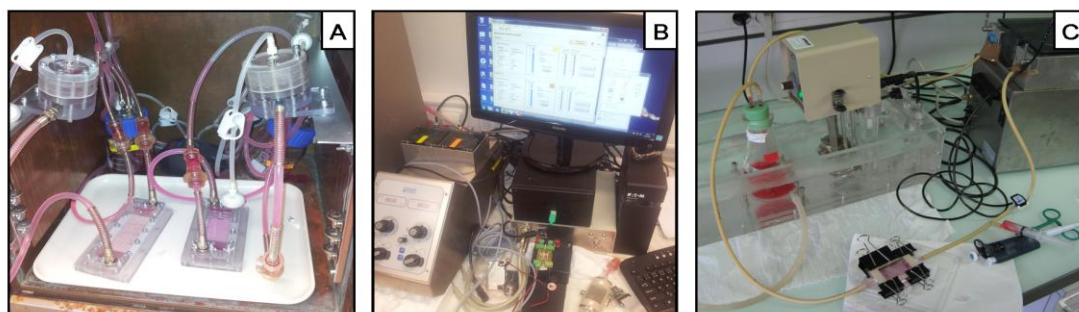


Figure 13. Hemodynamic bioreactor used for exposing endothelial cells to shear stress on 2D planar samples for vascular tissue engineering. **A-B.** Dept. of Biomaterials and Tissue Engineering, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, CZ. **C.** Université de Bordeaux, Bioingénierie tissulaire, INSERM U1026, Bordeaux, France.

5.3 Tubular 3D dynamic cell culture experiments *in vitro*

5.3.1 Human primary EC on PET/Collagen with adhesive protein assemblies under flow

The materials and methods are described in detail in Appendix 7 (Chlupac, J. *et al.* 2014c). Briefly, knitted tubular polyethylene-terephthalate (PET) vascular prostheses (6 mm) with commercial type I collagen (PET/Coll) were modified in the lumen by the adsorption of laminin (LM), coating with a fibrin network (Fb) or a combination of Fb and fibronectin (Fb/FN). Human patient-derived saphenous vein endothelial cells were seeded (1.50×10^5 /cm²) (Figure 14A), cultured for 72 h (Figure 14B) and exposed to laminar shear stress 15 dyn/cm² for 40 and 120 min in a hemodynamic circuit that mimics human arterial conditions (Figure 14C). The control grafts were excluded from shearing.

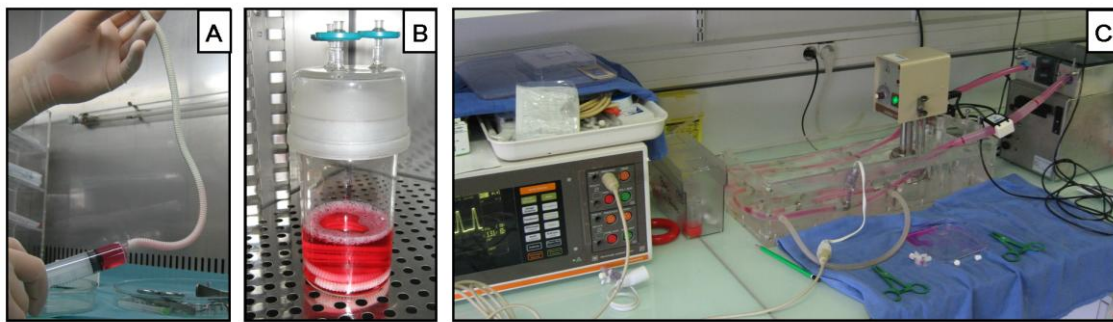


Figure 14. Seeding of human endothelial cells onto clinically-used polyethylene terephthalate (PET) vascular prostheses. **A.** Injection of cell suspension. (Subsequent horizontal rotational seeding not shown.) **B.** Maturation in culture medium. **C.** Hemodynamic bioreactor that simulates the conditions of human arterial circulation used for 3D tissue engineering of vascular grafts (Université de Bordeaux, Bioingénierie tissulaire, INSERM U1026, Bordeaux, France).

5.4 Tubular 3D animal (porcine) implantation experiments *in vivo*

5.4.1 Surgical anatomy

In this experiment, PET vascular prostheses with collagen (PET/Coll) 6 mm in diameter and 6-8 cm in length (VUP Joint-Stock Comp., Brno, Czech Republic) were implanted in a large animal model. The experiment was approved by the local ethics committee (Institute for Clinical and Experimental Medicine, Prague, Czech Rep.). Five animals were used to assess the surgical feasibility of the bypass graft model. An effort was made to mimic clinically implanted bypasses by suturing the anastomoses in end-to-side fashion. Two pig species were used for the surgery: domestic pig and minipig. There is an important difference in vascular anatomy in the aorto-iliac region between humans and pigs, and several hind-limb bypass settings were explored.

5.4.2 Double-stage seeding of autologous EC on PET/Coll with protein assemblies

The PET vascular prostheses with collagen 10 cm in length were modified on the inside by an adhesive matrix composed of fibrin and fibronectin. The surface-attached Fb gel was prepared by rinsing the prosthesis samples with Tris buffer (TB), followed by rinsing successively with solutions of fibrinogen (Fbg, human plasma, Sigma, 50 µg/ml TB) for 60 min, TB, Thrombin (Thr, human plasma, Sigma, 2.5 NIH U/ml) for 10 min, TB, Fbg (200 µg/ml) containing antithrombin III (AT III 0.5 U/ml) for 2 h, TB and PBS. A fibrin network was formed on the surface by catalytic action of the surface-attached thrombin on the ambient Fbg solution. Fibronectin (FN) was attached to the Fb network by incubating the sample with FN solution (50 µg/ml) in PBS overnight. A schematic drawing of the porcine experiment is given in Figure 15.

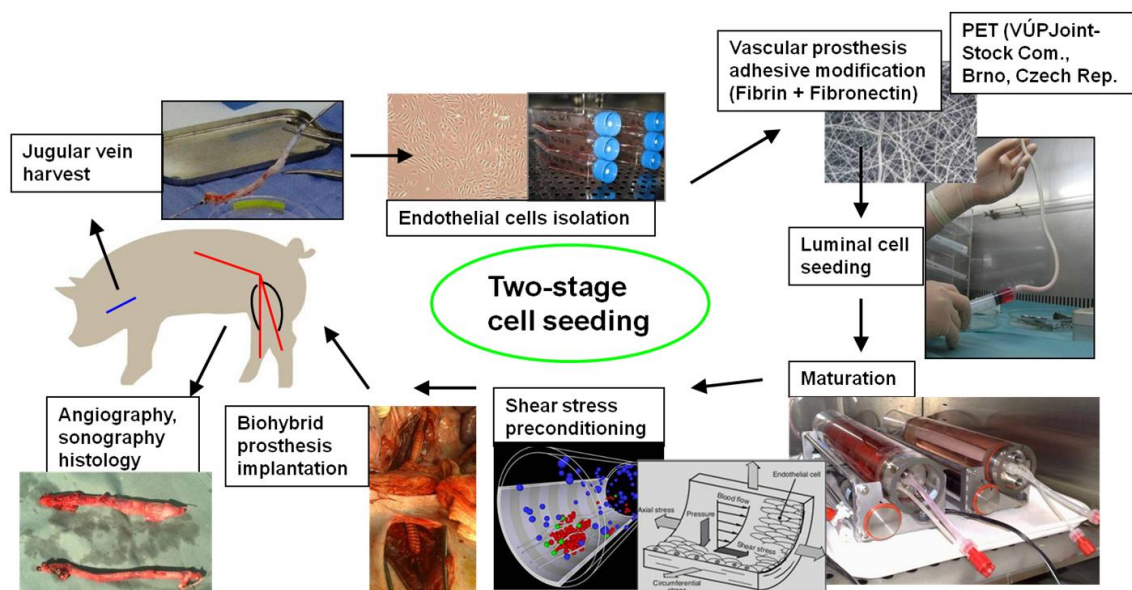


Figure 15. Scheme of the porcine experiment using the double-stage cell seeding method.

The internal jugular vein was removed from the neck of the animals under general anesthesia and was gently flushed with saline. Both ends and side branches were ligated, and the vein was cannulated and injected with collagenase solution for 30 min (Collagenase type 2-filtered, Worthington Biochemical Corp., NJ, USA, Cat. No 58D10513). Released autologous endothelial cells were harvested and mass expanded in Endothelial Cell Basal Medium-2 (EBM-2, Clonetics, MD, USA, Cat. No CC-3156) supplemented with 10% FBS. The EC were rotation-seeded in the lumen (6 rpm, 24h) and flow-preconditioned (1.41 ml/h, 48h) in a bioreactor (Provitro Co., formerly Oligene, Germany). An endothelialized bypass was implanted in one leg and a control unmodified bypass was implanted in the contralateral leg of the same animal under general anaesthesia, heparin 15 000 IU (Zentiva, Czech Republic) and antibiotic (Amoksiklav 1.2 g, Lek Pharmaceuticals d.d., Slovenia) administration. Bypass grafts were sutured to the clamped arteries by running polypropylene 6.0 sutures.

5.4.3 A comparison of the clinical performance of seeded and unseeded grafts

The patency of the bypass grafts was assessed by clinical status, angiography or ultrasound imaging. The prosthetic grafts were retrieved in the operating room under anesthesia, using the same techniques as for implantation. The grafts were removed *en bloc*, including anastomoses and adjacent arterial segments, and were fixed in 5% formaldehyde. The cells in the lumen were immunostained with primary rabbit anti-human von Willebrand factor IgG monoclonal antibody (Sigma) overnight and primary mouse anti- α -smooth muscle actin monoclonal antibody (Sigma) for 3 h. Both antibodies were diluted in PBS at a ratio of 1:200. After rinsing in PBS, the samples were incubated with goat anti-rabbit IgG (H+L) secondary antibody F(ab')₂ fragment, conjugated with Alexa Fluor 633 (red color, 2 mg/ml, Invitrogen, Cat. No. A21072) and goat anti-mouse IgG (H+L) secondary antibody, F(ab')₂ fragment, conjugated with Alexa Fluor 488 (green color, 2 mg/ml, Invitrogen, Cat. No. A11017). Both antibodies were diluted in PBS at a ratio of 1:400 and were incubated for 1.5 h at room temperature. The cell nuclei were counterstained with Hoechst 33342 (5 μ g/ml, Sigma). The cells were evaluated in a Leica TCS SP2 AOBS confocal microscope.

5.5 Statistical analysis

The data is presented as the mean and standard deviation (SD) of usually 4-12 measurements. In experiments performed in duplicate or in triplicate, the data is presented as mean and standard error of mean (SEM). The data was compared using either the unpaired Student *t*-test or one way ANOVA analysis (the Student-Newman-Keuls test or the Holm-Sidak test) for multiple comparison (SigmaStat 3.1 2004, Systat Software, Inc.). The difference between groups was considered significant at $p \leq 0.05$.

6. Results

6.1 *Planar static cell culture experiments in vitro:*

6.1.1 EC on fibrin assemblies

Detailed results including figures are given in Appendix 2 (Filova, E. *et al.* 2009). Briefly, the best initial cell spreading after 1 day was observed on 2D Fb and ultrathin 3D Fb. The highest concentration of vWF, a marker of EC differentiation, was observed after 3 days on thick 3D Fbs. The highest EC population densities after 7 days were observed on 2D Fb and on thick 3D Fb.

6.1.2 EC on fibrin assemblies with ECM proteins

Detailed results including figures are given in Appendix 3 (Filova, E. *et al.* 2014). Briefly, the highest number of initially attached cells was observed on a cross-linked thin 3D Fb layer. A relatively low initial number of EC was observed on the bulk Fb gel. The EC formed a confluent layer on all of the assemblies on day 7. Interestingly, the ECM protein coating significantly improved neither the cell attachment after 1 day, nor the EC proliferation during 7 day culture, in comparison with pure Fb assemblies. Increased numbers of longer and thicker focal adhesion plaques of vinculin were found on ECM protein-coated Fb assemblies and on thick Fb gel. Most of the Fb-based structures did not elevate the concentration of ICAM-1 and VCAM-1 in EC, and therefore their inflammatory activation.

6.1.3 EC on PET vascular prostheses with fibrin assemblies and VEGF

Unpublished data: Population densities of EC (CPAE line) which were grown on PET vascular prostheses with Fb assemblies are shown in Figure 16. The cell density on 1.Fb was significantly higher than the densities on other samples on day 1. The second highest density was observed on 2.Fb+VEGF and on 4.FbBulk+VEGF. The lowest densities were found on 3.FbBulk, on 5.FbBulk/VEGF and on control 6.PET. On day 4, significantly higher cell densities were observed on 2.Fb+VEGF, 3.FbBulk and on 4.FbBulk+VEGF than on 1.Fb, 5.FbBulk/VEGF and 6.PET. The final cell density on 1.Fb on day 7 was significantly higher than the densities on other surfaces. There was no difference in the cell numbers among the other samples on day 7, except for the cell population on 2.Fb+VEGF, which was significantly higher than on 6.PET. The cells on all of the samples increased in number significantly between day 1 and day 4 and between day 4 and day 7.

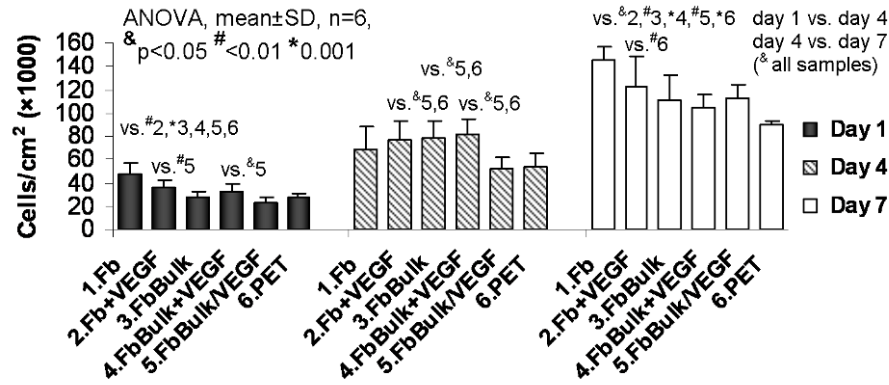


Figure 16. Growth of endothelial cells (bovine pulmonary artery, CPAE line) on patches of vascular prostheses made of polyethylene terephthalate (PET) with the following fibrin (Fb) assemblies: 1. Fb, thick 3D Fb network, 2. Fb+VEGF, thick 3D Fb network with incorporated VEGF, 3. Fb bulk, Fb 3D gel, 4. Fb bulk+VEGF, 3D Fb gel with incorporated VEGF, 5. Fb bulk/VEGF, 3D Fb gel with surface-attached VEGF, 6. PET. The seeding density was 5.0×10^4 cells/cm². Vascular endothelial growth factor (VEGF), versus (vs.).

The morphology of the EC (bovine pulmonary artery, CPAE line) grown on patches of vascular prostheses made of PET with Fb assemblies is shown in Figure 17. Cell confluence was achieved on all samples with Fb assemblies by day 4 (Figure 17A-E and G-K) and on the PET control by day 7 (Figure 17R). The cells seeded on Fb assemblies are well spread and show a typical cobble-stone morphology compared to the cells on the PET control, which grew more along the PET fibers (Figure 17F and L). Phenotypic maintenance can be seen on all of the samples since the cells are immunostained for β -actin.

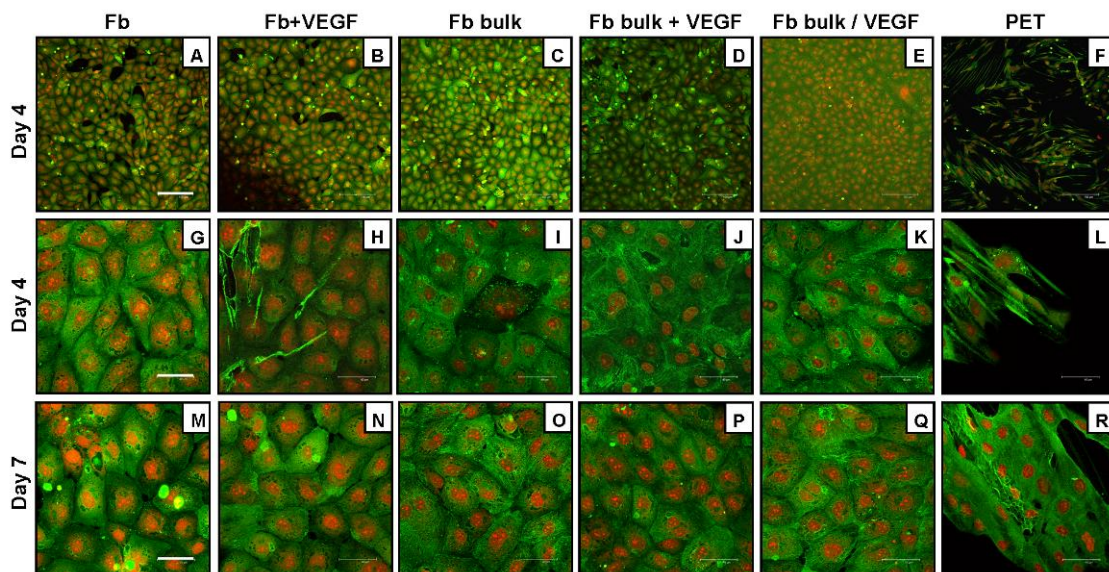


Figure 17. Morphology of endothelial cells (bovine pulmonary artery, CPAE line) on patches of vascular prostheses made of polyethylene terephthalate (PET) with fibrin (Fb) assemblies on

day 4 (A-L) and on day 7 (M-R). Samples: 1. Fb, thick 3D Fb network, 2. Fb+VEGF, thick 3D Fb network with incorporated VEGF, 3. Fb bulk, Fb 3D gel, 4. Fb bulk+VEGF, 3D Fb gel with incorporated VEGF, 5. Fb bulk/VEGF, 3D Fb gel with surface-attached VEGF, 6. PET. The seeding density was 5.0×10^4 cells/cm². Immunofluorescence of α -actin (green), cell nuclei counterstained with propidium iodide (red). Leica TCS SP2 AOBS confocal microscope, magnification 10 $\times$, scale bar = 150 μ m (A-F) or 40 $\times$ and zoom 2 $\times$, scale bar = 40 μ m (G-R). Vascular endothelial growth factor (VEGF).

6.1.4 EC and VSMC on PET, PET/Coll and ePTFE vascular prostheses

Unpublished data: Growth curves of EC (CPAE line) and VSMC (primary rat aortic), which were cultured on patches of vascular prostheses made of ePTFE, PET and PET with collagen (PET/Coll), are shown in Figure 18. The growth of EC on ePTFE was significantly lower than on the control surfaces (TCPS and glass) on each of the culture days. The cells did not increase significantly in number either between days 2 and 3 or between days 3 and 7 on any of the surfaces (Figure 18A). Similarly, the growth of VSMC on ePTFE was lower than on the control surfaces on days 2 and 3, though not significantly lower. However, the cell numbers of VSMC on ePTFE were significantly lower than the numbers on TCPS and on glass on day 7. The cells significantly increased in density on TCPS ($p < 0.01$) and on glass ($p < 0.05$) between days 3 and 7. This was not the case on ePTFE (Figure 18B). The densities of EC on PET and on PET/Coll were significantly lower than the densities on TCPS on any of the culture days. A significant increment in cell numbers was observed only on TCPS between days 1 and 5 ($p < 0.001$) (Figure 18C). The numbers of VSMC on PET and on PET/Coll were also significantly lower than the numbers on TCPS on days 5 and 7, but not on day 1. Again, significant cell growth was noted on TCPS between days 1 and 5 ($p < 0.001$), but not on PET or on PET/Coll (Figure 18D). Interestingly, the growth of both EC and VSMC was lower on PET/Coll than on PET. However, this difference was significant only in EC on PET on day 5 ($p < 0.05$) (Figure 18C).

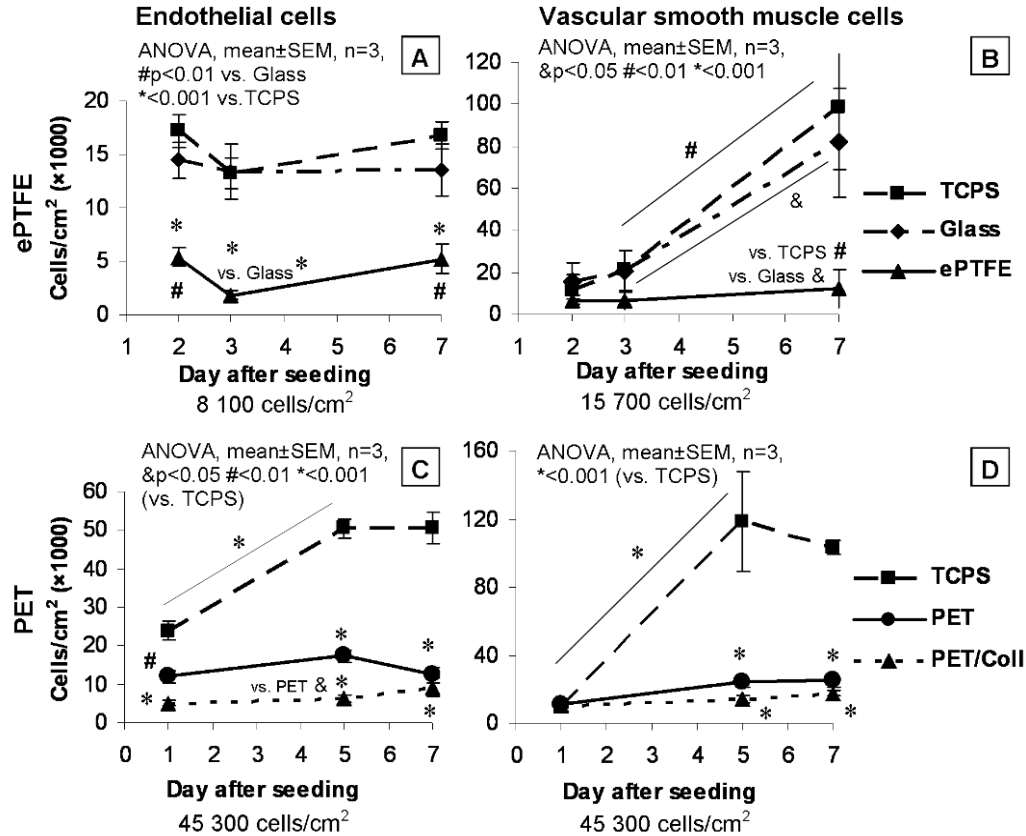


Figure 18. Endothelial cells (bovine pulmonary artery, CPAE line) (A and C) and vascular smooth muscle cells (primary rat aortic) (B and D) on patches of vascular prostheses made of expanded polytetrafluoroethylene (ePTFE) (A-B), polyethylene terephthalate (PET) (C-D) and PET with collagen (Coll) (C-D). Tissue culture polystyrene (TCPS), versus (vs.).

A direct comparison of EC and VSMC grown on PET and PET/Coll without a positive control surface is given in Figure 19. The numbers of EC on PET were significantly higher than the numbers on PET/Coll on day 1 ($p<0.01$) and on day 5 ($p<0.001$), but no longer on day 7. The densities of VSMC on PET were significantly higher than the densities on PET/Coll on day 5 ($p<0.001$) and on day 7 ($p<0.001$), but not on day 1. The populations of VSMC on PET were higher than the populations of EC on PET on day 5 ($p<0.001$) and on day 7 ($p<0.001$), but not on day 1. The numbers of VSMC on PET/Coll were significantly higher than the numbers of EC on PET/Coll on all days of culture: on day 1 ($p<0.05$), on day 5 ($p<0.01$) and on day 7 ($p<0.001$). The VSMC gradually increased in number on both PET and PET/Coll, significantly between day 1 and day 5 on PET ($p<0.05$). The EC increased in density non-significantly on PET/Coll, but significant growth was observed on PET between day 1 and day 5 ($p<0.05$). However, their density dropped significantly ($p<0.05$) between day 5 and day 7.

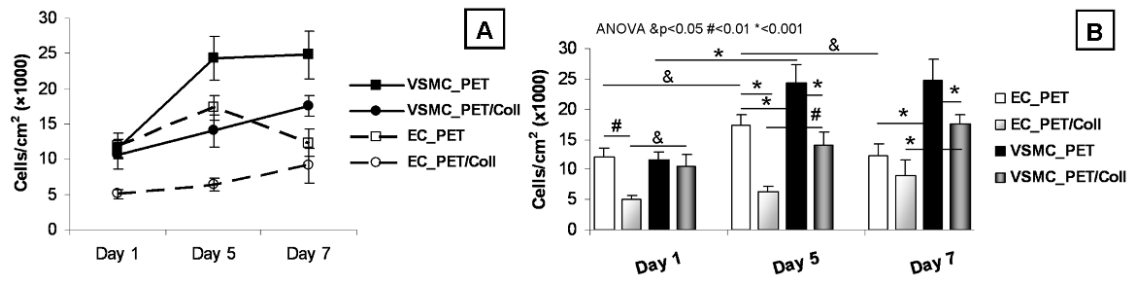


Figure 19. Growth curves (A) and cell numbers (B) of bovine pulmonary artery endothelial cells (EC) of the CPAE line and primary rat aortic vascular smooth muscle cells (VSMC) on patches of vascular prostheses made from polyethylene terephthalate (PET) and PET with collagen (PET/Coll). The seeding density was 45 300 cells/cm². Significances between groups are shown only in B.

The morphology of EC and VSMC cultured on patches of vascular prostheses made of ePTFE, PET and PET/Coll on day 7 is shown in Figure 20. The cell growth on vascular prosthetic samples is clearly inferior in comparison with TCPS, which served as a positive control. Both EC and VSMC display a round morphology on ePTFE (Figure 20A and C, respectively) and on PET (Figure 20E and H, respectively). Cells on PET/Coll are hardly seen (Figure 20F and I). The ECs on the control TCPS are well spread and form a sub-confluent layer or even a confluent layer with a typical polygonal morphology (Figure 20B and G, respectively). The VSMC on the control TCPS reached confluence (Figure 20D) and started to grow in a multilayer (Figure 20J).

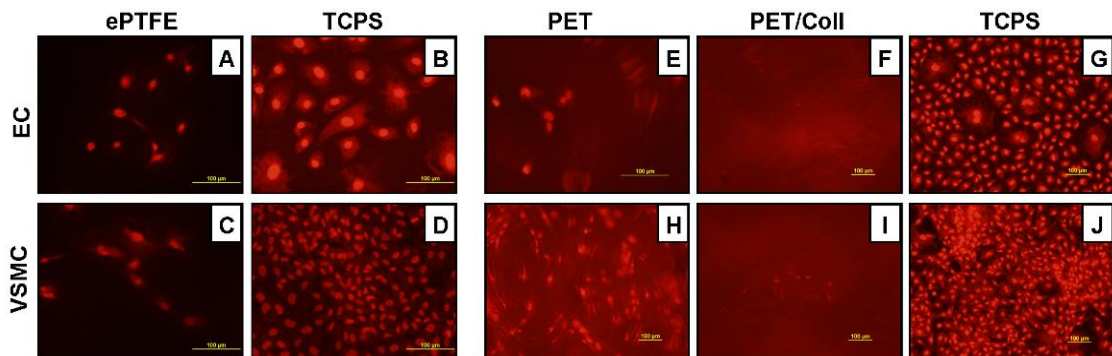


Figure 20. Morphology of bovine pulmonary artery endothelial cells (EC) of the CPAE line and primary rat aortic vascular smooth muscle cells (VSMC) on patches of vascular prostheses made of expanded polytetrafluoroethylene (ePTFE) (A and C), polyethylene terephthalate (PET) (E and H) and PET with collagen (PET/Coll) (F and I). Day 7 after seeding 8 100 cells/cm² (A-B), 15 700 cells/cm² (C-D) and 45 300 cells/cm² (E-J). Tissue culture polystyrene (TCPS) served as a positive control (B and D, G and J). Staining with propidium iodide, epifluorescence microscope (Olympus IX51) and digital camera (Olympus DP70, Japan), magnification 10× (A-J), zoom 2× (A-E), bar = 100 μm.

6.1.5 EC and VSMC on PET/Coll with adhesive protein assemblies

Unpublished data: Population densities of bovine EC of the CPAE line and primary rat aortic VSMC on PET/Coll vascular prostheses with adhesive protein assemblies are given in Figure 21. There was no difference in the growth of EC among samples with various adhesive protein assemblies on day 1. The cells were significantly more numerous on all of the samples on day 4 than on day 1, except for PET/Coll +Co/Fb/FN. The highest cell densities on day 4 were detected on PET/Coll +Co/FN and +Co/Fb. Interestingly, a significant increment in cell numbers between day 4 and day 7 was observed only on PET/Coll. The cell numbers on PET/Coll +Co/LM, +Co/Fb and Co/Fb/FN did not change, while the cell populations on PET/Coll +Co and +Co/FN even decreased between day 4 and day 7. The highest cell numbers on day 7 were reached on PET/Coll and on PET/Coll +Co/Fb (Figure 21A).

Unlike EC, there was no difference in the densities of VSMC among various protein assemblies on either of the culture days. The VSMC increased their densities non-significantly between day 1 and day 4, but increased their numbers significantly on all of the samples between day 4 and day 7 (Figure 21B).

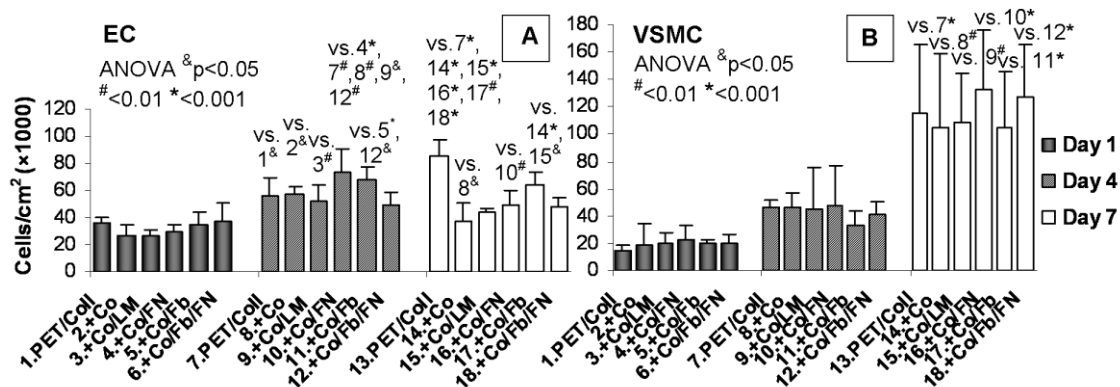


Figure 21. Population densities of bovine endothelial cells (EC) of the CPAE line (A) and primary rat aortic vascular smooth muscle cells (VSMC) (B) on PET/Coll vascular prostheses with adhesive protein assemblies. The seeding density was 5.0×10^4 cells/cm². Abbreviations: polyethylene terephthalate (PET), commercial bovine collagen type I impregnation (Coll), collagen type I (Co), laminin (LM), fibronectin (FN) and fibrin gel (Fb), versus (vs.).

A comparison of the cell densities of EC and VSMC on PET/Coll with adhesive protein assemblies is shown in Figure 22. On day 1, there was a higher cell number of EC on PET/Coll ($p<0.001$) and PET/Coll +Co/Fb/FN ($p<0.05$) in comparison with the cell numbers of VSMC. On day 4, higher cell density of EC was observed only on PET/Coll + Co/Fb ($p<0.05$). The situation was reversed on day 7: the populations of VSMC were higher than the populations of EC on all tested surfaces. However, this difference was not significant on PET/Coll and on

PET/Coll +Co/Fb. Statistical significance between numbers of EC and VSMC was detected on all other samples on day 7.

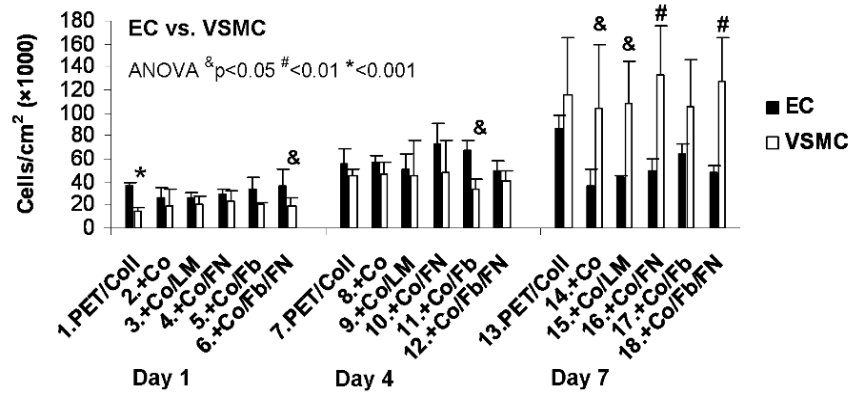
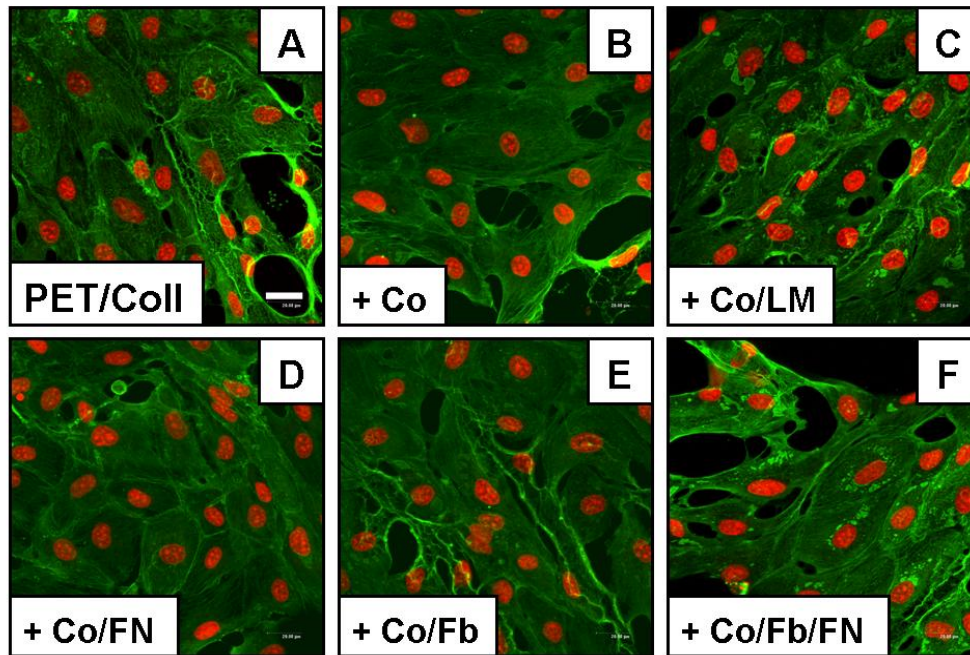


Figure 22. Comparison of population densities of bovine endothelial cells (EC) of the CPAE line and primary rat aortic vascular smooth muscle cells (VSMC) on PET/Coll vascular prostheses with adhesive protein assemblies. The seeding density was 5.0×10^4 cells/cm². Abbreviations: polyethylene terephthalate (PET), commercial bovine collagen type I impregnation (Coll), collagen type I (Co), laminin (LM), fibronectin (FN), fibrin gel (Fb), versus (vs.).

The morphology of both EC and VSMC seeded on PET/Coll vascular prostheses with adhesive protein assemblies is shown in Figure 23. The EC grew in an almost confluent monolayer with a well-developed β -actin cytoskeleton (Figure 23A-F), and the VSMC grew in a multilayer with a visible α -actin cytoskeleton (Figure 23G-L). Both cell types are able to span the interstices of the knitted structure of the prosthesis.

Endothelial cells



Vascular smooth muscle cells

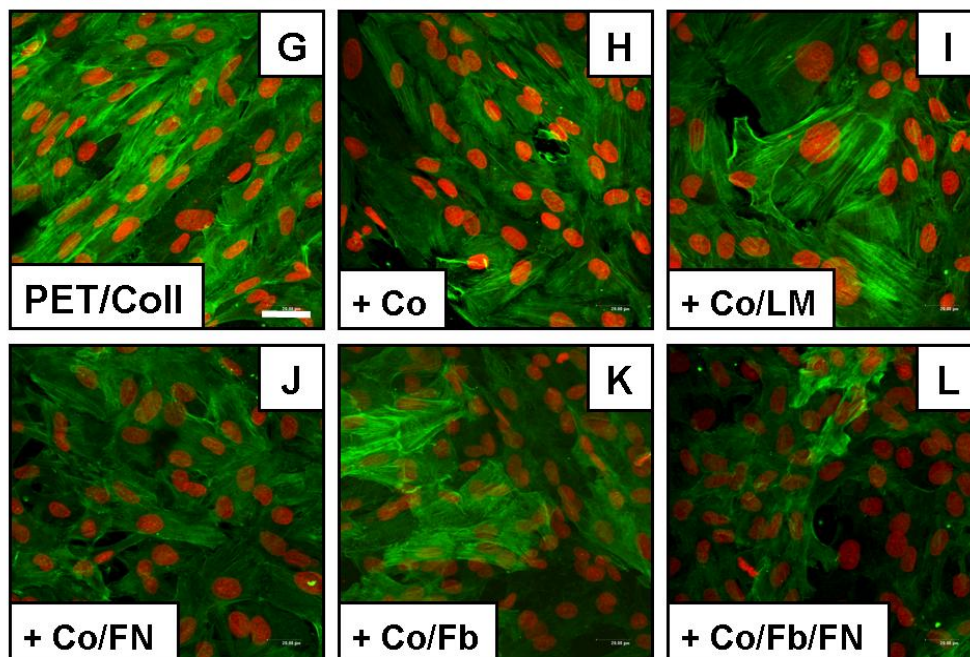


Figure 23. Morphology of bovine endothelial cells of the CPAE line (A-F) and primary rat aortic vascular smooth muscle cells (G-L) seeded on PET/Coll vascular prostheses with adhesive protein assemblies. Day 7 after seeding 5.0×10^4 cells/cm². Immunofluorescence of β-actin (green) (A-F), α-actin (G-L), cell nuclei counterstained with propidium iodide (red). Leica TCS SP2 AOBS confocal microscope, magnification 40×, zoom 2×, bar = 20 μm. Abbreviations: polyethylene terephthalate (PET), commercial bovine collagen type I impregnation (Coll), collagen type I (Co), laminin (LM), fibronectin (FN) and fibrin gel (Fb).

The morphology of the EC lower magnification is shown in Figure 24 and the morphology of the VSMC lower magnification is shown in Figure 25. The texture of the knitted vascular prosthesis is clearly visible in these figures.

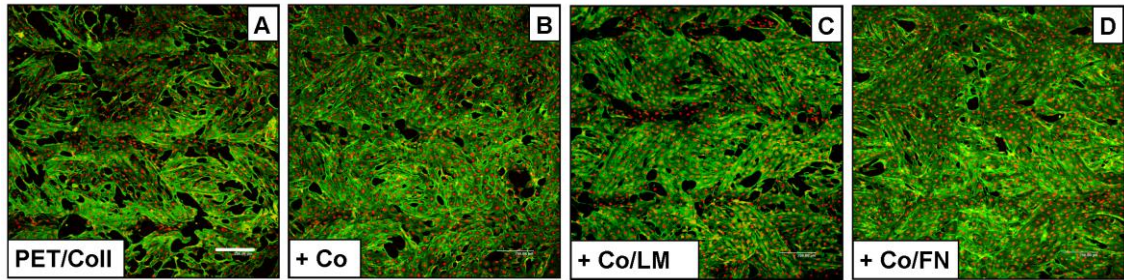


Figure 24. Morphology of bovine endothelial cells of the CPAE line seeded on PET/Coll vascular prostheses with adhesive protein assemblies. Day 7 after seeding 5.0×10^4 cells/cm². Immunofluorescence of β -actin (green), cell nuclei counterstained with propidium iodide (red). Leica TCS SP2 AOBS confocal microscope, magnification 10 $\times$, bar = 200 μ m. Abbreviations: polyethylene terephthalate (PET), commercial bovine collagen type I impregnation (Coll), collagen type I (Co), laminin (LM).

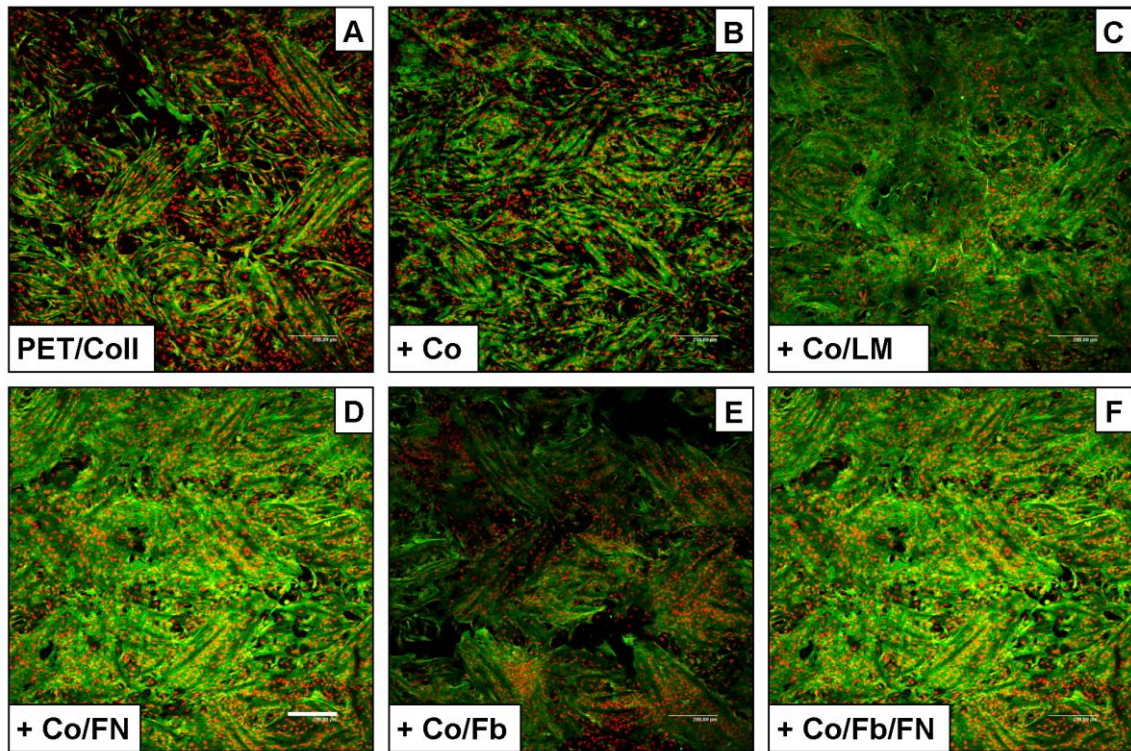


Figure 25. Morphology of primary rat aortic vascular smooth muscle cells seeded on PET/Coll vascular prostheses with adhesive protein assemblies. Day 7 after seeding 5.0×10^4 cells/cm². Immunofluorescence of α -actin, cell nuclei counterstained with propidium iodide (red). Leica TCS SP2 AOBS confocal microscope, magnification 10 $\times$, bar = 200 μ m. Abbreviations: polyethylene terephthalate (PET), commercial bovine collagen type I impregnation (Coll), collagen type I (Co), laminin (LM), fibronectin (FN) and fibrin gel (Fb).

6.1.6 EC on PET with degradable copolymers and protein assemblies

Detailed results including graphs and figures are given in Appendix 5 (Chlupac, J. *et al.* 2014a). Briefly, the endothelial cells reached confluence on all PET surfaces modified with adhesive protein assemblies, with the highest final density on +Co/FN. Impregnation of PET with degradable copolymers made it less adhesive for cells in the following order: PET > PET–PLG > PET–PLGC. However, additional coating with the protein assemblies enhanced the endothelial cell growth, especially on fibrin-containing surfaces. The morphology of the EC on PET, PLG– and PLGC–impregnated PET vascular prostheses is shown in Figure 26 (unpublished data).

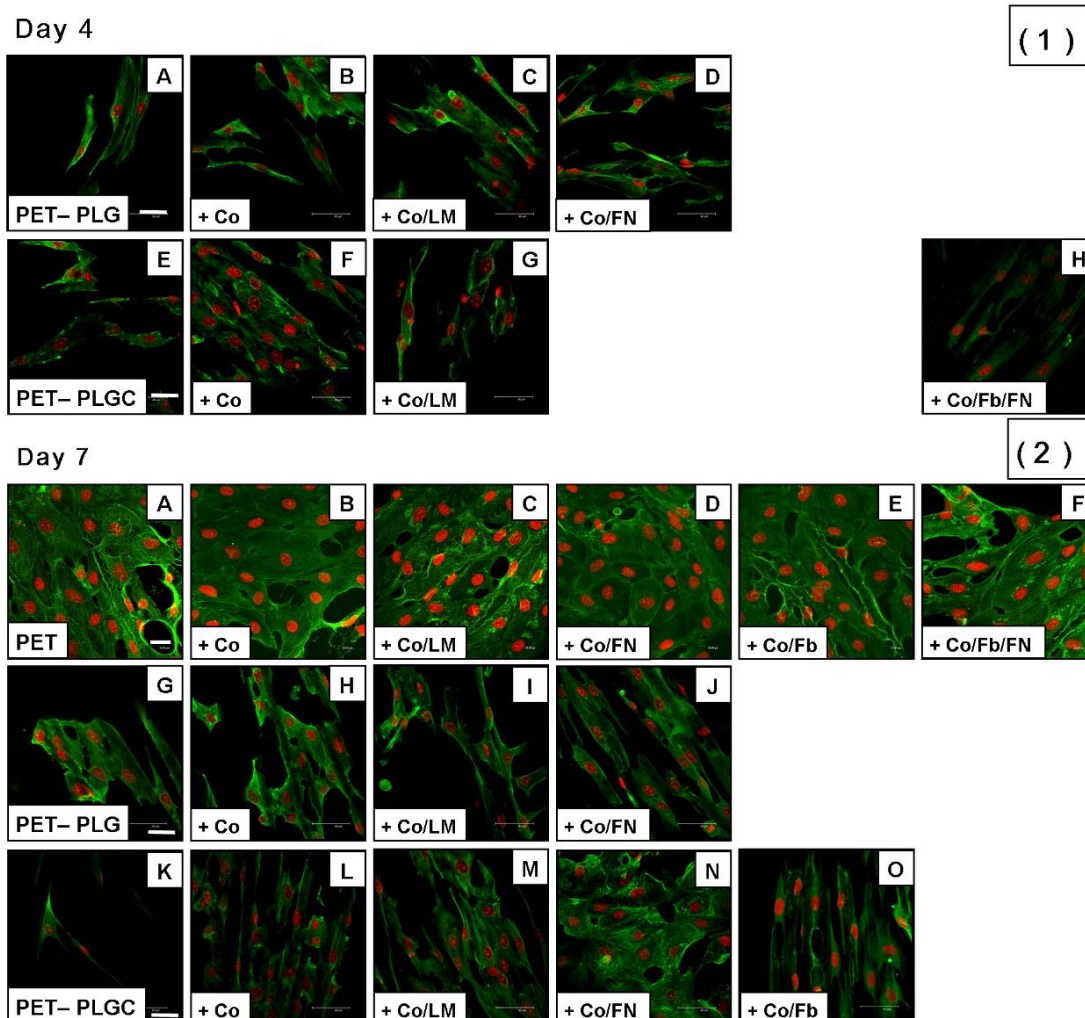


Figure 26. Morphology of bovine endothelial cells of the CPAE line seeded on PET, PLG–impregnated and PLGC–impregnated PET vascular prostheses and modified with adhesive protein assemblies. Day 4 (1) and day 7 (2) after seeding 5×10^4 cells/cm². Immunofluorescence of β -actin (green), cell nuclei counterstained with propidium iodide (red). Leica TCS SP2 AOBS confocal microscope, z-series of 50 images with a z-step of 1 μ m, magnification 40 $\times$, zoom 2 $\times$, bar = 20 μ m. The figure is incomplete due to technical errors. Abbreviations: polyethylene

terephthalate (PET), poly(L-lactide-co-glycolide) (PLG), poly(L-lactide-co-glycolide-co- ϵ -caprolactone) (PLGC), collagen type I (Co), laminin (LM), fibronectin (FN) and fibrin gel (Fb).

6.2 Planar dynamic cell culture experiments in vitro:

6.2.1 Phenotype of human primary EC on ECM proteins under flow

The results, including graphs and figures, are given in detail in Appendix 6 (Chlupac, J. *et al.* 2014b). Briefly, the cells reached confluence rapidly on all of the surfaces with similar growth curve patterns. The cells on Co/LM and Co/FN were resistant to flow up to 120min, but minor cell desquamation occurred on Co corresponding with temporary downregulation of VE cadherin and vinculin mRNA and decreased fluorescence of vinculin. The cells seeded on Co/LM initially more upregulated vinculin mRNA and also the inflammatory factor NF- κ B, and the cells plated on Co/FN changed the expression profile minimally in comparison with the static control. Fluorescence of VE cadherin and von Willebrand factor was enhanced on Co/FN. The cells cultured on Co/LM and Co/FN expressed more VE cadherin and KDR mRNA than the cells on Co. They also increased the vinculin fluorescence. The cells plated on Co/FN upregulated the mRNA of VE cadherin, CD-31 and MMP 1 to a greater extent than the cells on Co/LM and, moreover, they enhanced the fluorescence of VE cadherin, KDR and von Willebrand factor. Some of these changes sustained up to 6h of flow, as confirmed by immunofluorescence.

6.3 Tubular dynamic cell culture experiments in vitro:

6.3.1 EC on PET/Coll vascular prostheses under flow

The results including graphs and figures are given in detail in Appendix 7 (Chlupac, J. *et al.* 2014c). Briefly, the cell adherence after 4 h on PET/Coll, PET/Coll +LM, PET/Coll +Fb and PET/Coll +Fb/FN was 22%, 30%, 19% and 27% of seeding, respectively. Compared to static, the cell density on PET/Coll and PET/Coll +LM dropped to 61% and 50%, respectively, after 120 min of flow. The cells on PET/Coll +Fb and PET/Co +Fb/FN showed no detachment during 2 h of laminar shear stress.

6.4 Tubular animal (porcine) implantation experiments in vivo:

6.4.1 Surgical anatomy

The differences in the vascular anatomy of the aorto-iliac arterial bed between humans and porcines are shown in Figure 27. The human abdominal aorta is bifurcated in common iliac arteries, which further branch into larger external iliac arteries (supplying the lower legs) and

smaller internal iliac arteries (supplying the pelvic organs) (Figure 27A and B). In pigs, the abdominal aorta gives rise to smaller external iliac arteries and runs as a common trunk, which is then bifurcated into larger internal iliac arteries (Figure 27C and D).

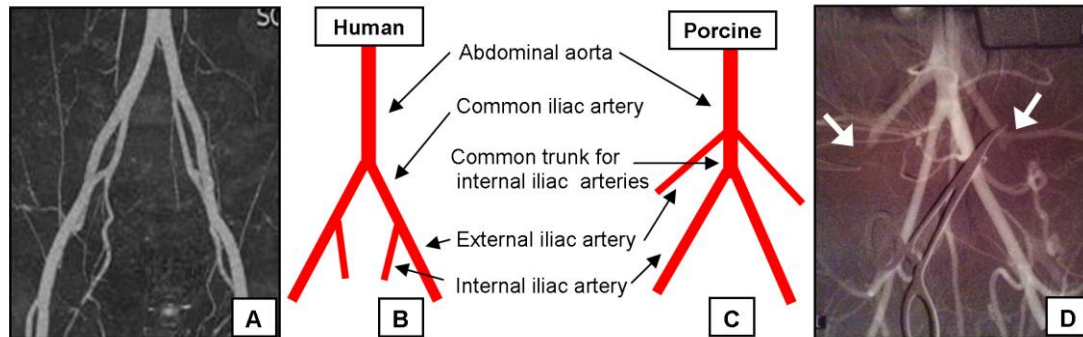


Figure 27. Vascular anatomy of aorto-iliac arteries in humans (**A** and **B**) and in pigs (**C** and **D**). The human abdominal aorta is bifurcated in common iliac arteries, which further branch into larger external and smaller internal iliac arteries (**A** and **B**). In pigs, the abdominal aorta gives rise to smaller external iliac arteries and runs as a common trunk, which is then bifurcated into larger internal iliac arteries (**C** and **D**). Angiogram pictures (**A** and **D**) are courtesy of Dept. of Radiology, Institute for Clinical and Experimental Medicine, Prague, Czech Rep.

Five animals were used for exploring the surgical anatomy without cell seeding. Three hind-limb bypass settings were examined: (1) an iliac-femoral bypass graft, (2) an aortic-external iliac bypass graft and (3) an aortic-internal iliac bypass graft (Figure 28). The bypassed artery and some of the adjacent arteries were ligatured to arrange for sufficient blood inflow into the tested prosthesis. The iliac-femoral setting of the bypass grafts suffered from compression by the inguinal ligament in the groin of growing animals and resulted in graft thrombosis (Figure 28A). Moreover, two animals showed clinical signs of spinal cord ischemia (paraparesis). The aorto-external iliac setting suffered from insufficient blood flow and graft thrombosis even when the internal iliac artery was ligated (Figure 28B, C). The best tolerated anatomical setting was the aorto-internal iliac bypass graft supplemented by ligature of the external iliac arteries (Figure 28D).

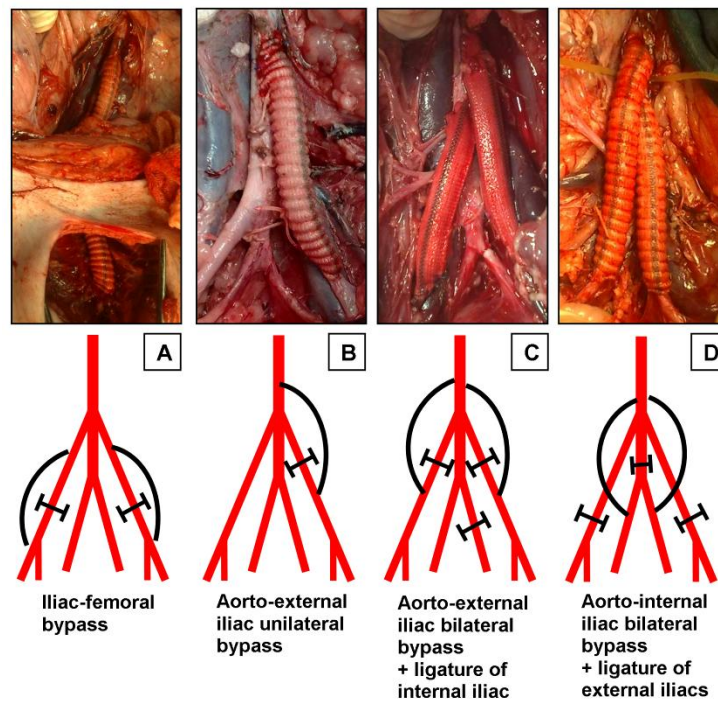


Figure 28. Various anatomical settings of lower leg prosthetic bypass grafts in a porcine model. The aortic-internal iliac bypass with ligature of external iliac arteries (**D**) was the most durable and best-tolerated anatomical setting.

The mean period of anesthesia (hour:min) was $1:45 \pm 0:43$ for vein explantation and $4:16 \pm 1:08$ for bypass implantation. The mean aortic or iliac clamping time was 31 ± 14 min. Minipigs were surgically more suitable because of the weight difference (domestic pig 57 ± 14 kg versus minipig 30 ± 10 kg at the time of vein harvest) and because of the substantial weight gain during the experiment in domestic pigs without a significant difference in the caliber of the vasculature, which complicated later implant surgery.

6.4.2 Double-stage cell seeding on a PET vascular prosthesis

Endothelial cells were harvested in 6 animals, resulting in 5 seeding procedures and 1 cell culture failure. The seeding procedures are summarized in Table 5. Contamination of smooth muscle cells had to be dealt with on three occasions: first, a co-culture of EC and VSMC was seeded (domestic pig 1), second, EC were isolated by a dilution method (domestic pig 2), and third, EC were isolated by a magnetic separation method (minipig 8). Isolated EC were characterized via immunocytochemistry and positive staining of von Willebrand factor – an EC specific marker (Figure 30A). Negative staining for smooth muscle cell specific marker α -actin indicated that purity of EC was achieved. The cells exhibited a cobble-stone morphology typical of EC. The mean incubation period between cell harvest and re-implantation surgery was 50 ± 16 days, and the mean cell gain totaled 2.3 ± 1.6 million cells. The mean cell seeding density onto the prosthesis lumen was 57 ± 35 000 cells/cm².

Table 5. Summary of the endothelial double-stage seeding procedures in a porcine model. Species (Spp.), endothelial cells (EC), vascular smooth muscle cells (VSMC), standard deviation (SD).

Pig	Spp.	Cell gain (million)	Seeding density (cells/cm ²)	Time (d)	Note	Bypass setting	Flow preconditioning
1.	D		20 000	30	Co-culture EC+VSMC	Iliac-femoral	-
2.	D		100 000	71	EC cloned by dilution	ditto	-
8.	M	1.53	33 000	59	EC cloned by separation	Aortic-iliac	+
10.	M	4.5	100 000	56		ditto	+
11.	M	0.8	33 000	36		ditto	+
Mean±SD		2.3±1.6	57±35 000	50±15			

6.4.3 Comparison of the seeded and the unseeded grafts

A summary of the animal porcine implantation experiments *in vivo* is presented in Table 6. From the 5 seeded grafts, two spontaneously occluded together with the control grafts within 22 and 38 days (one symptomatic and one asymptomatic, pig 1 and pig 11), one thrombosed due to early sepsis (pig 2), one due to a suspected poor outflow tract (pig 8), and one remained patent for 22 days while the control graft occluded (pig 10).

Table 6. Summary of tubular 3D animal porcine implantation experiments *in vivo*. Species (Spp.), domestic pig (D), minipig (M).

Pig	Spp.	Seeding	Bypass setting	Patency - endothelialized	Patency - control
1.	D	+	Iliac-femoral	?, chronic compression by growth within 38 days	?, chronic compression by growth within 38 days
2.	D	+	ditto	1 day, septic thrombosis	?, chronic occlusion within 72 days
3.	D	-	Cell culture failed	-	-
4.	D	-	Iliac-femoral, ligature of internal iliacs	-	- (paraparesis)
5.	D	-	Aortic-external iliac, ligature of internal iliacs	-	1 day, obesity complication
6.	M	-	ditto	-	1 day, unsuitable model
7.	M	-	Aortic-internal iliac, ligature of external iliacs	-	72 days, suitable model
8.	M	+	ditto	?, occlusion due to dissection	6 days, but fresh thrombus
9.	M	-	ditto	-	43 days, suitable model
10.	M	+	ditto	22 days	?, chronic occlusion 22 days
11.	M	+	ditto	?, chronic occlusion 22 days	?, chronic occlusion 22 days

Some representative explants of the porcine bypass grafts are shown in Figure 29. The seeded graft and the control unseeded graft are compared. Interestingly, there was a substantial macroscopic intimal hyperplasia in the unseeded grafts (Figure 29A), while there was only moderate intimal proliferation in the seeded graft that remained patent (Figure 29D).

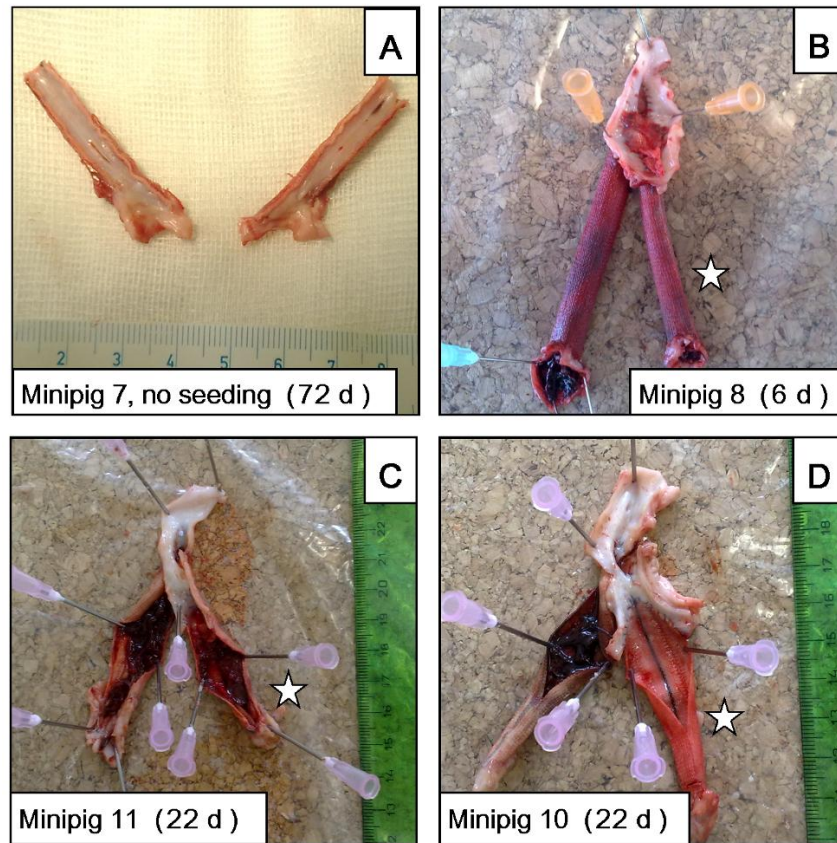


Figure 29. Explants of minipig lower limb bypass grafts. The anatomical setting was an aortic-internal iliac bypass with ligation of the external iliac arteries. The grafts seeded with autologous endothelial cells are indicated by an *asterisk. **A.** No seeding was performed, both grafts are sub-occluded by intimal hyperplasia with a narrow flow channel only. **B.** The seeded graft became occluded by a thrombus due to dissection of the outflow artery, the control graft became spontaneously thrombosed. **C.** Both grafts became occluded by a thrombus. **D.** The seeded graft remained patent while the control graft became thrombosed.

Confocal microscopy images of the explanted seeded graft in minipig 10 are shown in Figure 30. The presence of the seeded endothelial cells was confirmed by staining for an EC marker - von Willebrand factor (cells in red, Figure 30A). The endothelium can still be seen 22 days after implantation. However, there is a moderate intimal hyperplasia (α -actin positive cells in green) underneath the endothelium (Figure 30E-F).

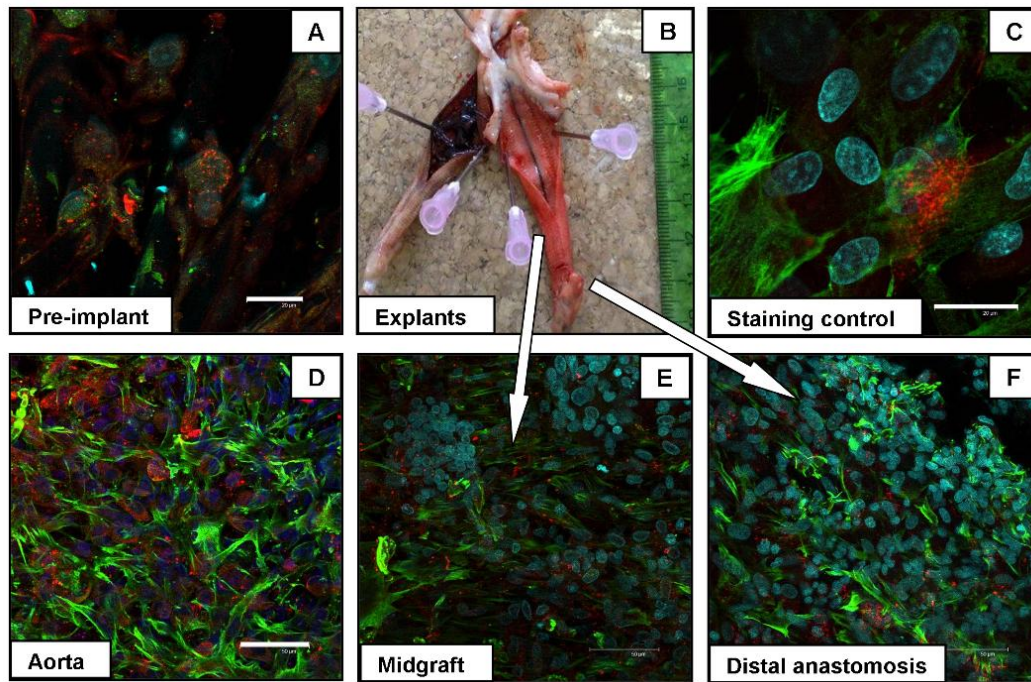


Figure 30. Porcine model of a bioartificial vascular prosthesis. Autologous endothelial cells seeded on a clinically used polyethylene terephthalate vascular prosthesis with collagen (PET/Coll) modified with adhesive fibrin/fibronectin assemblies, 5 days after seeding (**A**). Bypass graft explants 22 days after implant, the control bypass is occluded with a thrombus and the seeded bypass is patent (**B**). Control of staining, bovine pulmonary artery endothelial cells (CPAE line) and primary rat aortic vascular smooth muscle cells on glass (**C**). Aorta adjacent to proximal anastomosis (**D**), midgraft section (**E**) and distal anastomosis (**F**) of the seeded graft. Immunofluorescence of von Willebrand factor - marker of endothelial cells (red) - and α -smooth muscle actin – a marker of smooth muscle cells or fibroblasts (green). Cell nuclei are counterstained with Hoechst (blue). Leica TCS SP2 AOBS confocal microscope, magnification $63\times$ zoom $3\times$ (**A**), $63\times$ zoom $2\times$ (**C**), $63\times$ (**D-F**), scale bar $20\ \mu\text{m}$ (**A** and **C**) and $50\ \mu\text{m}$ (**D-F**).

7. Discussion

7.1. Planar 2D static EC culture experiments *in vitro*:

7.1.1. EC on fibrin assemblies (published)

Detailed discussion is provided in Appendix 2 (Filova, E. *et al.* 2009). Briefly, the maturation of EC on thick Fb gels (g, h, i) was positively correlated with the concentrations of both integrins α_v and vinculin, and also with the formation of confluent layers with relatively high cell population densities. At later culture intervals the cells became partially burrowed in the gel, so that the proportion of the cell membrane exposed to Fb might be greater than the proportion in the cells growing on thin 2D Fb layers. This partial burrowing of the cells resembled the embedment of cells in the physiological ECM or Fb clot, provided the cells with more cell-matrix contact, and might be a stimulus for cell maturation. A potential application of these thick Fb coatings is on the inner surface of polymeric vascular grafts in order to support the development of a confluent, semi-permeable layer of mature EC. These 3D Fb structures can also be essential for angiogenesis in engineering 3D tissues (Xue, L. and Greisler, H.P. 2002). In addition, the thick Fb gel supports accumulation of the ECM components produced by the cells and prevents their diffusion into the culture medium or blood (Ye, Q. *et al.* 2000). On the other hand, 2D Fb layers and surface Fb gels can be prepared on a substrate surface without the formation of an Fb gel in the solution. These films could therefore potentially be applied for modifying porous or fibrous scaffolds for tissue engineering without complete filling of the spaces inside the material, which would facilitate cell ingrowth and metabolism (Filova, E. *et al.* 2009).

7.1.2. EC on fibrin assemblies with ECM proteins (published)

Detailed discussion is provided in Appendix 3 (Filova, E. *et al.* 2014). In the experiment with EC on Fb/ECM assemblies, interestingly, ECM protein coating improved neither cell attachment nor proliferation in comparison with pure Fb assemblies. However, phenotypic maturation and more mature focal adhesion plaques were observed on those ECM protein-coated Fb assemblies without increasing non-specific immune activation of the cells. This may be due to the fact that LM and Co also supported cell differentiation, which is often reversely correlated to cell proliferation. The use of more than one component (Fb and ECM protein) in the construction of a provisional matrix for cell seeding makes the matrix more similar to natural ECM (Filova, E. *et al.* 2014). Finally, cell differentiation and the formation of mature focal contacts may be desired events in the endothelialization of body implants once the seeded cells reach confluence and should be exposed to dynamic conditions (Bacakova, L. *et al.* 2011).

7.1.3. EC on PET vascular prostheses with fibrin assemblies and VEGF

Despite some differences in population densities of EC on PET with Fb assemblies on day 1 and on day 4, the highest density was observed on 1.Fb (thick 3D Fb gel) in comparison

with other samples both on day 1 and on day 7. Cell confluence, however, was observed on all the Fb samples already by day 4, and the monolayer of EC was confluent and looked very similar on all of the Fb surfaces. The statistical differences observed would therefore probably be of less clinical significance when coating vascular prostheses with Fb assemblies, since achieving a rapid EC monolayer is the main objective (Seifalian, A.M. *et al.* 2002). The Fb assemblies used in this experiment (a thick 3D Fb gel and 3D Fb bulk) were previously shown to induce both cell proliferation and cell differentiation (Filova, E. *et al.* 2009). The main modification of the Fb structures in this experiment was the incorporation of the VEGF into the Fb network or onto the surface of the bulk Fb gel, which did not further enhance cell growth. Unlike the previous experiments with Fb structures, we did not, however, test the influence of VEGF on cell maturation or differentiation in this experiment.

7.1.4. EC and VSMC on PET, PET/Collagen and ePTFE vascular prostheses

We have proved that the growth of EC and VSMC is very low on vascular prostheses. Of course the growth may depend on the initial seeding density, which was quite low in our setting. However, a certain degree of colonization is possible. This is in accordance with the published literature (Feugier, P. *et al.* 2005; Berard, X. *et al.* 2009). Interestingly, the lowest cell growth was observed on PET/Coll. Impregnation of PET vascular grafts with collagen is performed by the manufacturer to prevent blood loss through the wall of the graft when it is implanted in patients with fully heparinized blood (Chakfe, N. *et al.* 1999). Collagen impregnation is derived from fragmented biological tissue, which is not created to optimize endothelial proliferation (Francois, S. *et al.* 2009). In addition, collagen as a component of bioprostheses has often been cross-linked by glutaraldehyde and related agents, which are cytotoxic and may cause calcification of the grafts (Jorge-Herrero, E. *et al.* 2010). Our experiments were performed in serum-containing media, which can enhance the cell adhesion and growth. We can assume that vascular grafts *in vivo* are exposed to body fluids that also contain adhesive proteins. Moreover, the difference in cell adhesion to polyester vascular grafts in a serum-containing medium and in a serum-free medium was previously assessed to be approximately only 10% (Pronk, A. *et al.* 1994). The proliferation rate of VSMC was slightly better than the rate of EC. This can roughly be related to the events of clinical restenosis of vascular prostheses, where the growth of VSMC cannot be regulated by missing EC (Chlupac, J. *et al.* 2009).

7.1.5. EC and VSMC on PET/Collagen prostheses with protein assemblies

The better growth of EC on pure PET/Coll than on PET/Coll with adhesive protein assemblies can be explained by the surface-smoothing effect of coating with adhesive proteins. This effect may have decreased the effective surface area for cell adhesion and growth in a static culture (Tunstall, A. *et al.* 1995). However, cells grown on pure PET/Coll without adhesive support would probably be less functional in terms of cell retention under dynamic conditions of

flow (Kaehler, J. *et al.* 1989). Importantly, when looking at cell morphology, EC reached confluent coverage of the prosthesis surface on day 7 on all of the tested surfaces with adhesive protein assemblies (Figure 23).

The lower densities of VSMC in comparison with EC on day 1 and day 4 can be explained by the fact that our adhesive protein assemblies were designed more for adhesion and growth of EC. Their composition was intended to resemble the natural ECM of EC. Perhaps the ECM for adhesion of VSMC would require the inclusion, for example, of elastin in the design of *tunica media* or ECM that more surrounds the embedded VSMC (Stegemann, J.P. *et al.* 2005). Higher population densities of VSMC in comparison with EC on day 7 can be attributed to lack of contact inhibition in VSMC that grow in a multilayer, whereas EC grow in a monolayer.

7.1.6. EC on PET with degradable copolymers and protein assemblies (published)

Detailed discussion is provided in Appendix 5 (Chlupac, J. *et al.* 2014a). Briefly, several research groups have reported the development of composite vascular grafts that combine non-degradable synthetic materials to provide mechanical support and a degradable adhesive matrix to provide a substrate for cell adhesion (Meinhart, J. *et al.* 1997; Tschoeke, B. *et al.* 2008; Thebaud, N.B. *et al.* 2010). Pioneering work was done by Weinberg and Bell, who produced the first tissue-engineered blood vessels from collagen, cultured vascular cells and an open Dacron mesh (Weinberg, C.B. and Bell, E. 1986). Previous reports studying cell behavior on modified PET vascular prostheses and/or on modified degradable polymers are listed in Table 7. Most of the studies show improved cell growth on modified surfaces.

Table 7. Cellular behavior on modified polyethylene terephthalate (PET) vascular prostheses and/or degradable polyester-based (co)polymers. Serum (S.) refers to the presence of serum in the medium at the time of cell seeding, enhanced (↑) or reduced (↓) cell adhesion or growth, *normalized for unit surface area, &tubular grafts, #migration, @flow conditions. Abbreviations: Arteria (a.), bovine aortic endothelial cells (BAEC), bovine pulmonary artery endothelial cells (CPAE), collagen type I (Co I), type III (Co III), days (d), endothelial cells (EC), endothelial progenitor cells (EPC), factor XIII (FXIII), fibrin gel (Fb), fibroblasts (FB), fibronectin (FN), hours (h), human coronary artery endothelial cells (HCAEC), human keratinocytes (HKC), human mesothelial cells (HMC), human saphenous vein endothelial cells (HSVEC), human umbilical cord progenitor-derived endothelial cells (PDEC), human umbilical vein endothelial cells (HUVEC), laminin (LM), non-significant (ns.), poly(caprolacton) (PCL), poly(glycolic) acid (PGA), poly(L,D-lactic acid) (PLDLA), poly(lactic) acid (PLA), poly(L-lactide-co-ε-caprolactone) (PLLACL), poly(L-lactide-co-glycolide) (PLG), poly(L-lactide-co-glycolide-co-ε-caprolactone) (PLGC), result (res.), tissue culture polystyrene (TCPS), vascular endothelial growth factor (VEGF), vascular smooth muscle cells (VSMC). References that are not cited

elsewhere (Miller, D.C. *et al.* 2004; He, W. *et al.* 2005; Atthoff, B. and Hilborn, J. 2007; Pankajakshan, D. *et al.* 2007).

	Prosthesis	Polymer	Modification	Cells, seeding, culture	S.	Control	Res
Pronk <i>et al.</i> 1994	knitted PET		FN	HMC, $2.5 \times 10^5/\text{cm}^2$, 1h	+	PET	↑
Tunstall <i>et al.</i> 1995	knitted or woven PET		medium+serum	HUVEC, $6-8 \times 10^4/0.3\text{cm}^2$, 5d	+	TCPS*	↓
Tjia <i>et al.</i> 1999		PLG	Co I+soluble FN	HKC, $2 \times 10^3/\text{cm}^2$, 2d	-	PLG	↑ [#]
Perego <i>et al.</i> 2003		PLLACL	hydrolysis+plasma	HMVEC, $2 \times 10^5/\text{cm}^2$, 4d	?	PLLACL	↑
	Dacron ^{&}	PLLACL	hydrolysis+plasma	HMVEC, 2d	?	PLLACL	↑
Miller <i>et al.</i> 2004		PLG	hydrolysis+casting	Rat EC, VSMC, $3.5 \times 10^3/\text{cm}^2$, 5d	+	PLG	↑
He <i>et al.</i> 2005		PLLACL	air plasma+Co I	HCAEC, $1.5 \times 10^4/\text{cm}^2$, 7d	+	PLLACL	↑
Sreerekha and Krishnan 2006	Dacron		Fb+FN+FXIII+ VEGF+gelatin	ovine EPC, $5 \times 10^4/\text{cm}^2$, 3d	?	Dacron	↑
Pankajakshan <i>et al.</i> 2007		PCL	Fb+FN+gelatin+ proteoglycans+ angiogenic factors	HUVEC, $2 \times 10^4/\text{cm}^2$, 3d	+	PCL	↑
Atthoff and Hillborn 2007		PET, PLA, PGA	hydrolysis+ Co I or Fb	mouse 3T3 fibroblasts, 5h	?	PET, PLA, PGA	↑
Bérard <i>et al.</i> 2009	knitted PET + CoI+III			PDEC, 0.2 or $2 \times 10^5/\text{cm}^2$, 9d	-	TCPS +gelatin	↓
François <i>et al.</i> 2009	PET	PLLA		BAEC, $4.25 \times 10^3/\text{cm}^2$, 15d	+	PET	↑
Tschoeke <i>et al.</i> 2009		PLDLA	autologous Fb ^{&}	ovine VSMC, FB, EC, 21d [@]	+	a.carotis	ns
Chlupáč <i>et al.</i> 2014c	PET/Co I ^{&}		LM, Fb/FN	HSVEC, $1.5 \times 10^5/\text{cm}^2$, 4h	+	PET/CoI	↑
Chlupáč <i>et al.</i> 2014a	knitted PET		Co/FN	bovine CPAE, $5 \times 10^4/\text{cm}^2$, 7d	+	PET	↑
	knitted PET	PLG or PLGC		dtto	+	PET	↓
	knitted PET	PLG or PLGC	protein multilayers, Co/FN	dtto	+	PET-PLG/ PLGC	↑

The advantage of combining a synthetic bioresorbable polymer with an ECM protein coating for EC attachment and functioning was demonstrated by He *et al.* (2006). They fabricated a nanofiber mesh (copolymer of PLA and PCL) modified by air plasma treatment and collagen type I coating (290 µg/ml). The adhesion, growth and spreading of human coronary artery EC (HCAEC, $3 \times 10^4/\text{cm}^2$, day 3) were improved in comparison with the unmodified polymer. In addition, the cells preserved their endothelial phenotype and their potential ability to remodel ECM (He, W. *et al.* 2006).

A prototype of a compound vascular prosthesis similar to that in our study was described by Perego *et al.* (2003). They synthesized a copolymer of L-lactide and ε-caprolactone (PLLACL), which ensures biocompatibility, and an external Dacron layer was applied for mechanical reinforcement. Surface activation for cell adhesion was provided by hydrolytic etching (NaOH/HCl) and argon/oxygen/air plasma treatment (in our study we used a coating with cell-adhesive protein assemblies). Human microvascular EC (HMVEC, $2 \times 10^5/\text{cm}^2$, day 4) did not adhere to untreated copolymer but formed an almost confluent monolayer after functionalization on PLLACL patches and also in tubular grafts (Perego, G. *et al.* 2003).

Sreerekha and Krishan (2006) cultured ovine blood endothelial progenitor cells (EPC, $5 \times 10^4/\text{cm}^2$) on patches of Dacron and ePTFE grafts coated with a composite matrix containing fibrin, FN, factor XIII, VEGF and gelatin. In addition, a composite matrix on Dacron contained

either low-density fibrin (2 mg/ml) or high-density fibrin (10 mg/ml) in the solution. An increase in cell number of about 50% was observed after 72 h on bare Dacron, a 2-fold increase was observed on low-density fibrin Dacron, and a 10-fold increase was observed on high-density fibrin-coated Dacron. There was a 6-fold increase in cell population on ePTFE grafts coated with high-density fibrin. Moreover, the cells on graft patches were able to produce nitric oxide, especially on high-density fibrin-coated Dacron, in both static and dynamic conditions (Sreerekha, P.R. and Krishnan, L.K. 2006).

This supports our concept that bi-component vascular grafts are a prerequisite for sufficient coverage and function of EC seeded onto the lumen. **Tri-component grafts** were prepared, in our study by adding cell-adhesive protein assemblies onto a PET graft impregnated with degradable polymers.

The number of cells in our study on day 7 was higher on pure PET than on PET modified with protein assemblies. The **effective surface area** of a PET vascular prosthesis is about 6 times greater than that of smooth surfaces (*e.g.* PET film or TCPS), due to the interstices present between the fibers (Tunstall, A. *et al.* 1995). In our work, pre-coating a PET prosthesis with collagen type I before the attachment of LM, FN or Fb decreased the surface area accessible for cells by reducing the dimensions of the interstices. Additional smoothing of the surface was probably achieved by the fibrin gel coating. The decrease in the effective surface area probably contributed to the decrease in the number of adhering and growing cells. The same effect could contribute to lowering the cell numbers on PET impregnated with PLG or PLGC. However, subsequent **functions of EC** on a vascular graft, such as strength of adhesion and resistance to flow, may be enhanced when the cells are seeded on ECM components (Kaehler, J. *et al.* 1989).

The number of EC on day 7 on PET–PLG or PET–PLGC was highest when seeded on a Co/FN matrix, though statistical significance was not reached. Interestingly, Tjia *et al.* (1999) showed that pretreatment of PLG substrate with Co type I ($5 \mu\text{g}/\text{cm}^2$) together with cell-secreted FN concertedly induced the migration of human keratinocytes to a much greater extent than in the case of FN or Co pretreatment only. They concluded that the roles of ECM proteins are not cross-functional, and that Co-FN interactions may present FN in a suitable **conformation**, thus promoting cell adhesion and migration (Tjia, J.S. *et al.* 1999). This supports the idea that **composite ECM assemblies** may be better substrata for adhesion, proliferation and retention of cells seeded on vascular grafts (Sreerekha, P.R. and Krishnan, L.K. 2006).

Degradable polymers, including PLG and PLGC, which were used in our study, have recently been evaluated in terms of cytocompatibility towards mesenchymal cells (murine fibroblasts L929 and human osteoblasts MG-63) and immune cells (murine macrophages RAW 264.7). PLG proved to be biocompatible, but PLGC reduced the adhesion of all cell types and enhanced the activation of macrophages. This undesired effect was attributed to the presence of

ϵ -caprolactone units that endow the polymers with low polarity and higher deformability (Scislowska-Czarnecka, A. *et al.* 2012; Scislowska-Czarnecka, A. *et al.* 2013). In our present study, PET prostheses with PLG and PLGC were seeded with bovine EC and, consistently with the previous tests, the lowest EC adhesion and proliferation occurred on PLGC and initially also on PET-PLGC+Co, +Co/LM, +Co/FN. However, cell adherence and growth on **fibrin-containing surfaces** PET-PLGC+Co/Fb and +Co/Fb/FN was not compromised in comparison with other vascular prosthesis types.

The approach to tissue engineering of vascular grafts may use (1) cells alone that secrete their own matrix, (2) biomaterials alone that are remodeled by ingrowing cells, or (3) a combination of these two approaches. The use of solid **non-degradable components** in the design of vascular prostheses is associated with several limitations, in particular reduced compliance, vasoactivity, and the possibility of foreign body reaction (Simionescu, A. *et al.* 2011). Nevertheless, firm mechanical reinforcement ensures superior safety by preventing potential graft rupture or aneurysm formation (Yu, T.J. *et al.* 1994). In addition, **degradable polymers** may be associated with increased local environment acidity (Pamula, E. and Menaszek, E. 2008); however, degradation products would be washed out in vascular dynamic conditions, and the advantage of replacing the polymer step-by-step by cell-secreted matrix is self-evident (Francois, S. *et al.* 2009).

A completely biological tissue-engineered vascular graft is an upcoming substitute for damaged blood vessels, but its clinical use is currently limited due to poor mechanical strength. Polymeric vascular grafts lined with endothelial cells may therefore be a more viable option at the present time.

7.2. Planar 2D dynamic cell culture experiments *in vitro*:

7.2.1. Phenotype of human primary EC on ECM proteins under flow (published)

Detailed discussion is presented in Appendix 6 (Chlupac, J. *et al.* 2014b). A healthy normal endothelium ensures a **blood-compatible surface** of cardiovascular bypass grafts (Deutsch, M. *et al.* 2009), and the combination of polymeric material and seeding of living cells onto a protein-based scaffold is still a prevailing option in vascular tissue engineering (Stegemann, J.P. *et al.* 2007; Williams, S.K. *et al.* 2011; De Visscher, G. *et al.* 2012).

Sreerekha and Krishnan (2006) seeded ovine endothelial progenitor cells (EPC) onto composite-coated polystyrene (CCPS) containing fibrin, fibronectin and gelatin. EPC grew and differentiated into EC much better on CCPS than on fibrinogen only. In addition, the improved functional status of the EC on engineered graft patches under flow was confirmed by an NO release assay (Sreerekha, P.R. and Krishnan, L.K. 2006). In our study, we did not show significantly better EC growth on protein composites than on Co only; however, cell retention under flow showed a tendency to be better (although non-significantly) on bi-layers, and some

adhesion genes were undesirably downregulated on pure Co, in comparison with Co/LM and Co/FN. This confirms our concept that cells can grow and, particularly, resist flow better on **composite matrices** (Prasad, C.K. and Krishnan, L.K. 2008) resembling the natural ECM, and show more physiological gene expression under SS, depending on the underlying matrix.

Balcells and Edelman (2002) seeded bovine aortic endothelial cells (BAEC) in serum-free media on tissue culture polystyrene (TCPS) coated with FN or LM or gelatin. The seeding density and the protein concentration in the solutions in our study were similar. They did not find significant differences in initial cell adhesion and subsequent proliferation until reaching confluence. They suggest that **pre-adsorbed proteins** non-specifically enhance cell adhesion but specifically influence **cell function**, as was shown by the increased prostacyclin production by BAEC on FN or LM compared to gelatin. The authors explain this observation by the existence of different types and/or affinity of the integrins that regulate cell function in dependence on adhesive support (Balcells, M. and Edelman, E.R. 2002). These findings are in full agreement with our study, where cell adhesion was similarly promoted on all three adhesive surfaces but the functional gene profile of the EC under flow proved to be better on samples Co/LM and Co/FN.

The main challenge for current vascular tissue engineering is to maintain the retention and the function of the endothelial layer. Exposing EC to physiological levels of long-term laminar shear stress (10-20 dyn/cm²) can provide an analogy with the native endothelial lining in the straight part of arteries. We used the level of steady non-pulsatile shearing of 12 dyn/cm² without stretching, which is consistent with other studies (Fernandez, P. *et al.* 2006; Fernandez, P. *et al.* 2007; Chien, S. 2007). Physiological laminar shear stress downregulates pro-atherogenic genes, while cells exposed to turbulent flow experience low levels of shear stress, producing a phenotype that is prone to atherothrombosis (Brooks, A.R. *et al.* 2004). These two different **endothelial phenotypes** (athero-protective versus athero-susceptible) were also confirmed in vivo (Miao, H. *et al.* 2005; Davies, P.F. 2008). Under normal circumstances, of course, endothelium does not go acutely from a static environment to a flow environment, as must happen in an experimental setting. Nevertheless, acutely changed gene expression in an *in vitro* model settles up over the course of hours to a given phenotype (Wasserman, S.M. *et al.* 2002; Wasserman, S.M. and Topper, J.N. 2004). We chose a 2-hour interval, since some of the early changes in mRNA levels related to endothelial dysfunction can be obvious after that period (Hsieh, H.J. *et al.* 1991). However, we are aware that a 2-hour-gene profile may represent an “acute onset” response to SS which may transiently be pro-inflammatory, and that chronic effects of normal shear stress (cell alignment and **steady state of gene expression**) would have been achieved several hours later. Last but not least, 2h is theoretically an acceptable time for potential seeding and preconditioning of human cells on body implants during surgery (Bordenave, L. *et al.* 2008).

We investigated nine genes which had previously been shown to be differentially expressed in cultured EC after submitting to flow. Although they play a clear role in vascular health, the reference data for comparison regarding the expression profiles of EC on engineered surfaces typically contain some contradictory results (Punchard, M.A. *et al.* 2007; Vara, D.S. *et al.* 2011).

In conditions of tissue engineering, the goal is to achieve rapid surface endothelialization of vascular implants, possible preconditioning by laminar flow and maintaining the function of the seeded cells, mainly their anti-thrombogenic properties (Fernandez, P. *et al.* 2007; Vara, D.S. *et al.* 2011). The contribution of our study consists in defining the short-term (up to 2h) effect of physiological laminar SS on phenotypic modulation of human patient-derived EC in close dependence on pre-coating composite ECM matrices for the purposes of vascular tissue engineering.

7.3. Tubular 3D dynamic cell culture experiments *in vitro*:

7.3.1. Human primary EC on PET/Coll with protein assemblies under flow

Detailed discussion on this topic is provided in Appendix 7 (Chlupac, J. *et al.* 2014c). Impregnation of textile vascular prostheses with a collagen sealant was proposed in 1960 in order to make the graft impermeable to blood at the time of implantation; however, impregnated prostheses are not optimal substrata for direct endothelialization (Chakfe, N. *et al.* 1999). To establish a cell monolayer on graft surfaces, optimal attachment and spreading of the cells is desired, together with the ability to withstand shear stress. Several research groups therefore investigated pre-coating vascular prostheses with cell-adhesive substrates and exposing the seeded cells to flow conditions (Remy-Zolghadri, M. *et al.* 2004; Bacakova, L. *et al.* 2011). EC seeding of rough-surfaced vascular prostheses with a tissue culture technique is problematic. Many authors have demonstrated the importance of providing a **protein matrix** to facilitate the adhesion, growth and retention of EC on prosthetic graft surfaces (Sentissi, J.M. *et al.* 1986; Gourevitch, D. *et al.* 1988; Seeger, J.M. and Klingman, N. 1988; Vohra, R. *et al.* 1992; Pronk, A. *et al.* 1994; Sreerekha, P.R. and Krishnan, L.K. 2006).

To summarize current knowledge, it is evident that human EC of various origins can colonize commercial PET vascular grafts *in vitro*. However, substantial **cellular detachment** still occurs even after short-time action of flow conditions that represent the clinical situation. The contribution of our experiment consists in confirming these observations in **clinically- used** tubular 3D bypass grafts submitted to flow in a hemodynamic chamber.

The advantages of using **fibrin** as a substrate for cell seeding are that it can be isolated from autologous blood, it contains recognition sites for growth factors and cell adhesion receptors, and it induces matrix synthesis and differentiation of EC (Filova, E. *et al.* 2009; Riedel, T. *et al.* 2009). Moreover, a combination of fibrin gel with additional ECM components

(e.g. FN) preserves EC adhesion and proliferation, and enables the formation of more mature contacts of focal adhesion (Filova, E. *et al.* 2014). This was demonstrated by the higher cell density on PET/Coll +Fb/FN than on PET/Coll +Fb.

Fibrin glue has previously been used as a support for endothelialization in arterial prosthetic grafts, both experimentally (Fernandez, P. *et al.* 2007) and in human trials, and it has been clearly shown that these conduits have better clinical performance (Meinhart, J.G. *et al.* 2001; Deutsch, M. *et al.* 2009); however, the underlying bypass graft material was **ePTFE** in most cases. A further exceptional contribution of our study is that we have adopted the approach of coating fibrin gel or fibrin/fibronectin assemblies onto clinically-used **PET/Coll** bypass grafts. These types of vascular prostheses have been clinically implanted for decades; however, the lack of an endothelial lining in human often results in thrombotic events with serious health complications. Our results are clinically relevant with potential direct clinical application, because we used **endothelial cells derived from patients** with cardiovascular disease, and our experimental coating of the prosthesis resulted in excellent shear stress resistance of the seeded cells.

7.4. Tubular 3D animal (porcine) implantation experiments *in vivo*:

7.4.1. Exploring the feasibility of various types of surgical anatomy

The principal advantage of using a bilateral aorto-iliac graft as an experimental model is its ability to serve as its own control, under the assumption of equal blood flows to both lower limbs. One may object that aorto-iliac vessels are a high-flow region in clinical applications, whereas the blood flow through our aorto-iliac grafts in minipigs (200-300 ml/min, data not shown) resembles the blood flow for small-caliber grafts in human patients. The conclusion that the best tolerated surgical setting was the aortic-internal iliac bypass graft with ligation of external iliac arteries is based purely on our observations from surgery on animals with unseeded grafts.

7.4.2. Double-stage seeding of autologous EC on PET/Coll with protein assemblies

Double-stage seeding of EC is a well-established method for creating a bioartificial bypass graft both in an experimental setting and in a clinical setting (Bordenave, L. *et al.* 2005). The largest group of patients reported to have received such a graft was implanted in Vienna, Austria. The ePTFE prosthesis was modified by a commercial fibrin preparation and was seeded with autologous endothelium of venous origin (Deutsch, M. *et al.* 2009). In our experiment, we chose PET vascular grafts produced in the Czech Republic, modified by controlled preparation of a thin 3D Fb gel with attached fibronectin.

We used PET because there is no clinically significant difference in long-term patency between PET and ePTFE bypass grafts (Roll, S. *et al.* 2008), and the **choice between PET and ePTFE** is usually left to the discretion of the implanting surgeon; however, some vascular

specialists prefer PET over ePTFE because PET has better handling characteristics, and there is less bleeding from suture holes and a lower tendency to form peri-graft seroma (Jonas, R.A. *et al.* 1987). The adhesive substrate Fb/FN was shown to support growth and differentiation of EC in our previous studies on planar 2D substrates (Filova, E. *et al.* 2009; Filova, E. *et al.* 2014), and also as on tubular 3D vascular prostheses (Chlupac, J. *et al.* 2014c).

We were able to reach a **harvest–implant period** of approximately 50 days. In two cases, we had to deal with smooth muscle cell contamination, and isolation of the EC further extended this time period. With an improved cell isolation technique we might be able to shorten the harvest–implant period. The harvest–implant period reported in the literature was a minimum of 2-4 weeks (Bordenave, L. *et al.* 2008). This method, of course, is not feasible in the setting of an acute vascular disease, when the vascular surgeons need to have the vascular grafts ready to use “off-the-shelf”. However, an **elective (planned) procedure** can be carried out in this way.

After our cell culture period, we were able to seed the endothelial cells on the prosthesis at a density of $57 \pm 35\,000$ cells/cm². The reported **seeding density** that is sufficient to achieve a monolayer of EC on the prosthesis is 100 to 200 000 cells/cm² (Fernandez, P. *et al.* 2006). Therefore our density might have been insufficient. However, in 2 of the 5 animals, the seeding density was 100 000 cells/cm², which is already a sufficient value. The presence of an EC monolayer was validated by microscopy prior to implantation, but it should be noted that the ECs were not always confluent. The presence of endothelium in the lumen of the prosthesis was again visualized at the time of explantation. However, we did not prove that this was the pre-seeded autologous endothelium. It could also have originated from spontaneous endothelial lining of a vascular prosthesis which, unlike in humans, is possible in young experimental animals (Zilla, P. *et al.* 2007). This may be another potential drawback of our study.

7.4.3. Comparison of seeded and unseeded grafts

A number of research groups have demonstrated that seeding of vascular prostheses in experimental animals resulted in complete endothelialization of the graft flow surface within 4-8 weeks after implantation. Moreover, autologous EC seeding in the lumen of a bifurcated Dacron grafts in humans resulted in significantly reduced thrombogenicity of synthetic prosthesis as shown by decreased deposition of radiolabelled platelets (Ortenwall, P. *et al.* 1990). However, existing **differences between species** regarding platelet behavior, blood coagulation, fibrinolytic capacity and proliferation potential of endothelium make the data from animal studies difficult to extrapolate to clinical situations (Ortenwall, P. *et al.* 1988).

There is also a concept of **bio-functionalization** of the prosthetic surface with adhesive substrate only without pre-implant cell seeding. Subsequent post-implant self-endothelialization is then promoted in an animal model. For instance, coating of polyester prosthesis with FN and stromal cell derived factor 1 α (SDF-1 α) resulted in improved endothelialization and reduced IH

in a sheep carotid interposition model (De Visscher, G. *et al.* 2012). Coating the lumen of 4 mm ePTFE prosthesis with FN (50 µg/ml) resulted in more rapid spontaneous surface endothelialization compared to plain ePTFE in a dog model. However, seeding the FN-coated grafts with autologous EC (3.4×10^4 /cm²) harvested from VJI resulted in the most rapidly formed EC coverage and 100% patency at 8 weeks (Seeger, J.M. and Klingman, N. 1988). Burkel *et al.* (1981) studied Dacron prostheses in dog. They found no difference between the seeded and control prostheses immediately after the clot, however, a differentiation became visible after 4 days and after 28 days post-implantation - the seeded prostheses were 80% lined with endothelium as compared to 10% in the control group (Burkel, W.E. *et al.* 1981).

Thus, **self-endothelialization**, unlike humans, is possible in animal models, but adhesive coating of vascular prosthetic grafts makes the endothelial lining more rapid. Moreover, **pre-seeding** the grafts with endothelial cells, although technologically more demanding, results in best endothelial coverage and reduced intimal hyperplastic response (Chlupac, J. *et al.* 2009).

In our study, together 5 prosthetic grafts pre-seeded with EC and 5 internal control (bare) grafts were implanted. Unfortunately, we could not determine the patency in 4 of these 5 grafts. First graft got occluded probably because of chronic compression by growth of the animal (domestic pig 1, iliac-femoral bypass, seeding density 20 000 cels/cm²), second graft got occluded at unknown timepoint during 22 days (minipig 11, aortic-iliac bypass, seeding density 33 000 cels/cm²) and third graft got closed also at unknown timepoint during 6 days, probably because of dissection of the outflow artery (minipig 8, aortic-iliac bypass, seeding density 33 000 cels/cm²). Fourth graft was likely to thrombose due to early postoperative sepsis during only 1 day (domestic pig 2, iliac-femoral bypass, seeding density 100 000 cells/cm²). Each of these 4 grafts got presumably occluded for the reason of a postoperative surgical complication, however, the apparently low initial seeding density of the EC might have had a negative impact on the outcome in 3 of them as well. Moreover, the seeded graft in pig 1 was subject to catheter thrombectomy due to intraoperative early thrombosis which could further damage the seeded endothelial cells. Finally, fifth endothelialized graft remained patent 22 days free of any unwanted events (minipig 10, aortic-iliac bypass, seeding density 100 000 cells/cm²). All of the control unseeded grafts were occluded at the time of explantation. It is difficult to apply any statistical analysis on the patency data (seeded graft patency 20%, n=5, control graft patency 0%, n=5, on day 22) due to low number of experiments and high number of unintended events, however, the main objective of this work was fulfilled - to establish a **clinically relevant hind-limb bypass model** in a large animal and to learn and to perform the double-stage method of autologous transplantation of endothelial cells.

8. Conclusion

8.1 Planar 2D static experiments *in vitro*

8.1.1 EC on Fb assemblies (published)

Two-dimensional Fbg/Fb layers (referred to as a, b) composed of homogeneous Fbg/Fb films containing individual thin Fb fibers can be prepared by repeating the incubation of a substrate successively with solutions of Fbg, Thr, and AT III-independent Thr inhibitors (hirudin and PPACK). Three-dimensional surface Fb gels (c-h) of various thicknesses can be prepared by the catalytic action of surface-attached Thr on an ambient Fbg solution (in the presence of Thr inhibiting complex heparin + AT III). Substrates can be coated with these techniques without the formation of bulk Fb gel. Two-dimensional Fbg/Fb layers effectively promoted initial spreading and proliferation of EC, but the maturation of EC on these surfaces was slow. Fast proliferation and maturation of EC, and a high concentration of α_V -integrins were attained on the thick 3D Fb networks (g-i) formed by thick Fb fibers. Apparently, the chemical composition of the 2D Fbg/Fb layers containing RGD cell-adhesive sequences is satisfactory for rapid proliferation of EC together with a closely packed arrangement of Fbg and Fb molecules, promoting good initial cell adhesion and spreading, while a flexible 3D network of fibrin fibers is necessary for successful EC maturation. Excessively rapid degradation of the very thin surface Fb gels formed by fine Fb fibers impairs cell proliferation with increasing time of cultivation.

8.1.2 EC on fibrin assemblies with ECM proteins (published)

Both 2D and 3D pure fibrin assemblies supported endothelial cell adhesion, proliferation and differentiation. Coating fibrin assemblies with type I collagen, laminin and fibronectin preserved excellent EC attachment and growth. In addition, these assemblies enhanced the formation of focal adhesion plaques containing vinculin, talin, and VE-cadherin and phenotypic maturation (measured by the concentration of von Willebrand factor and CD 31) in cultured endothelial cells. In addition, 2D fibrin coated with laminin and all 3D assemblies decreased non-specific immune activation, measured by the concentration of ICAM-1, compared to uncoated 2D assemblies.

8.1.3 EC on PET vascular prostheses with Fb assemblies and VEGF

Modification of PET prostheses with Fb assemblies generally promoted the growth of the seeded EC. The highest cell number was achieved on 1.Fb (thick 3D Fb gel). There was no difference among other samples, and confluence was achieved on all samples by day 4 except for the control PET. Including VEGF in the Fb structures did not further enhance the cell growth compared to Fb structures without VEGF.

8.1.4 EC and VSMC on PET, PET/Coll and ePTFE vascular prostheses

The growth of the EC and the VSMC on clinically-used vascular prostheses (ePTFE, PET and PET/Coll) did not reach the growth rate of the control TCPS surface. However, both EC and VSMC adhered to the prostheses and the VSMC showed a certain degree of proliferation. The prostheses are therefore not absolutely bioinert. The cell growth on PET/Coll was inferior to the cell growth on PET.

8.1.5 EC and VSMC on PET/Coll with ECM proteins

The EC generally grew on PET/Coll with adhesive protein assemblies until day 4, but growth arrest was observed between day 4 and day 7. This was not the case on pure PET/Coll, where the highest density of the EC was reached. However, the EC reached a confluent lining on all of the surfaces. The VSMC increased in number until day 7 without any notable difference among various adhesive protein assemblies. Initial cell growth was better in EC than in VSMC (day 1 and day 4), but the final cell densities were higher in VSMC than in EC on day 7.

8.1.6 EC on PET with degradable copolymers and protein assemblies (published)

In this study, tri-component vascular grafts were fabricated that comprise commercial semi-finished PET, to provide potential mechanical strength; copolymers PLG or PLGC, for potential semi-biodegradability; and cell-adhesive protein assemblies, to provide a lining with endothelial cells.

Bovine endothelial cells grew to confluence on PET and on all of the protein-coated PET surfaces. The highest final density was observed on +Co/FN. No marked differences in cell growth were detected among other protein multilayers. Modification of PET grafts by impregnation with degradable copolymers made them less adhesive for EC in the following order PET > PET-PLG > PET-PLGC. However, additional modification of the impregnated grafts with protein assemblies promoted the establishment of an endothelial lining, especially on fibrin-containing surfaces.

8.2 Planar 2D dynamic experiments *in vitro*

8.2.1 Phenotype of human primary EC on ECM proteins under flow (published)

Primary cultures of HSVEC rapidly formed a confluent monolayer on Co, Co/LM and Co/FN planar substrates. Despite the divergence in surface roughness and wettability, there were no differences in cell adhesion and growth. However, combined Co/LM and Co/FN matrices seemed to be more suitable for endothelial cell retention under flow than a Co surface alone. Moreover, a Co/FN matrix promoted a slightly more favorable cellular phenotype than Co/LM, as shown by the upregulated expression of some of the adhesion and metabolic genes under shear stress up to 2h and corresponding enhanced immunofluorescence of proteins under flow up to 6h.

8.3 Tubular 3D experiment *in vitro*

8.3.1 Human primary EC on tubular PET/Coll with protein assemblies under flow (published)

Human endothelial cells can sufficiently colonize the bare commercial knitted PET/Coll vascular prosthesis in a serum-containing medium; however, significant cell detachment occurs after flow is applied. Coating the inner surface with laminin improves the cellular adhesion, but the shear stress resistance remains poor. Conversely, the resistance of the adhering cells to dynamic flow conditions mimicking the blood flow is significantly enhanced when the cells are plated on prostheses modified by a fibrin network or by fibrin/fibronectin assemblies. These types of modifications may be suitable for the purposes of tissue engineering of vascular grafts.

8.4 Tubular 3D animal (porcine) implantation experiments *in vivo*

8.4.1 Surgical anatomy

A minipig is a surgically more suitable model than a domestic pig. The most durable surgical setting was a bilateral aortic-internal iliac bypass graft with ligation of the external iliac arteries.

8.4.2 Double-stage cell seeding

Endothelial cells can be harvested from the jugular vein, cultured and seeded onto a PET/Coll vascular prosthesis in a sufficient number by providing a Fb/FN substratum.

8.4.3 Comparison of seeded and unseeded grafts

Seeding own endothelial cells onto the vascular prosthesis may enhance the bypass graft patency in a minipig model.

9. Summary

Introduction: Cardiovascular disease has become a major problem of global health. Surgical repair is often necessary for advanced and multiple vascular lesions. Vascular surgery for atherosclerosis is confronted by the lack of a suitable bypass material. The gold standard material remains the patient's own artery or vein. However, this material may be unsuitable or unavailable. For more than 50 years, synthetic vascular prostheses have included two materials: polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE). They perform well as large-caliber replacements, but their long-term patency is discouraging in small-caliber applications (<6 mm), *e.g.* in coronary, crural or microvessel surgery. This failure is a result of an unfavorable healing process, the development of intimal hyperplasia and the lack of a spontaneous endothelial cell lining. This may result in graft thrombosis with serious health consequences. The search for an ideal small-diameter vascular graft has become a major focus of research. Tissue engineering strives to produce bio-artificial conduits with living cells to provide resistance to thrombosis.

Objectives: The objectives of the work were to modify clinically-used PET vascular prostheses with engineered bio-artificial surfaces in order to improve their bio-compatibility towards vascular smooth muscle cells (VSMC) and mainly towards endothelial cells (EC) for the purposes of vascular tissue engineering.

Materials and Methods: Engineered surfaces were fabricated on glass slides, on tissue culture polystyrene or directly on clinically-used PET vascular prostheses. Various techniques for coating synthetic surfaces with fibrin (Fb, blood coagulation protein) structures were tested: two-dimensional fibrin structures (2D Fb), three-dimensional fibrin networks (3D Fb) and ultra-thin 3D Fbs. Two-dimensional and 3D assemblies were further coated with extracellular matrix (ECM) proteins collagen (Co), laminin (LM) and fibronectin (FN). Cell-adhesive assemblies were prepared as combinations of ECM proteins and Fb, resulting in Co, Co/LM, Co/FN, Co/Fb and Co/Fb/FN samples. For the cell culture, bovine pulmonary artery EC of the CPAE line, primary rat aortic VSMC and human primary saphenous vein EC (HSVEC) harvested from cardiac patients were used. Four sets of cell culture experiments with increasing complexity were performed: (1) planar two-dimensional (2D) static experiments (*i.e.* with no flow of the culture medium), (2) planar 2D dynamic experiments (*i.e.* with flow of the culture medium to simulate blood circulation), (3) tubular 3D dynamic experiments and (4) animal porcine experiments *in vivo*. The cell adhesion, proliferation, differentiation, function and retention under flow were evaluated by means of cell number, morphology, concentration of intracellular proteins, light microscopy, immunofluorescence microscopy and quantitative real time polymerase chain reaction.

Results:

1. Planar 2D static experiments *in vitro*:

1.1. EC on fibrin assemblies (published)

The best initial cell spreading after 1 day was observed on 2D Fb and ultra-thin 3D Fb. The highest concentration of vWF, a marker of EC differentiation, was observed after 3 days on thick 3D Fbs. The highest EC population densities after 7 days were observed on 2D Fb and thick 3D Fb.

1.2. EC on fibrin assemblies with ECM proteins (published)

Immunofluorescence staining showed better assembly of talin and vinculin into focal adhesion plaques, and also more apparent staining of vascular endothelial cadherin on surface-attached 3D Fb and protein-coated Fb assemblies. On these samples, EC also contained a lower concentration of intercellular adhesion molecule-1, measured by enzyme-linked immunosorbent assay. Higher concentrations of CD31 (platelet-endothelial cell adhesion molecule-1) were found on 3D Fb coated with LM, and higher concentrations of von Willebrand factor were found on 3D Fb coated with type I collagen or LM in comparison to 2D Fb layers.

1.3. EC on PET vascular prostheses with fibrin assemblies and VEGF

The final cell density on 1.Fb (thick 3D Fb network) on day 7 was significantly higher than the densities on other surfaces. There was no difference in the cell numbers among the other samples on day 7, except for the cell population on 2.Fb+VEGF (thick 3D Fb network with incorporated VEGF), which was significantly higher than on the control sample 6.PET. Cell confluence was achieved on all samples with Fb assemblies by day 4, and on the PET control as late as by day 7.

1.4. EC and VSMC on PET, PET/Collagen and ePTFE vascular prostheses

The growth of EC on ePTFE was significantly lower than on the control surfaces (TCPS and glass). Similarly, the cell numbers of VSMC on ePTFE were significantly lower than the numbers on TCPS and on glass on day 7. The densities of EC on PET and on PET/Coll were significantly lower than the densities on TCPS on any of the culture days. The numbers of VSMC on PET and on PET/Coll were also significantly lower than the numbers on TCPS on days 5 and 7. Significant cell growth was noted on TCPS and on glass between days 1 and 5, but not on ePTFE, not on PET, and not on PET/Coll. Interestingly, the growth of both EC and VSMC was lower on PET/Coll than on PET. The populations of VSMC on both PET and PET/Coll were higher than the populations of EC on PET and on PET/Coll. The VSMC gradually increased in number on both PET and PET/Coll, significantly between day 1 and day 5 on PET. The EC increased in density non-significantly on PET/Coll, but significant growth was observed on PET between day 1 and day 5. However, their density dropped significantly between day 5 and day 7. Both EC and VSMC displayed a round morphology (*i.e.* poor adhesion) on ePTFE and on PET.

1.5. EC and VSMC on PET/Collagen prostheses with adhesive protein assemblies

There was no difference in the growth of EC among samples with various adhesive protein assemblies on day 1. The cells were significantly more numerous on all of the samples on day 4 than on day 1, except for PET/Coll +Co/Fb/FN. The highest cell densities on day 4 were detected on PET/Coll +Co/FN and +Co/Fb. Interestingly, a significant increment in cell numbers between day 4 and day 7 was observed only on the control PET/Coll. The highest cell numbers on day 7 were reached on PET/Coll and on PET/Co +Co/Fb.

Unlike EC, there was no difference in the densities of VSMC among various protein assemblies on either of the culture days. The VSMC increased significantly in density on all of the samples between day 4 and day 7.

On day 1, there was a higher cell number of EC on PET/Coll and PET/Coll +Co/Fb/FN in comparison with the cell numbers of VSMC. On day 4, higher cell density of EC was observed only on PET/Coll + Co/Fb. On day 7, the populations of VSMC were higher than the populations of EC on all tested surfaces. However, this difference was not significant on PET/Coll and on PET/Coll +Co/Fb.

The EC grew in an almost confluent monolayer with a well-developed β -actin cytoskeleton, and the VSMC grew in a multilayer with a visible α -actin cytoskeleton. Both cell types are able to span the interstices of the knitted structure of the prosthesis.

1.6. EC on PET with degradable copolymers and protein assemblies (published)

The cells reached confluence on all PET surfaces, with the highest final density on +Co/FN. Impregnation of PET with polymers made it less adhesive for cells in the following order: PET > PET-PLG > PET- PLGC. However, additional coating with the protein assemblies enhanced endothelial cell growth, especially on fibrin-containing surfaces.

2. Planar 2D dynamic experiments *in vitro*:

2.1. Phenotype of human primary EC on ECM protein assemblies under flow (published)

The cells on Co/LM and Co/FN were resistant to flow up to 120 min but minor desquamation occurred on Co, corresponding with temporary down-regulation of some of the adhesion genes. The cells plated on Co/FN upregulated the mRNA of VE cadherin, CD-31, and MMP 1 to a greater extent than the cells on Co/LM, and they enhanced the fluorescence of VE cadherin, KDR, and vonWillebrand factor. Some of these changes sustained up to 6h of flow, as confirmed by immunofluorescence.

3. Tubular 3D dynamic experiments *in vitro*:

3.1. Human primary EC on PET/Coll with protein assemblies under flow (published)

The cell adherence after 4 h on vascular prosthesis PET/Coll, PET/Coll +LM, PET/Coll +Fb and PET/Coll +Fb/FN was 22%, 30%, 19% and 27% of seeding, respectively. Compared to the static grafts, the cell density on PET/Coll and PET/Coll +LM dropped to 61% and 50%,

respectively, after 120 min of flow. The cells on PET/Coll +Fb and PET/Coll +Fb/FN showed no detachment during 2 h of shear stress.

4. Tubular 3D animal (porcine) implantation experiments *in vivo*:

4.1. Exploring the feasibility of various approaches to surgical anatomy

Three hind-limb bypass settings were examined: (1) an iliac-femoral bypass graft, (2) an aortic-external iliac bypass graft and (3) an aortic-internal iliac bypass graft. The best tolerated anatomical setting was the aorto-internal iliac bypass graft supplemented by ligation of the external iliac arteries. Minipigs were surgically more suitable, because of the substantial weight gain during the harvest-implant period in domestic pigs.

4.2. Double-stage seeding of autologous EC on PET/Coll with protein assemblies

Endothelial cells were harvested in 6 animals, resulting in 5 seeding procedures and 1 cell culture failure. The mean incubation period between cell harvest and re-implantation surgery was 50 ± 16 days. The mean cell seeding density onto the prosthesis lumen was 57 ± 35 000 cells/cm².

4.3. Comparison of the clinical performance of seeded and unseeded grafts

From the 5 seeded grafts, two spontaneously occluded together with the control grafts within 22 and 38 days (one symptomatic and one asymptomatic), one thrombosed due to early sepsis, one due to a suspected poor outflow tract, and one remained patent for 22 days while the control graft occluded. The endothelium could still be seen 22 days after implantation. Interestingly, there was a substantial macroscopic intimal hyperplasia in the unseeded grafts, while there was only moderate intimal proliferation in the seeded graft that remained patent.

Conclusion:

1. Planar 2D static experiments *in vitro*:

1.1. EC on fibrin assemblies (published)

2D Fbg/Fb layers are satisfactory for rapid proliferation of EC together with a closely packed arrangement of Fbg and Fb molecules, promoting good initial cell adhesion and spreading, while a flexible 3D network of fibrin fibers is necessary for successful EC maturation. Excessively rapid degradation of the very thin surface Fb gels formed by fine Fb fibers impairs cell proliferation with increasing time of cultivation.

1.2. EC on fibrin assemblies with ECM proteins (published)

The results indicate that ECM protein-coated 2D and 3D Fb assemblies can be used for versatile applications in various tissue replacements where endothelialization is desirable, for example, vascular prostheses and heart valves.

1.3. EC on PET vascular prostheses with fibrin assemblies and VEGF

Modification of PET prostheses with Fb assemblies generally promoted growth of the seeded EC. The highest cell number was achieved on a thick 3D Fb gel. Including VEGF in the Fb structures did not further enhance the cell growth compared to Fb structures without VEGF.

1.4. EC and VSMC on PET, PET/Collagen and ePTFE vascular prostheses

Both EC and VSMC adhered to the prostheses, and the VSMC showed a certain degree of proliferation. The prostheses are therefore not absolutely bioinert. The cell growth on PET/Coll was inferior to the cell growth on PET.

1.5. EC and VSMC on PET/Coll vascular prostheses with adhesive protein assemblies

The highest density of the EC was reached on pure PET/Coll. However, the EC reached a confluent lining on all of the surfaces. The VSMC increased in number until day 7 without any notable difference among various adhesive protein assemblies. The initial cell growth was better in EC than in VSMC (day 1 and day 4), but the final cell densities were higher in VSMC than in EC on day 7.

1.6. EC on PET with degradable copolymers and protein assemblies (published)

Tri-component vascular grafts composed of PET, copolymers and cell-adhesive assemblies were fabricated. Modification of PET grafts by impregnation with degradable copolymers made them less adhesive for EC in the following order PET > PET-PLG > PET-PLGC. However, additional modification of the impregnated grafts with protein assemblies promoted the establishment of an endothelial lining, especially on fibrin-containing surfaces.

2. Planar 2D dynamic experiments *in vitro*:

2.1. Phenotype of human primary EC on ECM protein assemblies under flow (published)

Combined Co/LM and Co/ FN matrices seem to be more suitable for EC seeding and retention under flow than a Co surface alone. Moreover, the Co/FN matrix promoted slightly more favorable cellular phenotype than Co/LM under SS of 2–6h.

3. Tubular 3D dynamic experiments *in vitro*:

3.1. Human primary EC on PET/Coll with protein assemblies under flow (published)

Pre-coating the clinically-used PET/Coll vascular prosthesis with LM or Fb/FN adhesive protein assemblies promotes the adherence of endothelium. Cell retention under flow is improved particularly on fibrin-containing (Fb and Fb/FN) surfaces.

4. Tubular 3D animal (porcine) implantation experiments *in vivo*:

4.1 Surgical anatomy

A minipig is a surgically more suitable model than a domestic pig. The most durable surgical setting was a bilateral aortic-internal iliac bypass graft with ligation of the external iliac arteries.

4.2 Double-stage cell seeding

Endothelial cells can be harvested from the jugular vein, cultured and seeded onto a PET/Coll vascular prosthesis in a sufficient number by providing an Fb/FN substratum.

4.3 Comparison of seeded and unseeded grafts

Seeding own endothelial cells onto the vascular prosthesis may enhance the bypass graft patency in a minipig model.

Importance of the work:

Artificial vascular prostheses have been made of non-degradable, non-compliant and **thrombogenic materials** for more than 50 years. Thus, they resemble passive tubing. Thrombosis of a vascular graft usually has serious health consequences such as heart attack, stroke, or amputation of a limb. Potential bio-activation by introducing living endothelial cells may approximate these materials to the native artery and make them less thrombogenic.

This work provides a method for facilitating the **growth of the cell lining** in the lumen of vascular prostheses via adhesive protein assemblies *in vitro*. Some of the experiments are of clinical significance because they were performed under **dynamic conditions** closely mimicking the blood flow in human arteries. Moreover, clinically-used **tubular 3D vascular prostheses** were also tested, since the results from planar 2D experiments cannot always be extrapolated into real 3D tissue-like situations.

Some of the experiments were performed using **primary human endothelial cells** harvested from real patients with cardiovascular disease. This fact makes the results even more clinically relevant.

Finally, an **animal implant** study was performed in order to try to confirm the positive results *in vivo*. To our knowledge, this was the first attempt to perform double-stage seeding of autologous endothelial cells into the lumen of a bio-artificial vascular prosthesis in the Czech Republic.

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11. Publications

11.1 Publications with impact factor

11.1.1 First author

1) Chlupáč J, Filová E, Bačáková L:

Blood Vessel Replacement: 50 years of Development and Tissue Engineering Paradigms in Vascular Surgery.

Physiological Research, 2009, 58 (Suppl. 2): S119-S139

IF = 1.505 (2009)

(Appendix 4)

2) Chlupáč J, Filová E, Riedel T, Brynda E, Pamula E, Bačáková L:

Endothelial Cell Lining of PET Vascular Prostheses: Modification with Degradable Polyester-based Copolymers and Adhesive Protein Multi-layers.

Journal of Tissue Science and Engineering, 2014, 5(2): 139: 1-10

IF = 2.17 (2012)

(Appendix 5)

3) Chlupáč J, Filová E, Riedelt T, Houska M, Brynda E, Pamula E, Remy-Zolghadri M, Bareille R, Fernandez P, Daculsi R, Bourget Ch, Bačáková L, Bordenave L:

The Gene Expression of Human Endothelial Cells is modulated by Sub-endothelial Extracellular Matrix Proteins: Short-term Response to Laminar Shear Stress.

Tissue Engineering A, 2014 (15, 16): 2253-2264

IF = 4.022 (2011)

(Appendix 6)

4) Chlupáč J, Filová E, Riedelt T, Houska M, Brynda E, Remy-Zolghadri M, Bareille R, Fernandez P, Daculsi R, Bourget Ch, Bordenave L, Bačáková L:

Adherence of Human Endothelial Cells to Polyester Vascular Grafts: Pre-coating with Adhesive Protein Assemblies and Resistance to Short-term Shear Stress.

Physiological Research, 2014, 63(2): 167-77

IF = 1.555 (2012)

(Appendix 7)

11.1.2 Co-author

1) Eduard Brynda, Tomáš Riedel, Jan Dyr, Milan Houska, Lucie Bačáková, Elena Filová, **Jaroslav Chlupáč**, Petr Lesný, Pavla Jendelová, Eva Syková:

Způsob přípravy regulovaných vrstev fibrinu na pevných površích. Patent č. 299687, cze – čeština, Obor: CE, Biochemie, Vydavatel: Úřad průmyslového vlastnictví ČR, rok uplatnění 2008.

Patent application 299687

(Appendix 1)

2) Filová E, Brynda E, Riedel T, Bačáková L, **Chlupáč J**, Lisá V, Houska M, Dyr JE.

Vascular endothelial cells on two-and three-dimensional fibrin assemblies for biomaterial coatings.

Journal of Biomedical Materials Research A, 2009, 90(1): 55-69

IF = 2.706 (2008)

(Appendix 2)

3) Filová E, Brynda E, Riedel T, **Chlupáč J**, Vandrovcová M, Švindrych Z, Lisá V, Houska M, Pirk J, Bačáková L.

Improved Adhesion and Differentiation of Endothelial Cells on Surface-Attached Fibrin Structures Containing Extracellular Matrix Proteins.

Journal of Biomedical Materials Research A, 2014, 102(3): 698-712

IF = 2.625 (2011)

(Appendix 3)

11.2 Publications without impact factor

11.2.1 First author

1) **Chlupáč J**, Filová E, Riedelt T, Brynda E, Remy-Zolghadri M, Bareille R, Fernandez P, Daculsi R, Bordenave L, Bačáková L:

Human endothelium on vascular prostheses modified by extracellular matrix proteins in a flow experiment.

Engineering of Biomaterials, 2006, 58-60: 10-13

2) **Chlupáč J**, Filová E, Riedel T, Brynda E, Pamula E, Lisá V, Bačáková L:

Endothelial Cells on PET Vascular Prostheses Impregnated with Polyester-based Copolymers and Coated with Cell-adhesive Protein Assemblies.

***Engineering of Biomaterials*, 2008, 81-84: 108-111**

3) Chlupáč J, Filová E, Bacáková L.:

Vascular prostheses: 50 years of advancement from synthetic towards tissue engineering and cell therapy.

***Rozhledy v Chirurgii*, 2010, 89(1): 85-94. Czech.**

11.2.2 Co-author

1) Matia I, Adamec M, Janoušek L, Lipár K, Marada T, Klein D, Baláž P, Varga M, **Chlupáč J**, Rokošný S.

Clinical experience with cold-preservation of venous and arterial allografts. Long-term outcomes.

***Rozhledy v Chirurgii*, 2010 89(1): 45-54. Czech.**

2) Janoušek L, Marada T, **Chlupáč J**, Lipár K, Baláž P, Rokošný S, Matia I, Adamec M:

Eversion carotid endarterectomy: evaluation of results after changing the operation technique.

***Časopis Lékařů Českých*, 2011, 150: 41-43. Czech.**

3) Baláž P, Rokošný S, **Chlupáč J**, Janoušek L, Adamec M:

Intraarterial peroperative thrombolysis in therapy of the acute limb ischemia.

***Rozhledy v Chirurgii*, 2011, 90(1): 55-58. Czech.**

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12. Conferences

12.1 International conferences

1. **Chlupáč J. et al.:** Endothelial Cells Seeded on Protein-coated Vascular prostheses under Experimental Shear Stress Conditions. *XIV International Symposium on Atherosclerosis, ISA (International Society for Atherosclerosis)*, Rome, Italy, 18.-22.6. 2006. **Oral presentation.**
2. **Chlupáč J. et al.:** Shear Stress Resistance of Human Endothelial Cells Seeded on Planar Surfaces and Tubular Vascular Grafts Coated with Extracellular Matrix Proteins. *20th European Conference on Biomaterials*, Nantes, France, 27.9.-1.10.2006. **Poster.**
3. **Chlupáč J. et al.:** Human endothelium on vascular prostheses modified by extracellular matrix proteins in a flow experiment. *XVI. Conference on Biomaterials in Medicine and Veterinary Medicine*, Rytro, Poland, 12.-15.10. 2006. **Oral presentation.**
4. **Chlupáč J. et al.:** Endothelial Cells Seeding and Shearing on the Extracellular Matrix Proteins-coated Vascular Prostheses. *8th World Biomaterials Congress*, Amsterdam, Netherlands, 27.5.-1.6. 2008. **Poster.**
5. **Chlupáč J. et al.:** Human Endothelium on Protein Multilayer Assemblies under Shear Stress. *ISACB (International Society for Applied Cardiovascular Biology) 11th Biennial Meeting*, Bordeaux, France 17.-20.9. 2008. **Poster.**
6. **Chlupáč J. et al.:** Endothelial Cells on PET Vascular Prostheses Impregnated with Polyester-based Copolymers and Coated with Cell-adhesive Protein Assemblies. *XVII. Conference on Biomaterials in Medicine and Veterinary Medicine*, Rytro, Poland, 13.-16.11. 2008. **Oral presentation.**
7. **Chlupáč J. et al.:** Adhesion, Proliferation and Shear Stress-induced Changes of Human Endothelial Cells on ECM Protein Bilayers. *ISACB 12th Biennial Meeting*, Boston, MA, USA, 22.-25.9. 2010. **Oral presentation.**
8. **Chlupáč J. et al.:** Endothelial Cells Phenotype on Extracellular Matrix Protein Bilayers under Dynamic Conditions. *3rd International Conference on Drug Discovery and Therapy*, Dubai, United Arab Emirates, 7.-10.2. 2011. **Poster.**
9. **Chlupáč J. et al.:** Endothelialization of Vascular Prosthesis in a Pig Model of Peripheral Bypass Graft. *9th World Biomaterials Congress*, Chengdu, China, 1.-5.6. 2012. **Oral presentation.**

10. **Chlupáč J. et al.:** Endothelial Cell Therapy for Potential Distal Arterial Reconstruction: Seeding of Autologous Endothelium on Planar Adhesive Assemblies and Vascular Prosthesis in an *in vitro* and *in vivo* Experiment. *15th World Congress of the International Union of Angiology*, Prague, Czech Republic, 1.-5.7. 2012. **Oral presentation.**
11. **Chlupáč J. et al.:** Porcine Model of Prosthetic Limb Bypass Graft Modified by Adhesive Protein Assemblies and Seeded with Autologous Endothelium. *ISACB 13th Biennial Meeting*, London, United Kingdom, 11.-14.9. 2012. **Poster.**
12. **Chlupáč J. et al.:** Porcine Model of Prosthetic Bypass Graft Lined with Autologous Endothelium. *First International Symposium on Vascular Tissue Engineering*, Leiden, Netherlands, 27.-29.5. 2013. **Poster – Best Poster Award.**
13. **Chlupáč J. et al.:** Vascular Prosthesis Lined with Autologous Endothelial Cells: Porcine Model of Modified Peripheral Bypass Graft. *49th Congress of the European Society for Surgical Research (ESSR)*, 21.-24.5.2014, Budapest, Hungary. **Oral presentation.**
14. **Chlupáč J et al.:** Partial Nephrectomy of Renal Allograft: a Report of 3 Clinical Cases. *European Transplant Fellow Workshop (ETFW), European Society for Organ Transplantation (ESOT)*, 19.-21.9.2014, Prague, Czech Republic. **Oral Presentation.**
15. **Chlupáč J. et al.:** Negative Pressure Therapy of Vascular Graft Infection: 5 Year Experience. *Annual Meeting of the European Society for Vascular Surgery (ESVS)*, 23-25.9.2014, Stockholm, Sweden. **Oral Presentation.**

12.2 Czech conferences

1. **Chlupáč J. et al.:** Adheze a růst cévních endotelových a hladkých svalových buněk na polytetrafluoroetylenových cévních protézách. *81.Fyziologické dny*, Košice, SR, 2.-4.2. 2005. **Oral presentation.**
2. **Chlupáč J. et al.:** Bioartifciální cévní náhrady. *Výjezdní zasedání FgÚ a ÚMCH AV ČR*, D. Jílovice 22.-25.9. 2005. **Oral presentation.**
3. **Chlupáč J. et al.:** Adhesion and growth of vascular endothelial and smooth muscle cells on artificial vascular prostheses and protein-coated surfaces. *Conference Cells VI/18.Biologické dny*, Č.Budějovice, 24.-26.10. 2005. **Poster.**
4. **Chlupáč J. et al.:** Innovation of clinically used vascular prostheses by protein assemblies on their luminal surface. *82. Fyziologické dny*, Praha 7.-9.2. 2006. **Oral presentation.**
5. **Chlupáč J. et al.:** Endothelial Cells Seeded on Protein Multilayer Assemblies under Shear Stress. *PhD Student Workshop*, Seč 2.-4.6. 2008. **Oral presentation.**
6. **Chlupáč J. et al.:** Lidské endotelové buňky na proteinových multivrstvách v dynamickém experimentu. *Konference Centra experimentálního výzkumu chorob srdce a cév*, Harrachov 30.9.-1.10. 2008. **Oral presentation.**
7. **Chlupáč J. et al.:** Biohybridní cévní náhrady v experimentu in vitro. *Konference Transplantcentra IKEM*, Jizerka 9.-11.1. 2009. **Oral presentation.**
8. **Chlupáč J. et al.:** Klinicky užívané cévní náhrady: od syntetických k biologickým *Konference Centra výzkumu chorob srdce a cév*, Harrachov, 19.-21.10. 2009. **Oral presentation.**
9. **Chlupáč J. et al.:** Tkáňové inženýrství cévních náhrad v experimentu in vitro. *XXIII. sjezd České společnosti kardiovaskulární chirurgie*, Brno, 6.-7.11. 2009. **Poster.**
10. **Chlupáč J. et al.:** Tissue Engineering of Vascular Grafts in Dynamic Culture System and in vivo Pig Model. *Konference Centra experimentálního výzkumu chorob srdce a cév*, Harrachov 10.-12.11. 2010. **Oral presentation.**
11. **Chlupáč J. et al.:** Adheze, proliferace a fenotyp lidského endotelu v dynamických podmínkách na konstruktech extracelulární matrix. *Konference Transplantcentra IKEM*, Jizerka 7.-9.1. 2011. **Oral presentation.**
12. **Chlupáč J. et al.:** Cévní protézy pokryté autologním endotelem v průtokovém systému a v prasečím modelu. *Studentská konference UK 2.LF*, Praha, 24.-25.4.2013. **Oral presentation.**
13. **Chlupáč J. et al.** Perivaskulární infekce a VAC terapie, *Symposium Hartmann*, Valtice, 24.-25.10.2013. **Oral Presentation.**

14. **Chlupáč J. et al.:** Negative Pressure Therapy of Vascular Grafts Infection: 5 Year Experience. 39. *Angiologické dny Praha*, 27.2.-1.3. 2014. **Poster.**
15. **Chlupáč J. et al.:** Podtlaková terapie hluboké perivaskulární infekce: 5-ti leté zkušenosti. *VI. Sjezd České společnosti kardiovaskulární chirurgie, Brno* 9.-11.11. 2014. **Oral presentation.**
16. **Chlupáč J. et al.:** Tissue Eng A 20 (15, 16): 2253-2264, 2014. **2. místo v soutěži o Prusíkovu cenu za nejlepší časopiseckou práci.** Česká angiologická společnost České lékařské společnosti J. E. Purkyně. 40. *Angiologické dny Praha*, 19.-21.2. 2015.

13. Awards

1. **Chlupáč J. et al.:** Porcine Model of Prosthetic Bypass Graft Lined with Autologous Endothelium. *First International Symposium on Vascular Tissue Engineering*, Leiden, Netherlands, 27.-29.5. 2013. **Poster – Best Poster Award.**



2. **Chlupáč J. et al.:** *Tissue Eng A* 20 (15, 16): 2253-2264, 2014. Czech Society for Angiology, Czech Medical Association of J. E. Purkyne, 40th *Angiological days*, Prague, Czech Republic, 19.-21.2. 2015. **2nd prize in Prusik Best Journal Publication Contest.**



Česká angiologická společnost
České lékařské společnosti J. E. Purkyně

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Vážený pan
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V Praze dne 13. 2. 2015

Vážený pane doktore,

dovolte, abychom Vás informovali, že odborná porota pro udělení výročních cen Angiologické společnosti vysoce ocenovala Vaši práci „*The Gene Expression of Human Endothelial Cells Is Modulated by Subendothelial Extracellular Matrix Proteins: Short-Term Response to Laminar Shear Stress*“, *TISSUE ENGINEERING: Part A Volume 20, Numbers 15 and 16, 2014*, a udělila Vám 2. místo v soutěži o Prusikovu cenu za nejlepší časopiseckou práci.

Bylo by nám ctí, kdybyste se mohl dostavit k předání příslušného diplomu a odměny, kterou se rozhodl výbor České angiologické společnosti mimořádně poskytnout. Předání by se mělo uskutečnit při slavnostním zahájení letošních 40. angiologických dnů dne 19. 2. 2015 v 9.00 hod. v hotelu Diplomat, Evropská 15, Praha 6 (v kongresovém sále Prague A+B).

S pozdravem


MUDr. Alena Broučková
předsedkyně odborné poroty


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14. Scholarships

1. Université Victor Segalen, INSERM-U577 Biomatériaux et Reparation Tissulaire, Bordeaux, France. *Subject:* Innovation of clinically-used vascular prostheses – planar and tubular dynamic experiments. *Tutor:* Prof. Laurence Bordenave, Ph.D.
 - 4.-11.6. 2005
 - 1.-15.10. 2005
 - 9.-27.9. 2006
 - 14.10.-3.11. 2007
2. Oligene (Provitro) Comp., Berlin, Germany. *Subject:* Tutorial on the use of FCS, TCS and PCS perfusion systems. *Tutor:* Andreas Rademacher, Ph.D.
 - 28.-29.4. 2006
3. Department of Biomaterials, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow, Poland. *Subject:* Innovation of clinically-used vascular prostheses with degradable copolymers. *Tutor:* Elzbieta Pamula, Ph.D., D.Sc.
 - 14.-24.3. 2007

15. Appendices

- 15.1 Appendix 1 Brynda *et al.*: Patent application, No 299687, 2008
- 15.2 Appendix 2 Filová *et al.*: J Biomed Mater Res A, 90 (1): 55-69, 2009 (IF=2.706)
- 15.3 Appendix 3 Filová *et al.*: J Biomed Mater Res A, 102 (3): 698-712, 2014 (IF=2.625)
- 15.4 Appendix 4 Chlupáč *et al.*: Physiol Res, 58 (Suppl. 2): S119-S139, 2009 (IF=1.505)
- 15.5 Appendix 5 Chlupáč *et al.*: J Tissue Sci Eng, 5 (2): 139, 2014 (IF=2.17)
- 15.6 Appendix 6 Chlupáč *et al.*: Tissue Eng, 20 (15-16): 2253-2264, 2014 (IF=4.022)
- 15.7 Appendix 7 Chlupáč *et al.*: Physiol Res, 63 (2): 167-177, 2014 (IF=1.555)