SCUOLA DI ALTA FORMAZIONE

Dottorato in Scienze e Biotecnologie Mediche

XXX Ciclo – S.S.D. B16

Conception and validation of a collagen-based Pleiotrophin controlled release system for vascular applications

Francesco Copes

Sotto la direzione congiunta di:

Professoressa Francesca Boccafoschi, PhD., Università del Piemonte Orientale
Professor Diego Mantovani, PhD., Laval University

Résumé

Lors de maladies cardiovasculaires avancées, telle que l'athérosclérose, les patients doivent subir une chirurgie, plus précisément un pontage artériel, afin de rétablir le flux sanguin. Cette opération consiste à remplacer l'artère malade, obstruée par des dépôts, par un substitut. Cependant, des complications post-implantation telles que la thrombose et l'hyperplasie intimale, subsistent et entrainent l'échec de la greffe vasculaire Pour palier à ce problème, l'approche proposée serait d'avoir une endothélialisation rapide du substitut vasculaire. Pour ce faire, la méthode proposée dans cette thèse est d'enrichir les substituts vasculaires avec une molécule pro-endothélialisation et de valider par la suite leurs propriétés biologiques. La pléiotrophine (PTN), une cytokine de croissance / différenciation, a été spécifiquement choisie dans ce travail, car elle est décrite comme un puissant facteur pro-angiogénique. Cependant, ses effets réels sur l'endothélialisation ne sont pas encore complètement connus. Aussi, afin d'avoir un effet efficace et à long terme, il apparait crucial de rechercher le meilleur moyen d'obtenir un substitut chargé en PTN, tout en conservant et maximisant son activité biologique. Les systèmes d'administration de médicaments à base de polymères naturels représentent une option intéressante pour une telle application. De plus, les gels de collagène de type 1 sont couramment utilisés comme échafaudages pour l'ingénierie des tissus vasculaires et pour le développement de systèmes à libération contrôlée grâce à leurs propriétés biologiques favorables. Pour mieux contrôler la libération de PTN, des interactions spécifiques non covalentes peuvent être utilisées pour stabiliser et immobiliser des médicaments dans l'échafaudage de collagène, grâce à l'utilisation d'agents de liaison. L'héparine apparait comme molécule de liaison idéale, déjà largement utilisée dans la formulation de systèmes d'administration de médicaments, en raison de sa capacité à séquestrer, à stabiliser et à protéger les facteurs de croissance et les cytokines.

En se basant sur les travaux précédents du Laboratoire des Biomatériaux et de la Bioingénierie de l'Université Laval, l'objectif de ce travail était donc de développer un système de libération contrôlée de PTN à base de gels de collagène de type I modifiés par l'héparine.

Dans un premier temps, les effets de la PTN sur la viabilité et la capacité de migration des cellules endothéliales ont été étudiés, et seront comparés aux effets de ceux du facteur 1, dérivé du stroma (SDF-1), facteur d'endothélialisation couramment utilisé lors de greffes

vasculaires. Ensuite, un gel de collagène de type I a été utilisé comme échafaudage pour le développement d'un système à libération contrôlée pour la PTN. Pour augmenter son interaction avec le gel et prolonger sa libération dans le temps, de l'héparine en solution a été ajoutée à la formulation de gel standard. Des évaluations mécaniques et structurelles ont été ensuite réalisées afin d'évaluer les effets de l'addition d'héparine sur les propriétés du gel de collagène. La PTN libérée à partir des gels de collagène modifiés par l'héparine a été d'abord quantifiée puis son effet sur la viabilité des cellules endothéliales et des cellules musculaires lisses a été évalué. Enfin, des tests d'hémocompatibilité ont été effectués pour analyser les effets combinés de l'héparine et PTN sur les propriétés thrombogènes des gels de collagène.

Abstract

Arterial bypass graft is the primary therapy for patients with advanced vascular occlusion diseases such as atherosclerosis. Post-implantation vascular graft failure is mainly caused by in-graft thrombosis and intimal hyperplasia. A fast endothelialization has the benefit of reducing these adverse events. Grafts enrichment with pro-endothelialization molecule has been proposed as an effective solution. Pleiotrophin (PTN) is a growth/differentiation cytokine that has been described as a potent pro-angiogenic factor. However, its pro-endothelialization effects have not been fully explored, and efficient ways to deliver PTN for graft enrichments have to be studied. Natural polymer-based drug delivery systems represent an interesting option for such an application. Type 1 collagen gels are commonly used as scaffolds for vascular tissue engineering and for the development of controlled release systems thanks to their favorable biological properties. To better control the release of PTN, specific non-covalent interactions can be used to stabilize and immobilize drugs within the collagen scaffold, through the use of binding agents. Heparin has been widely used in the formulation of drug delivery systems due to its ability to sequester, stabilize and protect growth factors and cytokines.

Based on previous work of the Laboratory for Biomaterials and Bioengineering at Laval University, the objective of this work was to develop a controlled release system for PTN based on a heparin-modified Type I collagen gels.

At first, the effects of PTN on the viability and migration ability of endothelial cells have been studied by comparing them with the effects exerted by stromal derived factor 1 (SDF-1), a known pro-endothelialization factor already used for vascular graft enrichment. Following, a type I collagen gel was used as scaffold for the development of a controlled release system for PTN. To increase its binding to the gel and to prolong its release over time, heparin have been freely added to the standard gel formulation. Mechanical and structural assessments were performed to evaluate the effects of the addition of heparin. Quantification of the released PTN from the heparin-modified collagen gels was studied along with the effects of the released PTN on the viability of endothelial and smooth muscle cells. Finally, hemocompatibility tests have been performed to analyze the effects of the addition of both heparin and PTN on the thrombogenic properties of the collagen gels.

Table des matières

Liste des figures

Liste des tableaux

Liste des abréviations, sigles, acronymes

ACE: Angiotensin-converting enzyme
ALK: Anaplastic lymphoma kinase
bFGF: Basic fibroblast growth factor
BM-MSC: Bone marrow mesenchymal stem cells
BMP2: Bone morphogenetic protein 2
CHU: Centre hospitalier universitaire
CTRL: Control
CVDs: Cardiovascular diseases
CXCL12: C-X-C motif chemokine 12
CXCR4: C-X-C chemokine receptor type 4
DDS: Drug delivery system
D-MEM: Dulbecco's modified Eagle's medium
EC: Endothelial cell
ECM: Extra cellular matrix
ELISA: Enzyme-linked immunosorbent assay
EPC: Endothelial progenitor cell
ePTFE: expanded polytetrafluoroethylene
FACIT: Fibril Associated Collagens with Interrupted Triple helices
FB: Fibroblast
FBS: Fetal bovine serum
FGF: Fibroblast growth factor
HB-EGF: heparin binding epidermal growth factor-like growth factor
HB-GAM: heparin binding growth associated molecule
HGF: Hepatocyte growth factor
HUASMC: Human umbilical artery smooth muscle cell
HUVEC: Human umbilical vein endothelial cell
IGF-1: Insulin like growth factor-1
LBB: Laboratory for Biomaterials and Bioengineering
NCPRM: NSERC CREATE program for regenerative medicine
NMR: Nuclear magnetic resonance
NSC: Neural stem cells
NSERC: Natural Sciences and Engineering Research Council of Canada
PBS: Phosphate buffer saline
PCNA: Proliferating cell nuclear antigen
PDGF: Platelet derived growth factor
PET: Polyethylene terephthalate
PGA: Polyglycolic acid
PTN: Pleiotrophin
Rac-1: Ras-related C3 botulinum toxin substrate 1
RER: rough endoplasmic reticulum
RPTP β/ζ: protein tyrosine phosphatase beta/zeta
RT: Room temperature
SDF-1: Stromal derived factor 1
SMC: Smooth muscle cell

SM-α-actin: Smooth muscle alpha actin
TGF-β: Transforming growth factor beta
TSR: Thrombospondin type I sequence
UPO: Università del Piemonte Orientale
VEGF: Vascular endothelial growth factor
VSMC: Vascular smooth muscle cell
VTE: Vascular tissue engineering
VWF: Von Willebrand factor
WHO: World health organization

A Mamma e Papà

Remerciements

Avant-propos

Vascular bypass/replacement surgery, despite the improvements observed over the last years, is still burden by thrombosis and neointima hyperplasia at the implantation site, leading to the ultimate failure of the implants. The fast re-establishment of a functional endothelial cells layer (ECs), along with the inhibition of the proliferation of smooth muscle cells (SMCs), is of crucial importance to reduce these adverse outcomes. The implants modifications with molecules and growth factors capable of speeding up the re-endothelialisation process and limiting the SMCs proliferation have been proposed over the last years. However, clinical trials of angiogenic factor delivery have been mostly disappointing, underscoring the need to investigate a wider array of angiogenic factors. In this work, a drug release system based on a type I collagen hydrogel has been proposed for the controlled release of pleiotrophin (PTN), a cytokine known for its pro-angiogenetic effects that has recently been shown to be a good candidate for vascular grafts enrichment compared to already used pro-endothelialization factors. Heparin, in virtue of its ability to sequester, protect and release growth factors, has been used to better control the release of PTN from the collagen gel. The biological performances of the PTN-based drug delivery systems on both the ECs and the SMCs have been investigated. The first part of this project was conducted at the Laboratory of Human Anatomy of the University of Piemonte Orientale led by Professor Boccafoschi and installed in the premises of Palazzo Bellini in Novara (Italy) while the second part was conducted at the Laboratory of Biomaterials and Bioengineering (LBB) of Laval University led by Professor Diego Mantovani and installed in the premises from the research center of Saint François d'Assise Hospital in Quebec City (Canada). This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the NSERC CREATE program for regenerative medicine (NCPRM) and the CHU de Québec Research Center.

The introduction of this thesis presents the general context in which this work fits, the vascular tissue engineering approaches to vascular grafts enrichments and the issues associated with them. Chapter 1 presents a literature review on the use of collagen for vascular medicine applications. The Chapters 2 and 3 present respectively the validation of Pleiotrophin as a pro-endothelialization molecules and the design and development of heparin-modified collagen-based delivery system for the controlled release of Pleiotrophin.

These chapters are the subject of two scientific papers, one of which has already been accepted for publication.

As the first author of these two publications, I conducted this work from their design to the validation and writing in collaboration with co-authors.

Chapter 2: Pleiotrophin: Analysis of the endothelialization potential

Authors: Francesco Copes, Martina Ramella, Luca Fusaro, Diego Mantovani, Mario Cannas, Francesca Boccafoschi

Journal: This article has been accepted for publication on Advances in Medical Sciences - Volume 64/1 due in March 2019.

For this article, I, Francesca Boccafoschi and Diego Mantovani conceived the design of the study. Under their supervisions and assistance, I performed all the experiments as well as the writing. Martina Ramella and Luca Fusaro helped me in the development of the protocols and the analysis of the results. Francesca Boccafoschi and Diego Mantovani contributed to the manuscript preparation and to its correction. The other authors have made their corrections to the manuscript.

Chapter 3: Collagen gels for controlled release of Pleiotrophin: Potential for vascular applications

Authors: Francesco Copes, Pascale Chevallier, Caroline Loy, Daniele Pezzoli, Francesca Boccafoschi, Diego Mantovani

Journal: This article has been submitted to Frontiers in Bioengineering and Biotechnology in the Biomaterials specialty.

For this article, I, Pascale Chevallier, Francesca Boccafoschi and Diego Mantovani conceived the design of the study. I performed all the experiments as well as the writing. Caroline Loy and Daniele Pezzoli helped me in the development of the protocols, the analysis of the results and the preparation of the manuscript. Pascale Chevallier, Francesca Boccafoschi and Diego Mantovani revised the manuscript. The other authors have made their corrections to the manuscript.

Chapter 4 presents a general discussion of the work done throughout this thesis, highlighting the main findings of the study, the challenges encountered and proposes the future perspectives.

Introduction

0.1 Context

Cardiovascular diseases are the leading cause of death in Western countries, accounting for 17.9 million deaths each year [1]. Among the different cardiovascular diseases, atherosclerosis, a condition in which plaques build up inside the arteries leading to the partial or complete obstruction of blood flow, is the major cause of deaths. The increase of risk factors associated with the pathology (obesity, diabetes, hypertension and smoking) coupled with the increase in average life expectancy has led to the urgent search for a durable and effective solution. Despite the advances made in the clinical treatment of this pathology over the last decades, endoluminal healing techniques (balloon angioplasty and stents) are not enough. Faced with the failure of such procedures, surgeons resorted to the replacement of the injured vessel (vascular bypass surgery). Autologous vessels are preferred as graft materials; However, this approach requires multiple surgical procedures and up to 40% of patients needing bypass surgery may not have healthy arteries suitable as autografts. Synthetic prostheses are the most established vascular substitutes because of their high availability, but the poor clinical efficacy of existing synthetic grafts for small diameter (<6 mm) artery surgery limits their use [2]. Since the 1980s, researchers have joined their knowledge in the fields of medicine, biology and materials science to develop artificial living tissues to be used as alternatives to autografts. This led to the development of vascular tissue engineering. The aim of vascular tissue engineering is to develop living vascular substitutes showing biological, structural and mechanical properties as close as possible to those of the native vessels.

This project is part of the activities of the Laboratory of Biomaterials and Bioengineering (LBB) at Laval University in Quebec City. One of the objectives of the LBB is to develop vascular substitutes developed from natural polymers able to present the same mechanical and biological behavior than that of the physiological healthy tissue. This work is based on previous work in the laboratory, which has enabled the development of a physiological-like tri-culture *in vitro* vascular wall model based on type I collagen gel featuring a multi-layered hierarchical organization composed of a fibroblast-containing adventitia-like layer, a media-like layer seeded with smooth muscle cells and an intima-like endothelial cell monolayer. The overall aim of the present research project is to enhance the endothelialization of the scaffolding during the regeneration of the vascular tissue by the enrichment with pleiotrophin, a pro-angiogenic cytokine, in order to

efficiently recruit endothelial cells and to promote their adhesion and growth in the matrix.

In this introductory chapter, after briefly introducing the cardiovascular system and its component, its most common diseases, their currently available treatment and the strategies under development will be presented and discuss.

0.2 The Cardiovascular System

The cardiovascular system, known also as circulatory system, is an organ system composed of the heart and blood vessels responsible for the circulation of blood in the human body. This ensure the transportation of nutrients, oxygen and signaling molecules to the body cells and allows the clearance of carbon dioxide and other waste products of cells metabolism, ultimately concurring in the maintenance of the homeostasis. The main components of the vascular system will be described hereafter.

0.2.1 The Heart

The heart is a muscular organ that resides in the mediastinal space within the thoracic cavity. Its main function is to ensure, through automatic and rhythmic contractions, blood circulation in two main closed circuits: systemic and pulmonary circulation. A third heart-specific circulation system, the coronary system, is also present to supply blood to the heart. The human heart consists of four distinct compartments: two atria, that collect blood and pump it into a pair of ventricles, which pump blood into the vessels. Cardiac valves, placed at the interface between atria/ventricles and ventricles/blood vessels, prevent the backflow of blood.

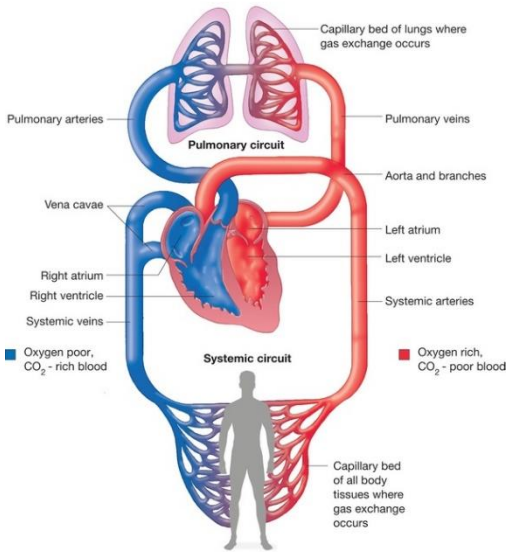

Figure 0.1*: Cardiovascular system. [A]*

The oxygenated blood is ejected from the heart by the left ventricle to the aorta and is then distributed to the organs by the systemic arteries. Following the gas exchange, the deoxygenated blood returns to the heart through the vena cava. It accumulates in the right atrium, then in the right ventricle where it is ejected in the pulmonary artery to be diffused towards the lungs. Once enriched with oxygen in the capillaries of the lungs, the blood returns to the heart, in the left atrium,

through the pulmonary veins (**Figure 0.1**). The slightest damage to this system can cause an alteration in the functioning of the heart and lead to the death of the organism.

0.2.2 Vascular System

The vascular system is a network of blood vessels that allow the circulation of blood. Vessels are divided into three main categories: Arteries, Veins and Capillaries. The different types of vessels are distinguished by thickness, cellular and protein composition of their walls. This heterogeneous, yet highly organized structure allows the blood vessels to effectively perform their vital functions [3]. Arteries are blood vessels that carry blood away from the heart. Blood carried by arteries is usually highly oxygenated, having just left the lungs on its way to the body's tissues. Arteries face high levels of blood pressure as they carry blood being pushed from the heart under great force. To withstand this pressure, the walls of the arteries are thicker, more elastic, and more muscular than those of other vessels. Capillaries are the smallest and thinnest of the blood vessels. They connect to arterioles on one end and venules on the other. Capillaries carry blood very close to the cells of the tissues of the body in order to exchange gases, nutrients, and waste products. The walls of capillaries consist of only a thin layer of endothelium that acts as a filter to keep blood cells inside of the vessels while allowing liquids, dissolved gases, and other chemicals to diffuse along their concentration gradients into or out of tissues. Veins are large return vessels of the body. Because the arteries absorb most of the force of the heart's contractions, veins are subjected to very low blood pressures. This lack of pressure allows the walls of veins to be much thinner, less elastic, and less muscular than the walls of arteries. Veins rely on gravity, inertia, and the force of skeletal muscle contractions to help push blood back to the heart. Some veins contain many one-way valves that prevent blood from flowing back. Arteries and veins are mainly composed of 3 tunics: Intima, Media and Adventitia (**Figure 0.2**).

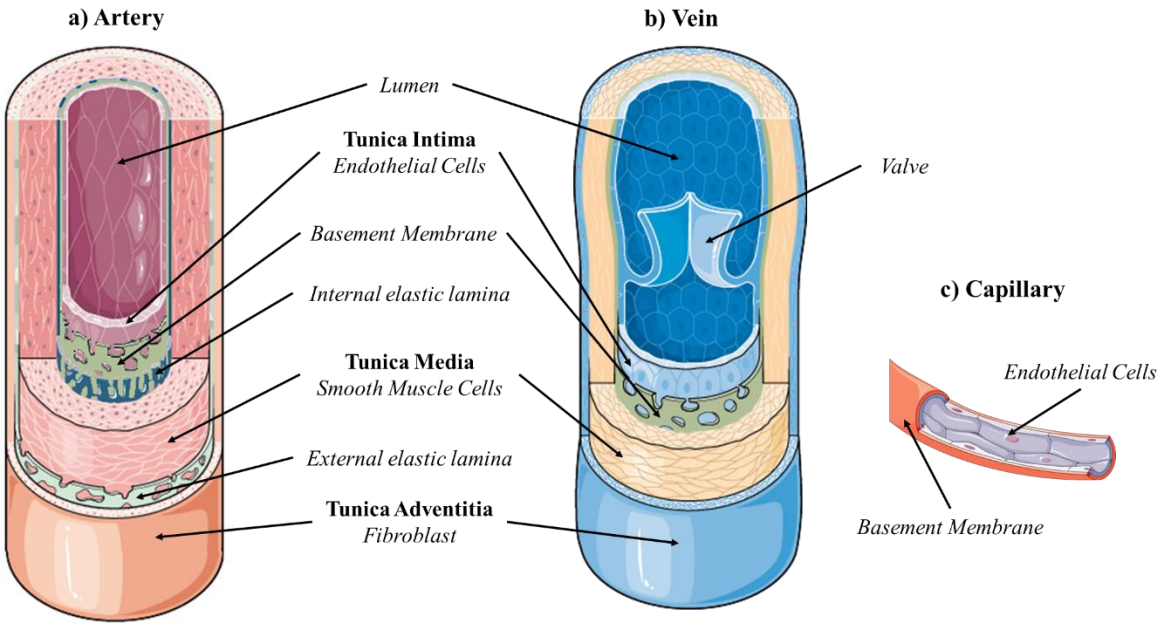

Figure 0.2*: Blood vessels structure: a) Artery; b) Vein; c) Capillary.*

a) The Intima

The tunica intima is the innermost lining of blood vessels. It is composed by the endothelium, which is almost always simple squamous epithelium. Endothelial cells (ECs) are flattened and polarized cells whose apical surface faces the lumen of the vessel. ECs have many important roles that ensure the functionality of the blood vessel [4]. These flat cells are tightly adherent and form a smooth surface that minimizes friction between the blood and the inner surface of the vessels. They are covered on their apical surface with a thin negatively charged layer called glycocalyx [5]. This surface is mainly composed of anticoagulant proteins secreted by endothelial cells. ECs also ensure mechano-transduction, the conversion by cells of a mechanical signal given by the blood flow into cellular physiological signals [6] acting on the ECs themselves (pro-inflammatory and proliferative signals) and on cells from other layers of the vessel wall (contraction of the media layer). In the arteries, a layer of elastic tissue called the internal elastic membrane marks the outer boundary of the tunica intima.

b) The Media

The tunica media is the middle layer of blood vessels. It consists of concentric sheets of contractile smooth muscle cells (SMCs) supported by an extracellular matrix (ECM) composed mainly of elastin, collagen type I and

III and proteoglycans [7]. The contraction of SMCs is controlled by the autonomic nervous system, hormones and local chemicals. Contraction of the SMCs is called vasoconstriction and results in a decrease in the size of the lumen and a decrease in blood flow. Relaxation of the SMCs is called vasodilation which has opposite effects. Small variations in the diameter of the vessels have marked effects on blood flow or pressure, thus the media plays an important role in the regulation of circulation in tissues and organs. Arteries sometimes have an outer layer of elastic tissue called the external elastic membrane. When present this marks the boundary between the tunica media and tunica adventitia.

c) The Adventitia

The adventitia is a connective tissue that forms the outermost layer of the vessel. It is composed mainly of collagen type III, elastin and fibroblasts (FBs). The composition of the ECM of this layer allow the blood vessels to stretch while preventing excessive dilation and often blend into the surrounding tissues. In thick-walled vessels, the adventitia may include nerve fibers and blood vessels to supply oxygen and nutrients to the outer layers. These vessels are called vasa vasorum [8].

0.2.3 The Blood

The primary function of blood is to deliver oxygen and nutrients to and remove wastes from body cells. The specific functions of blood also include immunological defense, distribution of heat, and maintenance of homeostasis [9]. Blood constitutes approximately 8% of adult body weight. Blood is a connective tissue and like all connective tissues, it is made up of cellular elements and an extracellular matrix. The cellular elements—referred to as the formed elements—include: 1) Red blood cells (also known as erythrocyte), which are anucleated biconcave disks containing hemoglobin whose primary function is to transport oxygen from the lungs to the body's tissues; 2) White blood cells (or leucocyte), which are a major component of the immune system implied in the defenses against infectious diseases and 3) platelets (or thrombocytes), cell fragments critical for the process of hemostasis, the stoppage of blood outflow from a damaged vessel (hemorrhage). The extracellular matrix, called plasma, makes blood unique among connective tissues because it is fluid. Plasma, which is mostly composed of water,

perpetually suspends the formed elements and enables them to circulate throughout the body within the cardiovascular system.

0.3 Main Pathologies affecting the Cardiovascular System

Cardiovascular diseases (CVDs) are one of the leading causes of death in the world. According to the World Health Organization (WHO), CVDs were responsible for 17,9 million deaths in 2017, almost 31% of the total global mortality [1]. CVDs include numerous pathologies, many of which are related to a process called atherosclerosis.

Atherosclerosis [10] affects arteries like the aorta, coronary, brain, pelvis, legs, arms or kidneys arteries. It is a condition that develops when a plaque builds up in the walls of the arteries, causing a partial or total occlusion (stenosis) of the artery lumen and thus reducing the blood flow (ischemia). If the oxygen supply to the heart muscle is reduced (hypoxia), a heart attack can occur. Lipids, cholesterol, foamy cells, cellular debris and calcium accumulate over time in the artery wall. Up today, the mechanisms underlying the insurgence of atherosclerosis are not yet fully understood. These substances stimulate SMCs to proliferate and produce other proteins and chemo-attractive biomolecules, resulting in the accumulation of more cells in between the intima and media layers of the artery where the atherosclerotic lesions form. The arterial wall becomes markedly thickened by these accumulating cells and surrounding materials, leading to ischemia (**Figure 0.3)**. Often, the rupture of the fibrous cap of the plaque will lead to the formation of a blood clot that will block the artery (thrombus), stopping the blood flow.

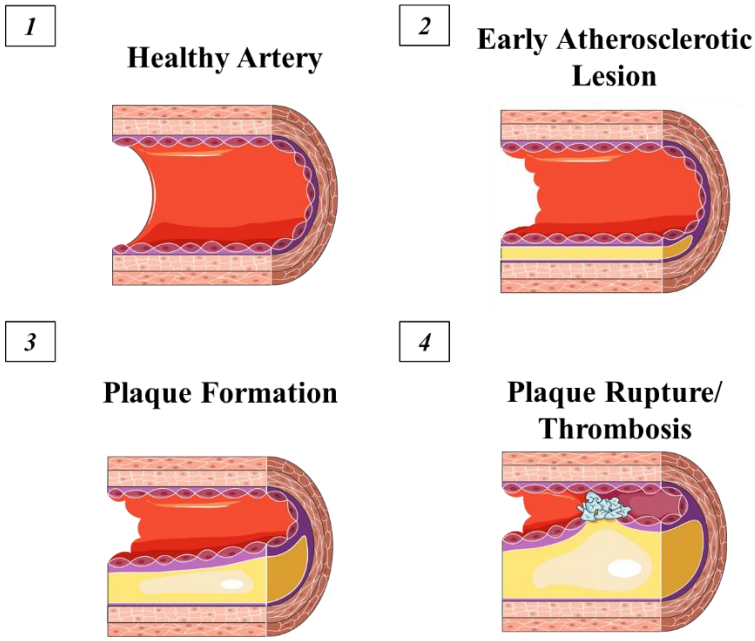

Figure 0.3*:* *Atherosclerosis*: Progression of the atherosclerotic lesion affecting an artery.

0.4 Current Clinical Treatments

Risk factors and the occurrence of a stenosis, or its aggravation, can be prevented. Heart-healthy lifestyle changes can help in preventing or limiting atherosclerosis. These include: Healthy eating, weight loss, managing stress, physical activity and quitting smoking. In the presence of a genetic predisposition or when the first clinical signs have already appeared, pharmaceutical drugs such as statins (to lower cholesterol levels) [11], angiotensin-converting enzyme (ACE) inhibitors and β-blockers (antihypertensive drugs) [12] are recommended.

In presence of severe atherosclerosis, a medical procedure or surgery to remove the atherosclerotic plaque is highly suggested. Balloon angioplasty is a procedure used to re-open blocked or narrowed coronary arteries. This procedure can improve blood flow to the heart and relieve chest pain. Through an incision in the femoral artery, a catheter on which is placed a balloon is inserted. Once inflated, the balloon compresses the plaque, re-opening the artery lumen [13]. Often a stent, a small mesh tube made of metal (stainless steel, magnesium alloy or titanium), is placed in the artery to keep it open after the surgical procedure [14](**Figure 0.4**).

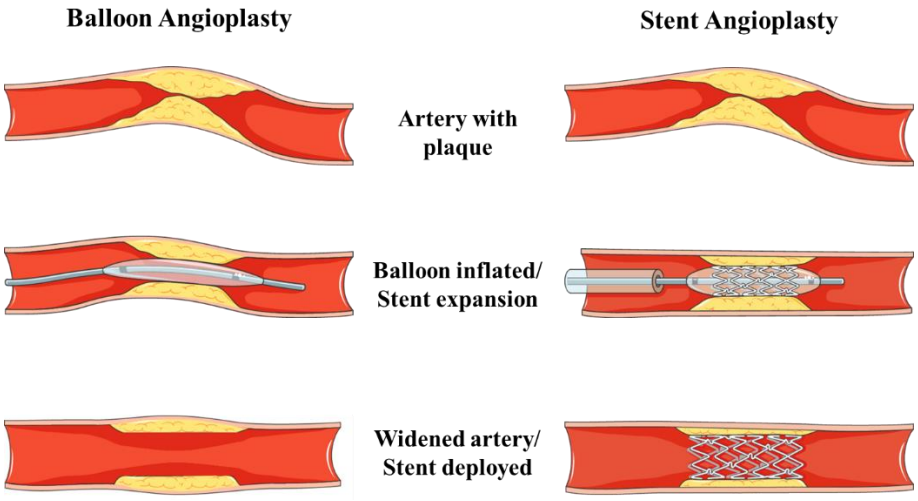

Figure 0.4*: Angioplasty:* Main steps of balloon and stent angioplasty procedure.

When the vessel's occlusion is severe and other treatments are not suitable, replacement or bypass surgery are the only current solution. During surgery, the diseased part of the artery is removed and replaced or bypassed by a graft or a prosthesis to restore blood circulation (**Figure 0.5**).

Autologous grafts are the ideal substitute solution with a success rate of 90% at one year [15]. Most often, the saphenous vein of the patient is used in this procedure. It is a vein running through the leg and its diameter and length make it a suitable substitute for

femoral, popliteal and carotid bridges and aorto-coronary bypass. In the absence of available natural substitutes, the simplest solution is to turn to synthetic substitutes.

The development of synthetic prostheses took off in the 1950s and opened a therapeutic alternative for the replacement of injured arterial segments. In 1952, the first synthetic vascular bypass was performed by Voohrees with the implantation of a porous textile prosthesis made of polyethylene terephthalate (PET), also known as Dacron® [16, 17]. These prostheses are indicated for the replacement of vessels of large calibers (> 10mm in diameter). Then in 1976, the first use of expanded polytetrafluoroethylene (ePTFE), also known as Teflon®, was reported [16, 18]. These prostheses are indicated in the replacement of medium-sized vessels, between 6mm and 10mm in diameter. No studies show the superiority of PET compared to ePTFE [19] .

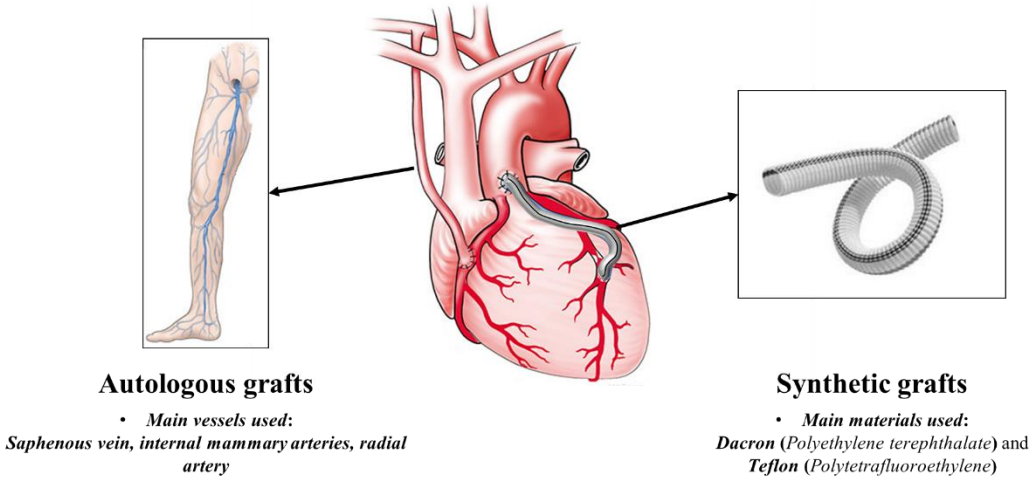

Figure 0.5*: Bypass surgery:* Use of autologous and synthetic grafts for bypass surgery.

Although many improvements have been made over the years, the clinical performance of these prostheses, especially for small diameter vessels bypass/substitution (Ø<6 mm) [20], is still hampered by high rate of graft lumen occlusion due to intra-graft thrombosis and intimal hyperplasia, especially at the anastomosis site, that are regularly observed in the months or years following surgery [2, 21]. The formation of a functional endothelial layer, known as endothelialization, in the lumen of the vascular substitutes would significantly improve small-diameter graft survival by: i) Preventing thrombus formation on the graft surface; ii) Enhancing internal healing and limiting intimal hyperplasia [22]. Developing prostheses for patients without suitable autologous vessels that can be used for bypass surgery following vascular pathologies has become necessary [23].

0.5 Vascular Tissue Engineering

Vascular tissue engineering (VTE) it is an inherently multidisciplinary area of research combining the know-how of several research fields with the aim of developing technologies for vascular medicine applications featuring biological and mechanical properties as close as possible to those of the native vessels, in order to ameliorate the integration and healing of the implanted substitutes. The ultimate goal of VTE is the development of artificial vascular substitutes. The ideal substitute must fulfil strict specifications such as: Absence of thrombogenicity and immunogenicity; Suitable mechanical properties; High availability, resistance to suture and infections and low costs [24-27]. The tissue engineering derived vascular substitutes can be classified according to various criteria. One of these criteria is the origin of the polymer used as a scaffold for the vascular substitutes. Polymers can be of synthetic or of natural origin. Synthetic polymers include biodegradable synthetic polymers, such as polyglycolic acid (PGA) [23], or permanent composite synthetic polymers, such as PET, ePTFE and polyurethane [23]. Natural polymers are usually proteins that constitute the original architecture of native ECM. The generation of protein polymers that mimic native structural proteins and adopt the characteristics of the arterial wall offers a unique approach to develop a vascular graft. There are three major types of natural vascular substitutes derived from tissue engineering:

- ***Decellularized matrices***, derived from living tissues that undergo chemical and mechanical treatments to remove cells from the tissue obtaining biological scaffolds that are expected to maintain the complex 3-D structure and biomechanical properties of the ECM [28, 29], allowing the re-cellularization with autologous cells to produce a new immunologically compatible tissue adequate for implantation [30].
- ***Self-assembly derived substitutes***, [31, 32] in which the scaffolds are obtained by wrapping cellular sheets made of cells and synthesized collagen. Cells (SMCs and FBs) are cultured to form cell sheets that are subsequently rolled around a mandrel. These tubular structures are further cultured for a maturation period before undergoing endothelialization.
- ***Scaffolding system based on natural polymers***, where cells (most often SMCs and FBs), are seeded in a protein solution which is then gelified [33]. This approach aims to create protein-bound cell matrices similar to vascular tissue.

Type I collagen is the most used protein because it is present in many tissues and can be isolated, solubilized and reconstituted according to numerous protocols [34]. Fibrin is also used for these applications [35, 36] as also elastin [37]. The use of gel solutions based on proteins makes it easy to inoculate cells. This cell suspension can then be molded to obtain the desired shape, for example tubular structures. The cells are uniformly dispersed and immobilized in the matrix during gelation. A reorganization of the matrix by the cells is then observed.

The clinical application of protein-based gels, however, is still limited owing to their low mechanical properties [38-44]. Numerous methods have therefore been developed to reinforce these matrices [45] avoiding the use of non-biological solutions, such as the use of chemicals or synthetic polymers.

Despite the progresses made in the last year in the development of artificial blood vessels, further work is still required to ensure success towards clinical translation. In fact, despite showing promising biological performances, these products still miss important characteristics, mainly mechanical properties, that will ensure a successful implantation of the tissue engineered vessels[46, 47].

However, the strategies developed in the pursue of an artificial blood vessel can still find application in vascular medicine, representing interesting in vivo model for study needing the complex hierarchy typical of the blood vessel architecture [48], or even provide material useful for the amelioration of existing vascular graft [49] or for the development of drug delivery systems (DDS) [50].

0.6 Vascular Grafts Enrichment

As previously stated, the lack of endothelialization is one of the major causes of small caliber vascular grafts failure. The absence of a complete and functional ECs layer on the luminal surface of the grafts leads to early thrombus formation and late restenosis, limiting the use of current vascular substitutes in clinic. Spontaneous endothelialization of vascular grafts or long segments of de-endothelialized arteries does not occur, leading to early thrombus formation, and the progressive SMC proliferation results in intimal hyperplasia, causing the occlusion (restenosis) of the grafted substitute. There is a critical need to control these phenomena by eliciting ECs ingrowth to optimize the physiologic functions of the vascular substitute. Physiological ECs ingrowth is regulated by complex interactions among growth factors and cytokines released by the cells and extracellular matrix within the local vascular microenvironment. For these reasons, the modification

of the grafts luminal surface to induce a faster re-endothelialization has been proposed. In recent years vascular substitutes have been additionally engineered by enrichment with growth factors, cytokines, regulatory proteins, bioactive peptide and genes able to efficiently recruit resident ECs and promote their adhesion and growth on the graft, ultimately guiding the optimal integration and functionality of the implant [51]. Several of these approaches have been shown to be able to promote endothelial cells recruitment for vascular grafts enrichment applications. In **Table 0.1** some of the most used molecules are listed.

Table 0.1: Bio-active molecules used for pro-endothelialization of vascular grafts.

Peptides			
Name	**Derived from:**	**Function**	**References**
RGD (Arg–Gly–Asp)	Fibronectin	Cell adhesion trough integrins interaction	[52, 53]
CAG (Cys–Ala–Gly)	Collagen	ECs selective binding tripeptide	[54, 55]
REDV (Arg–Glu–Asp–Val)	Fibronectin	ECs binding trough $\alpha_4\beta_1$ integrin	[56, 57]
YIGSR (Tyr–Lle–Gly–Ser–Arg)	Laminin	ECs binding trough 67LR receptor	[58, 59]
Growth Factors			
Name	**Function**		**References**
FGF	Fibroblast mitosis, EC migration; SMC proliferation; Vasculogenesis		[60-63]
VEGF	ECs migration and mitosis; Vasculogenesis and angiogenesis		[64, 65]
SDF-1	ECs proliferation; EPCs recruitment; Angiogenesis		[66-69]
Genes			
Name	**Encode for:**	**Function**	**References**
ZNF580	C_2H_2 zinc finger protein	ECs proliferation and migration	[70]

Abbreviations: FGF: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; SDF-1: Stromal derived factor; ECs: Endothelial cells; SMC: Smooth muscle cells; EPCs: Endothelial progenitor cells.

While the discovery of these effects initially had promising prospects for their use in therapy, clinical trials of angiogenic factor delivery have been mostly disappointing, underscoring the need for a wider array of angiogenic factors [71].

0.7 Pleiotrophin

Pleiotrophin (PTN), also known as "heparin binding growth associated molecule" (HB-GAM), is a growth/differentiation cytokine first discovered in 1989 that exert different biological effects on various cell types and is expressed mainly, but not exclusively, during embryogenesis [72]. The corresponding gene, *Ptn,* have been sequenced [73] and it encodes an 18 kDa protein of 168 amino acids. The conservation of PTN amino acid sequences among different species (human, bovine, rat, mouse, and chick) is the highest of any of the known cytokines [74-76]. PTN is known for its high affinity to heparin [72] and with extracellular matrix, from which it can be released into solution by heparin [74]. The binding sites of PTN to heparin were found to be located within the β-sheet domains of the protein, instead of the previously believed sites at the lysine rich N- and C-termini [77]. PTN has been shown to interact with and/or affect cell functions through several cell surface receptors such as syndecans [78], the anaplastic lymphoma kinase (ALK) [79], integrins [80], nucleolins [81] and neuropilin-1 [82]. However, the primary receptors for PTN is protein tyrosine phosphatase beta/zeta (RPTP β/ζ), whose activation can regulate multiple functions [83-86]. Over the years, PTN has been proved to be able to regulate multiple cell functions. The ability to promote cell growth was first described on FBs in 1989 [87]. After this first discovery, PTN has been found to be a mitogen for different cell types, including ECs, epithelial cells and different FBs cell lines [88-90]. PTN is also involved in cell motility [82, 91] and, by means of its structural similarity to the thrombospondin type I (TSR) sequence, PTN can mediate cell-to-extracellular matrix and cell-to-cell interactions [77]. It has been demonstrated to affect neural cells, stimulating neurite outgrowth from different cultured neuronal cell types [72, 92] and promoting process outgrowth when added to glial progenitor cells in primary cultures [93]. It also effects bone tissue: PTN has been found to take part in bone repair [94] and is also an osteocyte-derived factor that participate in mediating the osteogenic response to mechanical loading in bone [95]. Effects of PTN on skeletal muscle regeneration, mainly in the formation of neuro-musculature junctions, have been reported [96], as for the enhancement of cell survival and large-scale propagation of cultured human embryonic stem cells [97]. PTN is known for its effects on the immune system, like

promoting lymphocyte survival and driving immune cell chemotaxis [83], and has also been shown to exert beneficial effects in the hematopoietic niche, regulating the retention and self-renewal of hematopoietic stem cells in the bone marrow [98] and their regeneration [99].

PTN is also highly expressed in multiple human cancers, including breast, pancreas and lungs [84][14], in fact it is implicated in tumor angiogenesis and as proliferative driver and as factor in the resistance to apoptosis. PTN induce extracellular matrix remodeling, increase angiogenesis and stimulate the proliferation of stromal cells in the tumor microenvironment [100, 101].

- **Pleiotrophin and the Cardiovascular System**

Over the years, the effects exerted by PTN on the cardiovascular system, in particular on blood vessel and endothelial cells, have been widely studied. These effects of PTN on angiogenesis were firstly investigated in 1998, by virtue of its expression by ECs during ischemic brain injury healing process [102]. PTN was found to be able to stabilize the formation of capillary-like structures by cultured endothelial cells [103]. Subsequently, it was demonstrated that the induced overexpression of PTN in a rat model of myocardium ischemia was able to promote neovascularization of the infarcted area [104] and it can induce angiogenesis in adult and senescent rat aortic rings *ex vivo* [105]. In recent years, the involvement of PTN in physiological angiogenesis has been studied [106]. An important effect of PTN is the ability to chemotactically attract EPCs at angiogenic sites [107]. PTN seems to play a key role also in inflammation-induced neovascularization. The *in vitro* ability of PTN to induce a downregulation of monocytes/macrophages cell markers and an upregulation of endothelial cell characteristics, resulting in their transdifferentiation into functional endothelial cells has been reported [108] and further confirmed by other studies [109, 110][42, 43]. Moreover, PTN has been shown to be involved in the biosynthesis of catecholamine and in the regulation of the Renin/Angiotensin system in the mouse aorta [111, 112], thus, implying his involvement in the regulation of aortic blood pressure.

0.8 Collagen-based Releasing Systems

To efficiently deliver drugs or bioactive molecules, the choice of a suitable scaffold is of crucial importance. Among the different options available nowadays, natural polymers are one of the most interesting and promising candidates. One of such polymers is

collagen. Collagen comprises 25% (by dry weight) of total protein content *in vivo* [113]; In some types of collagen, the entire molecule is a triple helix (**Figure 0.6**), while in other types only a portion have this structure [114]. Of the various types of collagen, type I is by far the most prevalent form [115]. Mature type I collagen is composed of about 1000 amino acids and present a triple helix structure.

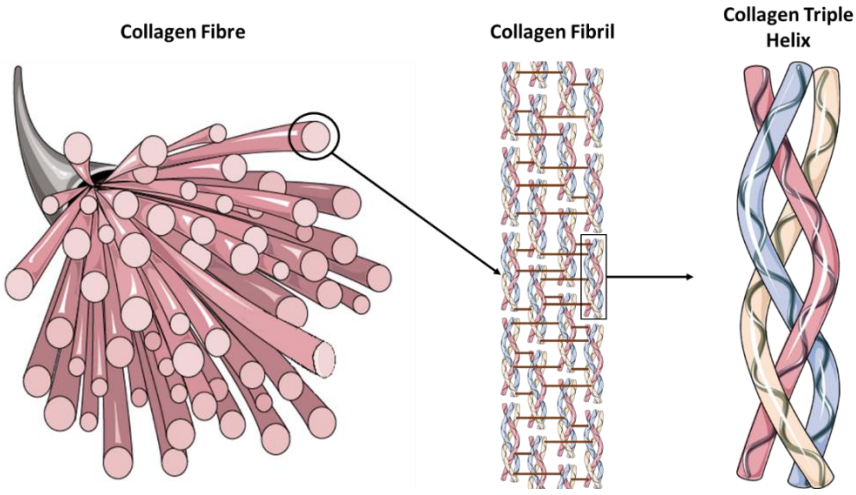

Figure 0.6*: Schematic representation of collagen structure*.

It is the main component of extracellular matrices, conferring their strength and shape to connective tissues such as bone, teeth, cartilage, tendons, ligaments and fibrous matrices of the skin and blood vessels. Type I collagen is widely chosen as a biomaterial for medical applications due to its ease of extraction, weak antigenicity, robust biocompatibility, and its ability to be physically and chemically modified for a variety of applications [116-118]. Among the applications the following can be mentioned: Substrate for cell cultures [119], suture material [120], tissue engineering scaffold for bladder reconstruction [121], bone tissue engineering scaffold [122], tendons reconstruction [123, 124], sponges are used to treat burns and other injuries (58) and scaffold for the regeneration of vascular tissue [125]. Due to its favorable biological properties, collagen-based matrices have been thoroughly investigated as a releasing system for therapeutic drug delivery applications. These investigations date back to the ‘70s [126] and over the years collagen-based drug delivery systems, including injectable microspheres, implantable collagen gels, interpenetrating networks of collagen and collagen membranes, have been studied for the delivery of growth factors, proteins, drugs, genes and cells [50] for application in several tissues such as bone [127], eye [128], ischemic heart [129], ischemic brain [130] and others. In **Table 0.2** some of the use of collagen-based releasing systems are reported.

Table 0.2: Collagen-based matrices/scaffolds for drug, cell and gene delivery used in different tissue engineering applications.

	Scaffold structure	Medical application	Biomolecule used	Cells seeded	Animal model used	Reference
Growth factors /Drugs	Collagen Sponge	Cartilage repair	bFGF	chondrocytes	Nude mice	[131]
	Collagen gels	Vascularization	VEGF	/	Chorioallantonic membrane	[132]
	Collagen sponges	Wound healing	VEGF	/	Rabbit anterior cricoid cartilage wound	[133]
	Collagen sponges	Tissue regeneration	bFGF, HGF, PDGF-BB, VEGF; IGF-1, HB-EGF	/	Mice subcutaneous implantation	[134]
	Collagen sponge	Antibacterial	Gentamicin	/	Horse tarsocrural joint	[135]
Genes	Collagen gels	Bone/Cartilage regeneration	BMP2 (gene)	BM-MSC	Mouse femoral muscle	[136]
	Collagen gels	Skin wound repair	PDGF A and B (gene)	/	Rabbit dermal ulcer/ Porcine dermal wound	[137]
Cells	Electrospun collagen	Bone	/	BM-MSC	/	[138]
	Collagen-Glycosamminglycans scaffold	Cardiovascular	/	BM-MSC	Rat myocardial infarction	[139]
	Collagen sponges	Brain	/	NSC	Rat cerebral ischemia	[140]
	Collagen sponges and hydrogels	Intervertrebal discs	/	Human intervertebral disc cells	/	[141, 142]

Abbreviations: bFGF: Basic fibroblast growth factor; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor; PDGF-BB: Platelet derived growth factor-BB; IGF-1: Insulin like growth factor-1; BMP2: Bone morphogenetic protein 2; HB-EGF: Heparin binding epidermal growth factor-like growth factor; BM-MSC: Bone marrow mesenchymal stem cells; NSC: Neural stem cells.

Among the different structure used, collagen gels are widely used for soft tissue applications. As already mentioned in the introduction, collagen gels are flowable, allowing to mold them to obtain different shape, like tubular structures, or to easily inject them. The most readily available forms of such collagen gels are suspensions of collagen fibers. However, fibrillar collagen gels have an effective pore size too large to allow a controlled release of the loaded protein-based drugs, such as growth factors and cytokines, by hindered diffusion. To control release, it is necessary to rely on binding of the active agent to collagen. Over the years, many types of drug delivery systems based on collagen have been developed for the control release of small molecule and protein-based drugs for biomedical applications [143]. The use of specific non-covalent interactions to stabilize drugs and immobilize them within a biocompatible matrix, thus protecting their biological activity and slowing their diffusion from the matrix, has been widely investigated in the last years. To achieve this result, several approaches have been used to modify the collagen scaffold.

- **Heparin**

One of such approaches rely on the modification of the collagen scaffold with heparin [144]. Heparin is a linear polysaccharide synthesized only in mast cells, where it is cleaved from the core protein serglycin [145]. Heparin polymer chains are made up of repeating disaccharides, the most common are 2-O-sulfated iduronic acid and glucosamine with varying degrees of sulfation and N-acetylation (**Figure 0.7**).

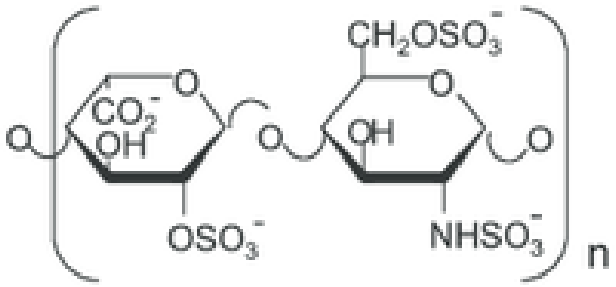

Figure 0.7*: Heparin major disaccharide repeating units [F].*

Heparin is best known for its anticoagulant properties, but has also been shown to promote cell adhesion, inhibit smooth muscle cell proliferation and to moderate inflammation [146]. Moreover, heparin is also known to sequester, stabilize and protect growth factors and

cytokines [144] and has been widely used in conjunction with different scaffolds to enhance their retention ability (**Table 0.3**).

Table 0.3: Heparin-modified scaffolds for growth factor delivery in different tissue engineering applications

Polymer/Scaffold Structure	Growth factor used	Application	Reference
Collagen gel	HB-EGF	Tissue engineered corneal equivalent	[147]
Collagen coating	VEGF	Porous orbital enucleation implants	[148]
Collagen-based demineralized bone matrix	PDGF	Tissue regeneration and wound repair	[149]
Collagen gel	BMP2	Bone repair	[150]
Heparin-conjugated fibrin-filled collagen sponges	BMP2	Bone repair	[151]
Polyethylene glycol hydrogels	VEGF	Vascularization of tissue engineering scaffolds	[152]

Abbreviations: HB-EGF: Heparin binding epidermal growth factor-like growth factor; VEGF: Vascular endothelial growth factor; PDGF: Platelet derived growth factor; BMP2: Bone morphogenic protein-2.

Heparin interactions with proteins are largely electrostatic, however, there are clearly contributions from hydrophobic effects and hydrogen bonding, as well as promoting secondary structure in the proteins binding to heparin, which imparts some selectivity and specificity [153]. Finally, of importance for its use in cardiovascular applications, heparin itself has been shown to be able to stimulate angiogenesis [154, 155].

0.9 Strategy and Structure of the Project

Vascular bypass/replacement surgery is the primary clinical therapy for patients with advanced vascular occlusion diseases such as atherosclerosis. For this application, the need for functional small-caliber grafts is highly demanded. Autologous replacement vessels are the gold standard for this application [156, 157]. However, because of the usual bad condition of the vascular system in the patient these vessels are not always available to be used [21, 158, 159]. In this scenario, the only alternative is the use of an artificial vascular substitute. Despite the improvements observed over the last years, the use of synthetic grafts is still burden by high rates of implants failure. After implantation, the low patency of the vascular substitutes is mainly related to early in-graft thrombosis, caused by platelet deposition and

blood coagulation, and to late intimal hyperplasia near the anastomotic regions [160, 161]. Failure of vascular grafts is mainly determined by the lack of endothelial cells, as these cells effectively inhibit thrombosis and intimal hyperplasia [162, 163].

Therefore, the rapid establishment of an endothelial lining on the luminal surface of a vascular prosthesis would be beneficial to prevent early thrombosis and failure and for the long-term patency of the grafts. Several approaches have been used to achieve the formation of an ECs monolayer by seeding with harvested ECs or endothelial progenitor cells (EPCs) followed by *in vitro* maturation steps, resulted in an improvement of the patency [164, 165]. Despite these positive results, *in vitro* endothelialization involves multiple specialized procedures and cell cultures require long incubation period, making it cost ineffective, inconvenient and limited to adequately-equipped facilities.

In recent years, to overcome the problem, implants modifications with bioactive molecules (growth factors, cytokines and other regulatory proteins) capable to efficiently recruit resident ECs, to promote their adhesion and growth speeding up the re-endothelialization process and, ultimately, guide the optimal integration and functionality of the grafted vessel [166-169] have been proposed over the last years. However, clinical trials of pro-endothelialization enrichment of vascular grafts have been mostly unsatisfactory, thus the need to investigate a wider array of angiogenic factors and new approaches for this application [71].

Pleiotrophin (PTN) is an 18-kDa growth/differentiation cytokine able to regulate multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability [170] and has been described to be a potent enhancer of angiogenesis and neovasculogenesis [104, 105].

However, the concentration of biomolecules must be fine-tuned in order to accomplish the desired effects on the migration and proliferation of ECs, thus the need to release these molecules in a controlled way. The development of drug delivery systems (DDS) capable of controlling the release of therapeutic agents have been widely explored in recent years. The use of collagen as a scaffold for DDS applications is justified by several favorable properties that characterized this natural polymer [116-118]. To further modulate the release of the loaded proteins, specific non-covalent interactions can be used to stabilize drugs and immobilize them within the collagen scaffold [143]. These systems help in protecting the

biological activity of the loaded proteins while further controlling their diffusion from the gel. Heparin, a polysaccharide mainly known for its anticoagulation properties, has been used in DDS to better control the release of bioactive molecule in virtue of its ability to sequester, protect and release growth factors and cytokines [144]. Moreover, PTN is known to have a high affinity for heparin [102].

The general hypothesis of this research project is:

A fast endothelialization of vascular grafts, by means of their modification with pro-endothelialization molecules, will help in limiting the adverse events that lead to the implant failure

In this context, the specific hypothesis to this research project are:

- ***a)*** *PTN, due to his known properties towards ECs, can be used as an effective endothelialization enhancer.*
- ***b)*** *The use of a collagen-based drug delivery system capable of prolonging the release of PTN while preserving its biological properties towards ECs.*

Stated the hypothesis, the general objective of the research project will be:

The development of a drug delivery system for the administration of pro-endothelialization drugs,

With the specific objective being:

- **a)** Assess the biological effects exerted by PTN on the viability and migration ability of ECs.
- **b)** Develop a DDS for the controlled release of PTN based on a type I collagen gel modified with the addition of heparin to increase the retention and thus prolonging the release over time of PTN from the scaffold.

This PhD research project is part of the activities of both the Laboratory for Biomaterials and Bioengineering (LBB) of Laval University and the Laboratory of Human Anatomy of the University of Piemonte Orientale (UPO) and was carried out in a joint PhD activity.

The Laboratory of Human Anatomy main fields of interest concern tissue engineering with special attention to the cardiovascular applications, the effects of mechanical stress applied to cell cultures (mechano-transduction) and materials bio- and hemocompatibility. The

expertise of the laboratory in biological processes linked to tissue engineering applications will be used for the characterization of the pro-endothelialization properties of PTN.

To evaluate these properties, the effects of PTN on ECs have been compared with the ones exerted by SDF-1, a well-known chemokine which plays an important role in angiogenesis and neovascularization [171, 172] already used for vascular grafts enrichment. Viability, cell migration ability and the expression of molecular marker related to the aforementioned process have been analyzed.

The LBB has chosen to work on vascular tissue regeneration from a scaffolding system based on the use of collagen as a natural polymer. One of the short-term objectives of the LBB is to develop in vitro models of vascular walls with the long-term mission of create knowledge leading to a tissue engineered vascular substitutes. Since 2002, type I collagen has been extracted from rat tail tendons and stored according to a protocol developed in the laboratory [173, 174]. The obtained collagen solution can be used to obtain collagen gels that seeded with vascular cells that can be shaped during gelation by means of molds to give them a flat or cylindrical shape [34]. In recent years, a tubular multi-layer tri-culture in vitro model based on a cellularised collagen gel has been developed at LBB [175]. This gel features a multi-layered organization characterized by an adventitia-like layer populated with FBs, a media-like layer seeded with SMCs and an intima-like ECs monolayer. Despite the significant progress that has been made, this tissue engineered model still lack the biological and mechanical complexity required to mimic a healthy human artery for vascular substitution application. However, it can be readily effective as a pseudo-physiological vascular model to study vascular cell interactions, as well as for drug testing and the investigation of physiological and pathological processes. Moreover, the collagen scaffold can be used for other cardiovascular applications, such as the development of pro-endothelialization DDS.

The type I collagen gel developed in the LBB of the Laval University [176] has been used as a scaffold for the DDS. To increase the binding to the gel and prolong the release of PTN over time, heparin has been added to the gel formulation. The PTN-heparin-collagen gels have been characterized both for their mechanical and biological properties. Stress/relaxation unconfined compression tests and immunofluorescence microscopy have been used to assess the mechanical properties of the modified collagen gels. Enzyme-linked immunosorbent assay (ELISA) has been used to characterize the release kinetic of PTN from the heparin-

modified collagen gels. The biological performance on ECs and SMCs viability and migration ability of the gel-retained and released PTN have been analyzed. Moreover, the effects of the modification with heparin on the hemocompatibility properties of the collagen gels have been evaluated.

Chapter 1: Collagen-based biomaterials for vascular tissue engineering

1.1 Introduction

Vascular bypass/substitution surgery represent the most common solution for the treatment of progressively occlusive cardiovascular diseases such as arteriosclerosis. The gold standard for this kind of application are autologous blood vessels, such as saphenous veins or radial arteries, that present the best structural, mechanical and biological properties. However, the use of these substitutes is not always possible, due to the multiple surgical procedures required. Moreover, a large part of the patients needing bypass surgery, may not have healthy arteries useful as autograft. The usual old age of the patients needing treatments, vascular diseases preventing the use of autologous vessels or previous harvesting for other surgical treatments are some of the limiting factor for the use of autograft. Synthetic grafts have been developed as alternatives to autografts, but their low patency owing to short- and intermediate-term failure still limits their clinical application, prompting the need for a viable alternative for this application. Tissue engineering is a multidisciplinary domain aimed to develop biologically-based tissues that can be used in the clinical treatments of diseases. Tissue engineering products have already shown to be effective in different applications, ranging from burns treatment to osteo-regeneration. The success obtained by this approach in other medical fields has opened the door for its use in vascular reconstruction. The use of scaffolding system based on natural polymers is one of the strategies used in vascular tissue engineering. Cells are seeded in a polymeric solution which is then jellified [33], with the aim to create protein-bound cell matrices similar to the vascular tissue. The ideal scaffold should be able to mimic the native vascular extracellular matrix (ECM) and the highly complex organization of the artery wall, showing important biological and mechanical characteristics, such as non-thrombogenicity, hemocompatibility, biocompatibility, non-immunogenicity along with tensile strength and viscoelasticity.

Among the natural polymers currently used for vascular scaffolding, collagen is the most used. It is one of the main components of the vascular ECM. Its main function is to guarantee structural resistance upon elongation of blood vessels under pressure and to provide suitable attachment for vascular cells [12] and several favorable biological features make it a perfect

candidate for its use in scaffold-based vascular tissue engineering. In this review, the main characteristics of the collagen molecule, along with the different types, will be presented. Moreover, the different use of collagen in vascular tissue engineering will be detailed, focusing on its use as a scaffold for the study of vascular cells in 3D-environments and its use for the development of artificial blood vessels. Collagen-based vascular substitute coatings will be then presented and, finally, the use of collagen for the development of drug delivery systems (with a focus on the ones with vascular applications), will be discussed.

1.2 Collagen

1.2.1 Structure and biosynthesis

Collagen is the most abundant protein in animals, therefore in the human body [177]. It accounts for one third of the total protein content and it is the main component of the ECM. To date, 28 different collagen types have been identified in vertebrates, and the discovery of collagen in dinosaurs bone fossils make it the oldest protein ever detected [178, 179]. Collagens can be divided into two main categories: Fibrillar and non-fibrillar. These collagens form elongated fibril structures and are known for their structural role of mechanical support in most animal tissues [180]. Non-fibrillar collagens are characterized by interruptions in their collagenous domains and are known to form different types of structures such as networks, beaded filaments or anchoring fibrils. The main type of both categories of collagens, along with their distribution and composition are listed in **Table 1.1**.

Table 1.1: Main Collagen types and their distribution in the human body. Modified from [177].

Structure	Type	Composition	Chains	Distribution
Fibrillar Collagens	**I**	Heterotrimer	$[\alpha1(I)]_2\alpha2(I)$	Skin, cornea, blood vessels, bone, ligaments and tendons
	II	Homotrimer	$[\alpha1(II)]_3$	Cartilage, intervertebral discs
	III	Homotrimer	$[\alpha1(III)]_3$	Skin, blood vessels
	V	Heterotrimer	$[\alpha1(V)]_2\alpha2(V)$ or $\alpha1(V)\ \alpha2(V)\ \alpha3(V)$	Skin, cornea, blood vessels, bone, ligaments and tendons
	XI	Heterotrimer	$\alpha1(XI)\ \alpha2(XI)\ \alpha3(XI)$	Cartilage, intervertebral discs
FACIT	**IX**	Heterotrimer	$\alpha1(IX)\ \alpha2(IX)\ \alpha3(IX)$	Cartilage
	XII	Homotrimer	$[\alpha1(XII)]_3$	Ligaments and tendons
Network-Forming	**IV**	Heterotrimer	$[\alpha1(IV)]_2\alpha2(IV)$	Basal lamina
	VII	Homotrimer	$[\alpha1(VII)]_3$	Under stratified epithelium

Abbreviations: FACIT: Fibril Associated Collagens with Interrupted Triple helices

All collagens, being them fibrillar or not, are characterized by the same molecular structures, that is composed of three α chains. These chains can either be identical, thus originating an homotrimer, or be a combination of 2 or 3 distinct α chains forming an heterotrimer. Each α chain contains three basic amino acids, which are glycine, proline and hydroxyproline, and is characterized by the presence of at least one collagenous domain, consisting of a repeating Gly-Pro-X triplet [181]. X is usually an hydroxyproline, however it can be any amino acid, conferring specific functions for the collagen (**Figure 1.1**).

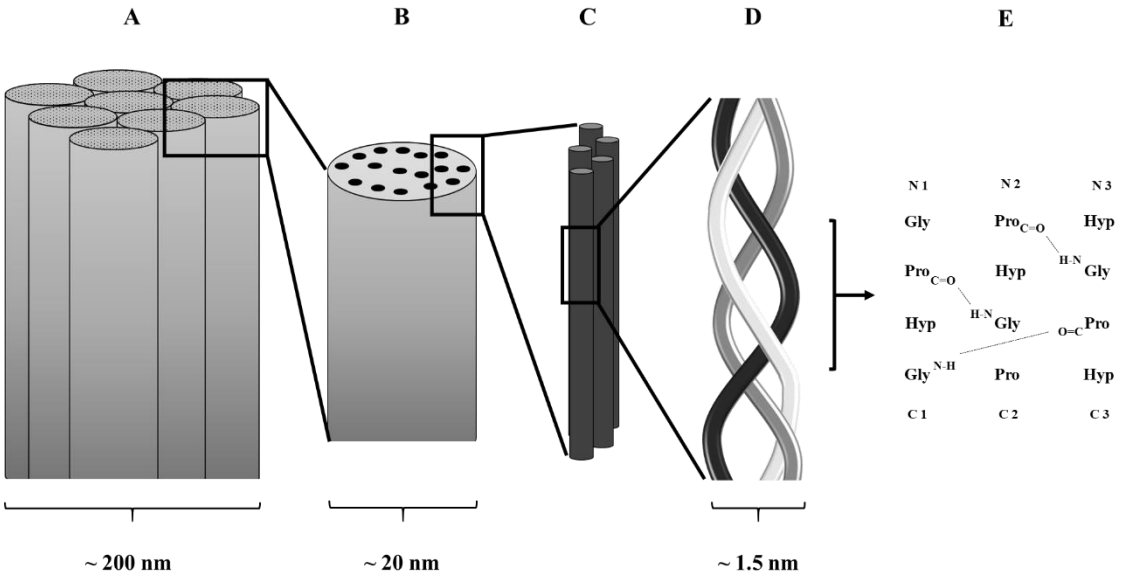

Figure 1.1*: Schematic collagen structure*. A) Collagen fiber formed by assembled collagen fibrils. B) Collagen Fibrils. C) Assembled tropocollagen. D) Collagen triple helix. E) Hydrogen bond in between collagen α chains.

Fibrillar collagens are the most used in the production of collagen-based biomaterials, with Type 1 representing the gold standard being the most abundant collagen in the human body [182]. During the synthesis of a fibrillar collagen molecules, alpha chains are formed by ribosomes present on the surface of rough endoplasmic reticulum (RER). These pre-procollagens present registration peptides and a signal peptide that, once the chains are released in the lumen of the RER, is cleaved to form pro-collagen chains. At this point, the pro-collagens go through several modifications (mainly hydroxylation of the lysine and proline residues and glycosylation of specific hydroxylysines) and they are finally assembled in triple helical structures. These pro-collagen triple helixes are then transferred to the Golgi apparatus to be encapsulated and secreted by exocytosis. Once in the extracellular environment, the registration peptides present on the pro-collagen are cleaved and tropo-collagen is formed. Trough cross-linking, several tropo-collagen molecules are assembled to produce collagen fibrils. In turn, collagen fibrils assemble to form collagen fibers [183].

1.2.2 Collagen as a biomaterial

As previously mentioned, collagen is the most used natural polymer for tissue engineering applications due to its presence in the ECM of almost every human tissue. The use of collagen as a biomaterial date back to the early decades of the XX century, when the first characterization of the interaction between cells and extracted collagen were investigated

[184, 185]. The use of collagen is prompted by several characteristics that make it a good material for biomedical applications: Weak antigenicity and robust biocompatibility, [116, 118, 186, 187], promotion of cell adhesion through cell receptors that recognize specific peptide sequence within collagen molecules [188-190] and biodegradability [191-193]. As an added value, collagen can be isolated from several sources, being one of the most abundant and best conserved proteins among vertebrates. Usual sources for collagen extractions are bovine skin and tendons [194], porcine skin [195] and rat tail tendons [174, 185, 196], but collagen has been also extracted from other organisms such as sponges [197], fishes [198], kangaroos [199] and alligators [200], making it a cost-effective solution for scaffold based tissue engineering.

Collagen-based biomaterials are mainly used for the treatment of burn and as wound dressing [201]. Due to their structure, porosity and surface properties collagens sponges have long being used for wound dressing application [202, 203]. Moreover, they can be loaded with therapeutic agents such as growth factors [204] or antibiotics [205] that greatly improve the healing process once implanted. Another common application for collagen products is as an osteogenic scaffold and filling material in orthopedy [206, 207]. Type 1 collagen scaffolds modified with hydroxyapatite have been used osteochondral scaffold to improve bone and cartilage regeneration [208] and collagen scaffolds can be used as injectable mineralized bone substitutes [209]. Collagen has been widely used also for dentistry applications, such as for the production of membranes for periodontal and implant therapy to improve cells proliferation [210]. Another field of application for collagen is in ophthalmology as corneal shield [211, 212] and as eye implants for post-operative recovery [213] and corneal implantation [214]. Finally, the use of collagen as a scaffold for the development of drug delivery system as attracted the attention of many researchers all over the world [50] for several applications such as bone regeneration, eye, cardiac and brain medicine [127-130] since the ‘70s [126].

1.3 Collagen as a material for vascular tissue engineering

1.3.1 Collagen scaffolds for vascularization and artificial blood vessel development

Over the years, collagen has been used as a pro-vascularization scaffold for several application. In fact, the ability of collagen scaffold to promote angiogenesis and the formation

of neo-vasculature has been demonstrated [215]. Collagen scaffolds have been first used as an in vitro model for the study of the angiogenetic process [216], but their use has been shortly translated to the clinic [217] for several application. In 2008, Shen et al. showed how a VEGF-modified collagen scaffold was able to efficiently promote penetration, proliferation and assembly of ECs in the scaffold [218]. In 2016, Chan and colleagues developed a 3-D scaffold from Type 1 bovine collagen able to support capillary formation *in vivo* and vascularization once implanted in animal models [219]. Similarly, other groups demonstrate how implanted collagen scaffold were able to promote EC infiltration and vascularization [220, 221]. Interestingly, the joint use of other ECM components along collagen, like elastin or glycosaminoglycans, have been shown to exert different effects on the vascularization of collagen scaffolds [222].

As already stated, collagen is one of the most abundant protein in vascular ECM. There, collagen fibers limit the distension of the vessel and provide attachment for smooth muscle cells, allowing them to transmit circumferential forces to the vessel wall, ultimately conferring excellent mechanical support to the blood vessels wall [223]. Therefore, the use of collagen, in particular Type 1, as a scaffold in the development of tissue engineered vascular substitutes has been largely explored. The first use of collagen gels to manufacture a vascular substitutes dates back to 1986, when Weinberg and Bell attempted to reconstitute a blood vessel [125]. Their method consisted in the production of a multilayered tubular construct made of collagen seeded with smooth muscle cells and fibroblasts and of the endothelialization of its lumen. Despite showing very low mechanical properties and the impossibility to be used for clinical purposes, this work marked a major advance in the field of cardiovascular tissue engineering, with several groups following in the footsteps [34, 224, 225] and trying to improve the system. One of the main problems related to this kind of construct is their limited mechanical properties. Different variants of the methodology from Weinberg and Bell such as winding leaflets around a mandrel to promote compaction of collagen [39], magnetic pre-alignment of collagen fibers to increase tensile strength [226], cross-linking of collagen scaffolds by glycation [40] or ultraviolet radiation [227] have been developed to improve the mechanical properties of the substitutes. However, the extent of these improvements still does not allow the implantation and, thus, the use in the medical practice of these grafts. The seeded cells play an important role too: SMC have been

demonstrated to actively influence the compaction of the collagen scaffold [41, 45] and to align along the direction of the collagen fibers [224], helping in increasing the mechanical properties of the substitutes. The biological properties have also been studied. Different molecules have been used to modulate the cellular response towards these scaffolds. The addition of insulin and growth factors such as TGF-β make it possible to increase collagen production by the seeded cells [228] and the addition dermatan sulfate has been able to increase the endothelialization of the lumen and, as a results, to reduce platelet adhesion and activation [38].

In recent years, hybrid collagen vascular substitute containing both synthetic [229-231] and natural polymers, such as fibrin [232] and elastin in particular [233-235], have been developed to further increase the mechanical and biological properties of the collagen-based vascular grafts, aiming to obtained an artificial vessel as close as possible to the natural ones.

1.3.2 Collagen coatings for vascular grafts

One of the main problems related to the use of synthetic vascular grafts, and especially with ones made of polyethylene terephthalate (Dacron), is linked to their high porosity. While this characteristic allows tissue ingrowth, insuring a better integration of the implanted grafts and a faster healing, on the other side cause excessive bleeding, creating a serious complication for the patients. Thus, the walls of the grafts must be rendered impervious in order to avoid this outcome. For this reasons, pre-clotting is usually performed on the Dacron grafts. This technique consists in the conversion of the porous wall of the prosthesis into one that has been rendered impervious by reaction with blood [236]. Despite helping in limiting bleeding, this technique is hampered by several disadvantages such as the creation of a rough luminal surface of the implanted grafts, increasing the occurrence of turbulent blood flow and thrombus formation, and the increase in the rigidity of the graft, that conversely lose their pliability.

The impregnation of porous Dacron vascular grafts with collagen was first proposed in the early ‘60s [237] as an alternative to pre-clotting. Striking improvements were obtained years later by Scott and colleagues in 1987 [238]. Their bovine collagen-coated grafts did not require pre-clotting or special preparation and did not bleed once implanted in a canine model. The luminal surface of the grafts showed neointima formation and the collagen coating was completely resorbed and substituted by native tissue after 3 months of

implantation. Moreover, the collagen was non thrombogenic and antigenic. That opened the door for the use of collagen-impregnated vascular grafts in the surgical treatment of aneurysms and for arterial bypass [49, 239, 240], proving to be a viable alternative to the previously used pre-clotting technique, being able to compete equally against other proposed techniques and materials [241].

Nonetheless, these collagen-coated grafts have been demonstrated over the year not to be free from complications: Variable inflammatory response and tissue adhesion [242], need of sustained chest drainage [243] and initiation of the immune response in the patients [244] in the treated patients. Moreover, they showed no added value for the replacement of small caliber arteries [245]. However, the performances of the collagen-coated vascular grafts have stood the test of time, resulting being one of the most used vascular grafts for medium and large diameter arteries substitution.

1.3.3 Collagen-based drug delivery systems

Biological signaling represents an important point in cell-driven tissue regeneration and providing signaling molecules greatly improve this process. However, when administering molecules and drugs, is of crucial importance to reach the appropriate dose at a specific site and for the necessary period of time, in order to accomplish the desired effects. Thus, the need to release these molecules in a controlled way.

The development of collagen-based drug delivery systems (DDS) for the release of pro-angiogenetic factors for wound healing applications and pro-endothelialization factors for vascular implants functionalization is highly sought after. Collagen has been widely studied as a biomaterial for DDS [246] and has found several uses in a variety of applications (**Table 1.2**).

Table 1.2: Collagen-based drug delivery systems.

	Scaffold structure	*Medical application*	*Biomolecule used*	*Cells seeded*	*Reference*
Growth Factors/Drugs	Collagen sponges	Wound healing	VEGF	/	[133]
	Collagen sponges	Tissue regeneration	bFGF, HGF, PDGF-BB, VEGF; IGF-1, HB-EGF	/	[134]
		Antibacterial	Gentamicin	/	[135]
Genes	Collagen gels	Skin wound repair	PDGF A and B (genes)	/	[137]
Cells	Electrospun collagen	Bone	/	BM-MSC	[138]
	Collagen-Glycosaminoglycans scaffold	Cardiovascular	/	BM-MSC	[139]
	Collagen sponges	Brain	/	NSC	[140]
	Collagen sponges and hydrogels	Intervertebral discs	/	Human intervertebral disc cells	[141, 142]

Abbreviations: bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; PDGF-BB: platelet derived growth factor-BB; IGF-1: insulin like growth factor-1; HB-EGF: heparin binding epidermal growth factor-like growth factor; BM-MSC: bone marrow mesenchymal stem cells; NSC: neural stem cells.

The use of collagen-based DDS for vascular application has been explored in recent years. Most of the studies performed are aimed to increase the affinity for the collagen scaffolds toward ECs. The enrichment of collagen matrices with several pro-angiogenetic growth factors, such as vascular endothelial growth factor (VEGF) [132, 247, 248] and stromal derived factor-1 alpha (SDF-1α) [249], basic fibroblast growth factor (bFGF) [250] has shown promising results in terms of controlling the release of the loaded molecules and the

angiogenesis induction, resulting effective during wound repair and for tissue engineering applications.

As stated in the Introduction section of this review, the use of synthetic vascular grafts for the treatment of occlusive vascular diseases is still burden by grafts failure, mainly caused by thrombosis and neointima hyperplasia. Implants modifications using pro-endothelialization molecules and growth factors with the aim of speeding up the re-endothelialization process has been proposed over the last years to guide the optimal integration of the grafts and to overcome the aforementioned problems. The use for vascular grafts enrichment has also been investigated. In their work from 2000, Wissink and colleagues developed an heparinized, crosslinked collagen matrix for the controlled release of basic fibroblast growth factor (bFGF) to improve the endothelialization of vascular grafts [251]. They were able to improve the binding of the loaded bFGF to the heparinized crosslinked matrix and to release it in a controlled way over time, leading to an improvement in the proliferation of treated EC *in vitro*.

The occurrence of infections in newly implanted synthetic vascular graft is one of complications that may arise, hampering the functionality of the prosthesis. Conventional treatments of vascular graft infections consists in the excision of the infected graft with extra anatomic bypass grafting [252]. To avoid the need of surgical operation to treat the infected grafts, the use of DDS has been proposed. In particular, collagen-based matrices have been demonstrated to be effective in delivering antibiotic agents to limit and treat bacterial infections in implanted synthetic vascular grafts [253, 254], avoiding the need of subsequent surgical intervention.

1.4 Conclusions

Collagen-based scaffolds have been proven to possess excellent biocompatibility and sufficient mechanical properties and have gained great achievements in vascular tissue engineering, proving to be a versatile biomaterial for vascular applications. Future perspectives are focused on further ameliorate the characteristics of these molecule, in order to obtained collagen-based scaffolds with performances able to mimic the one of natural collagen, both in structural and biological properties.

Chapter 2: Pleiotrophin: analysis of the endothelialisation potential

Francesco Copes[a,b¶], Martina Ramella[a], Luca Fusaro[a], Diego Mantovani[b], Mario Cannas[a], Francesca Boccafoschi[a¶*]

a) Laboratory of Human Anatomy, Department of Health Sciences, University of Piemonte Orientale, Novara, Italy

b) Laboratory for Biomaterials and Bioengineering, Canada Research Chair Tier I in Biomaterials and Bioengineering for the Innovation in Surgery, Department of Min-Met-Materials Engineering & CHU de Quebec Research Center, Laval University, Quebec City, Quebec, Canada.

This chapter has been accepted for publication on Advances in Medical Sciences - Volume 64/1 due in March 2019.

2.1 Résumé

Un des problèmes majeurs dans le domaine vasculaire sont la non-endothélialisation des substituts vasculaires, phénomène induisant des complications cliniques, allant jusqu'à l'échec des implants. Pour pallier ce problème, des promoteurs d'endothélialisation sont souvent utilisés, tel que le SDF-1 (stromal cell-derived factor 1). L'alternative proposée dans ce travail, est d'utiliser la pléiotrophine, cytokine de croissance / différenciation, et facteur pro-angiogénique. Bien que cette molécule soit déjà connue pour exercer des effets bénéfiques sur différents types de cellules, son potentiel en tant qu'inducteur de prolifération et de migration des cellules endothéliales n'a pas été encore étudié, à notre connaissance. Ce travail consiste donc à comparer les effets de la pléiotrophine sur la prolifération et la migration des cellules endothéliales par rapport à ceux observés avec le SDF-1.

Pour ce faire, les effets de la pléiotrophine (50 ng / ml) ou du SDF-1 (50 ng / ml) ont été testés sur une lignée de cellules endothéliales, la EA.hy926.. Dans un premier temps, la viabilité cellulaire a été évaluée par dosage MTT, puis des essais de migration cellulaire ont été réalisés dans des chambres Transwell. Pour finir, le potentiel de cicatrisation de plaies induites par égratignure a été évalué et l'expression de différents facteurs tels que CXCR4, RPTP β /, PCNA et Rac1 a été déterminée par Western Blot.

Les résultats ont montré que la viabilité et la migration des cellules endothéliales traitées par la pléiotrophine ont considérablement augmenté comparativement à celles traitées par du SDF-1. L'analyse Western Blot a démontré aussi que le traitement avec de la pléiotrophine induit une plus grande l'expression des facteurs RPTP β / ζ, PCNA et Rac1 par rapport à celle obtenue avec du SDF-1.

En conclusion, les résultats démontrent très clairement les effets bénéfiques de la pléiotrophine sur les cellules endothéliales, tant au niveau de leur viabilité, leur capacité à migrer mais aussi à induire leur régénération. De plus, ces effets sont nettement plus marqués sur les cellules traitées par la pléiotrophine par rapport au SDF-1. La pléiotrophine apparait donc comme une molécule intéressante et prometteuse pour favoriser la ré-endothélialisation des substituts vasculaires

Mots-clés: pléiotrophine, SDF-1, prolifération des cellules endothéliales, migration des cellules endothéliale.

2.2 Abstract

Purpose: endothelialisation of vascular substitutes, in fact, remains one of the most unsolved problems in cardiovascular diseases treatment. Stromal Derived Factor 1 (SDF-1) has been largely investigated as an endothelialisation promoter and pleiotrophin is a promising alternative. Although it has been known to exert beneficial effects on different cell types, its potential as an inducer of proliferation and migration of endothelial cells was not investigated. Therefore, this work is aimed to compare the effects of Pleiotrophin on proliferation and migration of endothelial cells with respect to SDF-1.

Materials/methods: endothelial cell line EA.hy926 was treated with Pleiotrophin (50ng/ml) or Stromal Derived Factor 1 (50ng/ml). Cell viability was evaluated by MTT assay and migration assays were performed in Transwell chambers. Wound healing potential was evaluated by scratch wound assay. CXCR4, RPTP β/ζ, PCNA and Rac1 expression was detected by Western Blot.

Results: Interestingly, Pleiotrophin significantly increased the viability of the treated endothelial cells with respects to SDF-1. The migratory ability of the endothelial cells was also improved in the presence of Pleiotrophin with reference to the SDF-1 treatment. Moreover, Western Blot analysis showed how the treatment with Pleiotrophin can induce an increase in the expression of RPTP β/ζ, PCNA and Rac1 compared to SDF-1.

Conclusion: Due to the significant effects exerted on viability, migration and repair ability of endothelial cells compared to SDF-1, Pleiotrophin can be considered as an interesting molecule to promote re-endothelialisation.

Keywords: *Pleiotrophin, SDF-1, endothelial cells proliferation, endothelial cells migration*

2.3 Introduction

Cardiovascular diseases (CVDs) are one of the leading causes of death worldwide. According to the World Health Organization (WHO), CVDs are responsible for 15 million deaths in 2015, almost 30% of the total global mortality [1]. Among the different diseases, vascular occlusion remains the leading cause of death in Western countries. Arterial bypass graft remains the primary effective surgical therapy for patients with advanced vascular occlusion. Autologous grafts are the ideal substitutes with a success rate of 90% at one year. However, often patients needing bypass surgery may not possess healthy available arteries useful as autograft [255]. In the absence of available natural substitutes, the simplest solution is the use of synthetic substitutes. Although many improvements have been made over the years, the clinical performance of these prostheses for small arteries bypass remains quite disappointing. In fact, lumen occlusion due to blood coagulation and platelet deposition under the relatively low flow conditions along with restenosis, that is commonly observed in the months or years following surgery [2, 21], hamper their use in the clinic. The rapid formation of a functional endothelial layer on the luminal surface of vascular substitutes would significantly improve small-diameter graft patency by: preventing thrombus formation on the graft surface, enhancing internal healing and limiting intimal hyperplasia [22]. In recent years, the enrichment with bioactive molecules such as growth factors and cytokines able to efficiently recruit resident endothelial cells (ECs), promote their adhesion and growth has been proposed as an approach to overcome this problem [256, 257]. Several molecules have been shown to be able to promote angiogenesis or endothelial cells recruitment. Among them, SDF-1 has been studied. It is known as C-X-C motif chemokine 12 (CXCL12), a chemokine protein that in humans is encoded by the *CXCL12* gene. SDF-1 is ubiquitously expressed in many tissues and cell types and its receptor, C-X-C chemokine receptor type 4 (CXCR4), is widely expressed in different tissues like blood vessels, particularly by endothelial cells [258]. SDF-1 is released into the circulation in response to ischemia [259] and is an initiating signal in the angiogenesis process, promoting endothelial cells recruitment. For these reasons, SDF-1 has been widely used for the biological functionalization of vascular substitutes [67, 260-264]. However, even if initially the application of SDF-1 on vascular prosthesis was regarded as very promising, clinical trials

of angiogenic factor delivery have been mostly disappointing, underscoring the need to investigate a wider array of angiogenic factors [71].

Pleiotrophin (PTN) is an 18-kDa growth/differentiation cytokine with high affinity for heparin and it is structurally related to Midkine, the only other member of its protein family [265]. It has mitogenic, differentiating and angiogenic properties for various cell types and is expressed mainly, but not exclusively, during embryogenesis. Through the activation of its receptor, protein tyrosine phosphatase beta/zeta (RPTP β/ζ), PTN is able to regulate multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability [170]. PTN was described as a potent pro-angiogenic factor acting on ECs during healing from ischemic brain injury, and was found to stabilize the formation of tube structures by cultured capillary endothelial cells [105]. Interestingly, recent studies have also pointed out a PTN-induced transdifferentiation of monocytes into functional ECs suggesting a role for PTN in inflammation-mediated neovascularization [108] and a role in the recruitment of endothelial progenitor cells (EPC) during angiogenesis [107].

Considering these evidences, herein the *in vitro* effects of PTN on proliferation, migration and repair ability of the endothelial cells were investigated and compared with those exerted by SDF-1, finally aiming to unravel the PTN potential as an endothelialisation enhancer.

2.4 Materials and methods

2.4.1 Cell Culture

Human umbilical vein cell line EA.hy926 [266] was used in this study. Cells were supplied by the Vascular and Endovascular Surgery Unit, Research Laboratory of Experimental and Clinical Vascular Biology, DISC, University of Genoa, Italy. No Ethical Approval was needed for the use of this cell line. Briefly, EA.hy926 cells were cultured in a cell culture medium so composed: Dulbecco's modified Eagle's medium (D-MEM) with 10% foetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100U/ml) and L-glutamine (2mM) (all products from Euroclone, Milan, Italy). This medium, that will be referred to as complete D-MEM (C-D-MEM), has been used in our experiments along with a serum free version of it (SF-D-MEM). The cells were maintained at 37 ° C in a saturated atmosphere at 5% CO_2. Cell culture media were renewed every two days. When 85% - 90% of confluence was

reached, cells were then enzymatically detached from the plate (0.05% trypsin) and then reseeded at a ratio of 1:10.

2.4.2 Cell Viability Assay

Cell viability was evaluated by MTT Assay. EA.hy926 cells were seeded at a density of 25000 cell/cm^2 in 96 well culture plates (5 wells for each condition). After an overnight incubation at 37°C in a saturated atmosphere at 5% CO_2, cells were treated respectively with: 1) C-D-MEM (CTRL); 2) C-D-MEM enriched with 50ng/ml SDF-1 (Sigma-Aldrich, Milan, Italy); 3) C-D-MEM enriched with 50ng/ml PTN (Sigma-Aldrich, Milan, Italy). The concentration used for the PTN has been chosen following a dose-response curve obtained in the preliminary steps of the study (data not shown). SDF-1 concentration was chosen accordingly to data present in literature [267, 268]. After 24 hours, 3 and 7 days, cells were incubated with the MTT reagent (Sigma-Aldrich, Milan, Italy) for 3 hours at 37°C. After the incubation, the formazan product obtained by the reduction of MTT reagent by the way of the mitochondrial activity was solubilized using dimethyl sulfoxide (Carlo Erba Reagents, Milan, Italy). Than the absorbance at 570 nm was measured with a SpectraCount Absorbance microplate reader (Packard, Connecticut, USA). Absorbance is proportional to cell viability.

2.4.3 Wound Healing Assay

Wound Healing Assay was performed to assess cell migration and reparatory ability *in vitro* in presence of PTN and SDF-1. Briefly, cells were seeded in 6-well multi-plate to obtain a confluent monolayer. Then, cell monolayer was scraped in a straight line to create a "scratch" with a 200µL pipet tip. Medium containing cellular debris was removed, and cells were washed 2 times with sterile PBS 1X and then treated as follow: 1) SF-D-MEM (CTRL), 2) SF-D-MEM enriched with SDF-1 (50ng/ml) or 3) SF-D-MEM enriched with PTN (50ng/ml). Markings were created to be used as reference points close to the scratch. Images at a magnification of 20X were collected at 0, 6, 12 and 24 hours of incubation (n=5). To quantify the scratch-area reduction over time, images acquired from each sample were further analysed by using ImageJ software (ImageJ 1.49v; Wayne Rasband; National Institute of Health, USA). Briefly, for each time point, the outline of the cell-free surface of the scratch was drawn and the inner area was calculated using the software. The percentage of wound

closure was calculated by comparing the areas measured at the different time points with the area at T_0.

2.4.4 Transwell Migration Assay

To verify the migratory response of EA.hy926 cells to SDF-1 and PTN treatments, the transwell migration assay was used. $9x10^4$ cells were seeded in the upper compartment of 24 well-format transwell with 8µm pores (Corning, Amsterdam, the Netherlands) in 250µL of D-MEM without FBS. In the lower compartment, the different treatment compounds were added: 1) SF-D-MEM (CTRL); 2) SF-D-MEM enriched with 50ng/ml SDF-1; 3) SF-D-MEM enriched with 50ng/ml PTN. Cells were incubated at 37°C in a saturated atmosphere at 5% CO_2 for 6 hours. After the incubation, cells on either faces of the porous membrane were fixed by incubation with formalin 4% for 20 minutes at room temperature. Then, cells were stained with 1% Crystal Violet for 20 minutes at room temperature. Once stained, cells on the upper side of the porous membranes were gently removed using a cotton swab. The transwell inserts were then placed under a phase-contrast microscope and images of different fields (n=5) were collected at 20X magnification. To assess the migration rate, for each condition stained cells were quantified.

2.4.5 Western Blot

Cells treated with C-D-MEM (CTRL), C-D-MEM containing 50ng/ml PTN and C-D-MEM containing 50ng/ml SDF-1 were lysed in adequate RIPA lysis buffer (Hepes 50mM pH 7.4; NaCl 150 mM; SDS 0,1%; Triton X-100 1%; Na Deoxycolate 1%; Glicerol 10%, $MgCl_2$ 1,5 mM; EGTA 1 mM; NaF 1mM) additioned with protease and phosphatase inhibitors (Sigma-Aldrich, Milan, Italy). Quantification of the protein extract was carried out using the BCA Protein Assay Kit (Thermo-Scientific, Rockford IL, USA) according to the manufacturer's instructions. Electrophoretic analysis of 30µg of total proteins was performed using 10% SDS–polyacrylamide gel. Gels were blotted onto nitrocellulose blotting membrane (GE Healthcare Life Sciences, Milan, Italy). Then, membranes were probed with the following primary antibody: mouse monoclonal anti-PCNA (1:1000; Millipore, Darmastadt, Germany), mouse monoclonal anti-Rac1 (1:1000), mouse monoclonal anti-PTPz (1:1000), rabbit monoclonal anti-CXCR4 (1:100) (all from Abcam, Cambridge, UK). For the analysis of PCNA and Rac-1, lysates were obtained by cells with both free receptors for PTN and

SDF-1 and with blocked receptors. In the un-blocked receptor group, cells have been grown as previously described. In the blocked receptor group, the receptors for PTN and SDF-1 has been blocked to impeach the interaction between the molecules and their receptors. To block PTN receptor, RPTP β/ζ, cells were incubated with its primary antibody (anti-PTPz, 1:100, Abcam, Cambridge, United Kingdom). CXCR4, SDF-1 receptor, was blocked with 25µg/ml AMD3100 octahydrochloride (Abcam, Cambridge, United Kingdom), a selective inhibitor of CXCR4. Primary antibodies were detected with species-specific horseradish peroxidase-conjugated secondary antibodies (Perkin Elmer, Milan, Italy). The bands were visualized using Western Lightning® Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, Milan, Italy). Results were revealed on membranes and acquired using the VersaDoc MP5000 System (Bio-Rad, Milan, Italy). The intensity of protein bands was measured with ImageJ software. Results were normalized to tubulin.

2.4.6 Statistical Analysis

A n=5 samples for each experiment has been used. Each of the experiment performed was repeated three times. The data shown are means ± standard deviation (SD). Data have been analysed by two researchers through established protocol used in the laboratory. Statistical significance of the presented results was calculated using ANOVA non-parametric Kruskal-Wallis method through the software InStat ™ (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ or less were considered significant.

2.5 Results and Discussion

The formation of a functional endothelial layer in newly implanted vascular grafts is of crucial importance for their functionality [165]. EC seeding on prosthetic materials was proposed as a solution, but several studies demonstrated a significant cell loss after implantation and exposure to blood flow [269]. The enrichment of vascular grafts with molecules acting as chemoattractant for ECs has been proposed as a method to speed-up the re-endothelialisation of the implants *in situ*. Herein we propose PTN as a promising molecule capable of promoting and fastening the formation of a functional endothelial layer. PTN has been shown to be an effective angiogenic agent both *in vitro* and *in vivo*. Therefore, we have investigated whether PTN is able to exert beneficial effects on EC behaviour with respect to SDF-1, a chemokine known to play a key role in angiogenesis and EC chemo-attraction [171,

172, 270]. In our experiments, the human umbilical vein cell line EA.hy926 was used. This cell line demonstrates highly differentiated functions characteristic of human vascular endothelium, while offering the advantages of immortality, stability through passage number thus allowing a more consistent responses to specific variables and greater reproducibility of data [271-274].

Our results show that the treatment with PTN can significantly improve EC viability *in vitro* with respect to SDF-1 and to CTRL cells. The MTT assay showed that after 24 hours, the treatment with both PTN (absorbance at 570 nm: 0.176±0.009) and SDF-1 (0.174±0.015) was able to significantly increase cell viability compared to CTRL (0.145±0.012; $p<0.01$ vs. CTRL). This effect was enhanced at longer time, especially for the treatment with PTN that after 3 days (0.404±0.072) significantly increased cell viability with respect to both SDF-1 (0.243±0.036; $p<0.001$) and CTRL (0.223±0.033; $p<0.001$). After 7 days, again PTN (1.429±0.151) significantly increased cells viability with respect to CTRL (0.664±0.061; $p<0.001$) and SDF-1 (1.197±0.203; $p<0.05$) (**Figure 2.1**). Contrary to the results presented by Palmieri et al. [110], where the treatments with PTN was not able to induce a better viability of the treated ECs, in our experiments PTN was able to efficiently increase the viability of the treated ECs, confirming the effects observed by Brewster et al. with the use of a chimeric PTN fusion protein [275, 276]. Moreover, the treatment with PTN has been shown to obtain better results compared to the treatment with SDF-1.

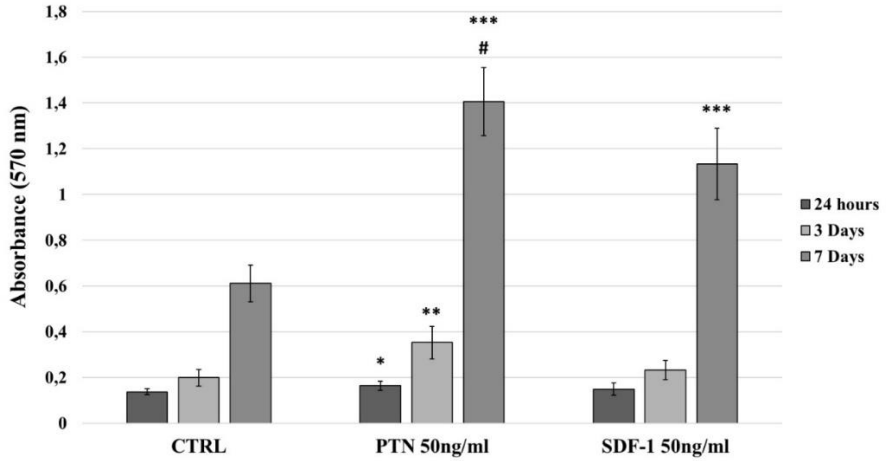

Figure 2.1*: Viability Assay***.** Ea.hy926 cells were treated up to seven days with: basal growth medium (CTRL); growth medium enriched with 50 ng/ml PTN (PTN 50ng/ml); growth medium enriched with 50 ng/ml SDF-1 (SDF-1 50ng/ml). Cell viability was measured after 24 hours, 3 and 7 days with MTT Assay. The graphic shows the mean absorbance recorded for each condition. * $p<0.01$ vs. 24 hours CTRL; ** $p<0.001$ vs. 3 days CTRL and $p<0.01$ vs. 3 days SDF-1 50ng/ml; ***$p<0.001$ vs. 7 days CTRL; # $p<0.01$ vs. SDF-1 50ng/ml.

The expression of CXCR4, SDF-1 receptor, and RPTP β/ζ, PTN receptor, was evaluated by Western Blot analysis (**Figure 2.2**). Concerning the expression of CXCR4, after 24 hours of incubation no significant differences were shown. However, after 3 days, the expression of CXCR4 in presence of SDF-1 (2.48±0.31) significantly increased with respect to both CTRL (0.93±0.14; $p<0.001$). Interestingly, the treatment with PTN (1.79±0.25; $p<0.05$) was able to significantly increase the expression of CXCR4 compared to CTRL ($p<0.05$) (**Figure 2.2A**). Concerning RPTP β/ζ, after 24 hours PTN showed an increased expression (1.32±0.17) of the receptor with respect to SDF-1 (0.95±0.13; $p<0.01$). After 3 days, the expression of RPTP β/ζ in presence of PTN (1.53±0.21) was significantly increased compared to CTRL (0.71±0.13; $p<0.001$) and SDF-1 (0.93±0.12; $p<0.001$). (**Figure 2.2B**). The results here obtained show that the treatment of EC with PTN not only induce, an increase in the expression of PTN own receptor RPTP β/ζ, but also an increase in the expression of CXCR4, suggesting a possible role of PTN in potentiating the response of EC to SDF-1 by means of increasing the expression of its receptor. Similar effects of PTN induction of the overexpression of other growth factor /chemokine receptor has already been reported [110], suggesting a role for PTN in potentiating the pro-endothelialisation effects of other endothelial cells enhancer molecules.

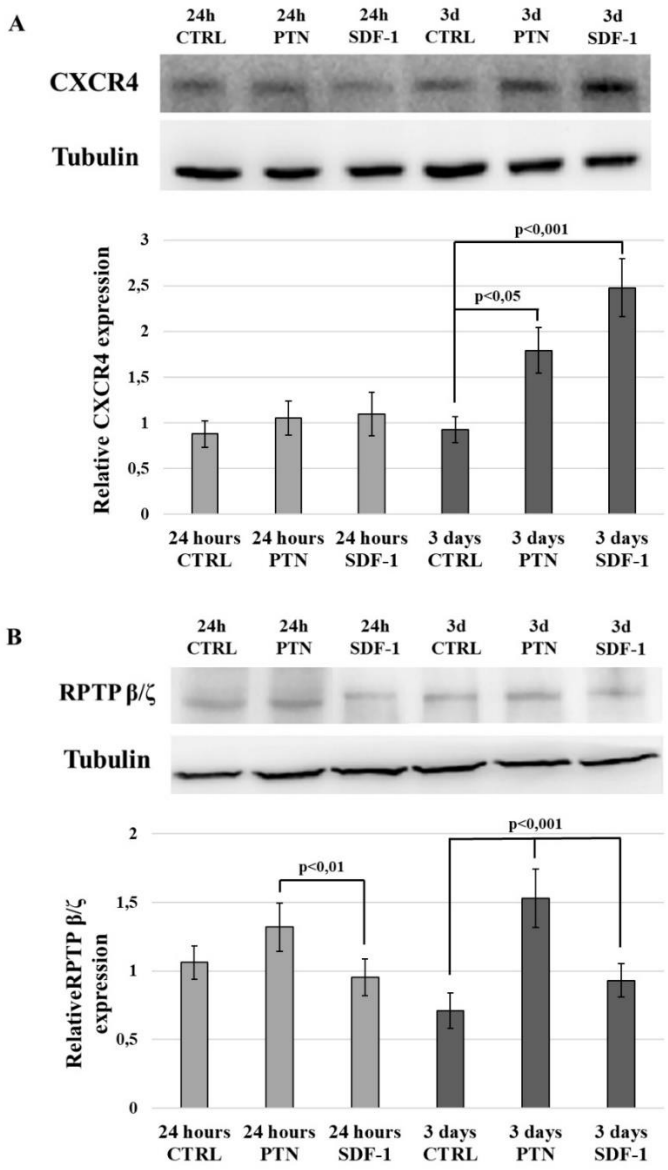

Figure 2.2*: Western Blot Analysis for RPTP β/ζ and CXCR4***.** The images show the results of the Western Blot analysis for the expression of the two receptor in samples obtained by EA.hy926 cells treated with: C-D-MEM (CTRL), C-D-MEM containing 50ng/ml PTN (PTN 50ng/ml) and C-D-MEM containing 50ng/ml SDF-1 (SDF-1 50ng/ml). Lysates were collected after 24 hours (24h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **A**) Expression of SDF-1 receptor CXCR4. **B**) Expression of PTN receptor RPTP β/ζ.

The migration ability of EA.hy926 cells following the treatments with PTN and SDF-1 have been tested by the Wound Healing assay. As shown in **Figure 2.3A**, CTRL showed a 14.7±7.9% scratch reduction after 6 hours, 19.2±0.4 after 12 hours and a 43.3±5% scratch reduction after 24 hours. The enrichment with PTN and SDF-1 significantly increased the rate of wound closure compared to CTRL: with PTN, 27.1±6.3% scratch reduction after 6

hours; 52.4±7.1% after 12 hours ($p<0.001$ vs CTRL) and 75±5% after 24 hours ($p<0.001$ vs. CTRL and $p<0.01$ vs. SDF-1) was achieved; SDF-1 induced a scratch reduction of 17.5±4.8% after 6 hours, a 44.5±2.4% after 12 hours ($p<0.001$ vs. CTRL) and 63.1±7.4% after 24 hours ($p<0.001$ vs. CTRL) (**Figure 2.3B**).

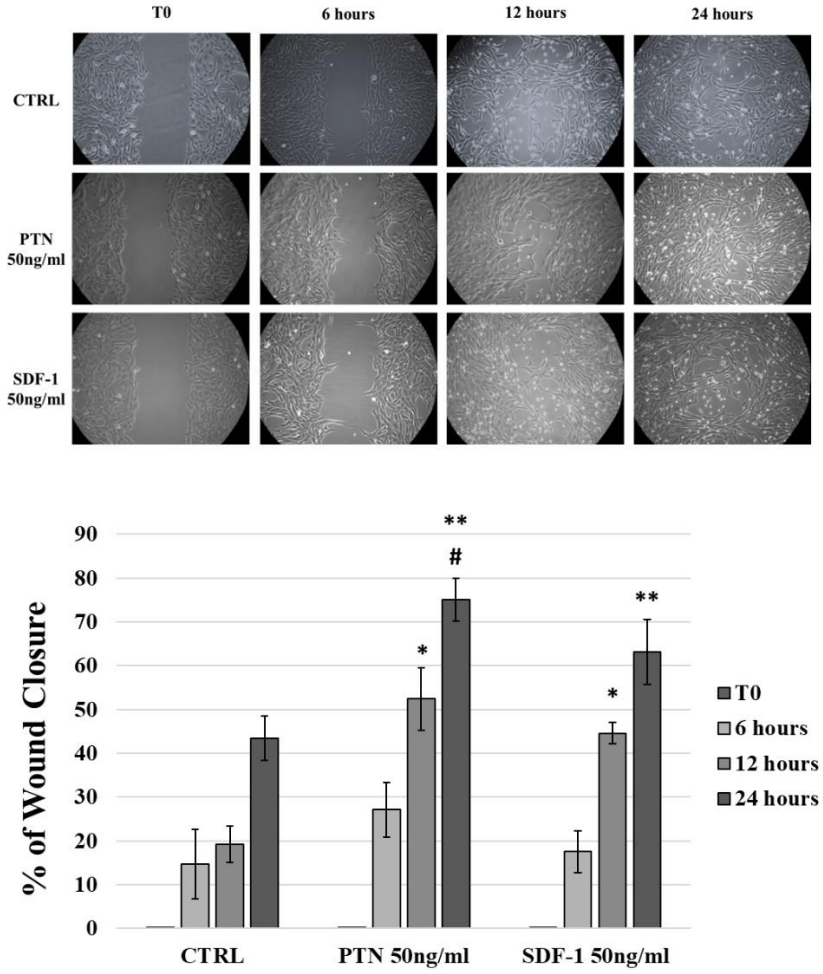

Figure 2.3*: Wound Healing Assay.* **A)** Migration of EA.hy926 induced by treatment with: SF-D-MEM (CTRL), SF-D-MEM enriched with SDF-1 (SDF-1 50ng/ml) or SF-D-MEM enriched with PTN (PTN 50ng/ml). The pictures were acquired right after the scratch (T0) and 6, 12 and 24 hours after the scratch. (magnification: 20X). **B)** Percentage of wound closure. Graphic represents the area as mean ± SD of the percentage reduction of original wound at T0, 6, 12 and 24 hours with the different treatments. * $p<0.001$ vs 12 hours CTRL; ** $p<0.001$ vs. 24 hours CTRL; # $p<0.01$ vs. 24 hours SDF-1 50ng/ml.

EC migration was further analysed by Transwell migration assay. After 6 hours, the average number of migrated cells per field counted for CTRL was of 54.6±6.2. In response to the treatment with PTN or SDF-1, the average number of migrated cells was significantly higher with respect to CTRL: in presence of PTN, the average number of migrated cells per field

was 73.7±11.8 ($p<0.01$ vs. CTRL) while in presence of SDF-1, an average number of 68.6±8 cells ($p<0.05$ vs. CTRL) was counted. After 24 hours, the average number of migrated cells was not significantly modified for CTRL, with an average of 60.3±9.5 cells per field. Instead, both PTN (95.3±9.2 cells/field; $p<0.001$ vs. CTRL) and SDF-1 (84.2±6.7; $p<0.001$ vs. CTRL) significantly increased the number of migrated cells (**Figure 2.4**). These results show how the treatment with PTN is able to improve the migration rate and repair ability of treated ECs compared to CTRL, has shown by other groups [82, 103, 110]. Of interest, the effects on migration exerted by PTN resulted being higher than the one obtained with the treatment with SDF-1, once again suggesting that the use of PTN for the enrichment of vascular substitute may play an important role in the re-endothelialisation process.

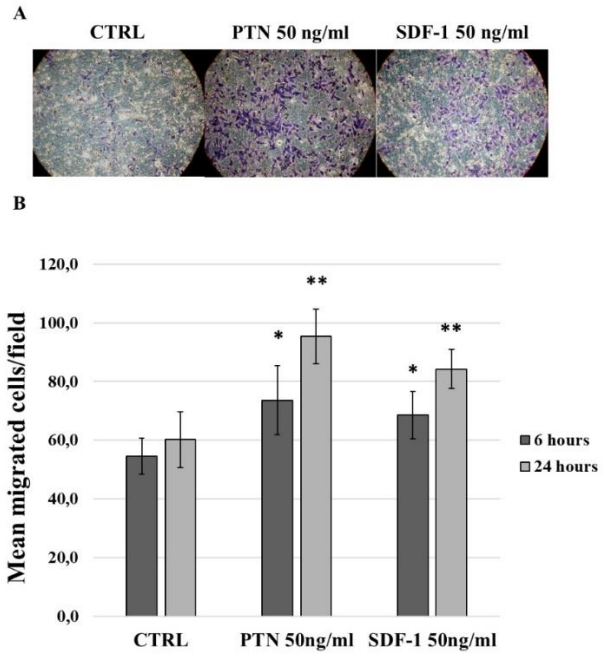

Figure 2.4*: Transwell Migration Assay.* **A)** Brightfield images showing the migrated cells, stained with Crystal Violet, after 24 hours of incubation with different treatments: SF-D-MEM (CTRL); SF-D-MEM enriched with 50ng/ml SDF-1; SF-D-MEM enriched with 50ng/ml PTN. **B)** Quantitative analysis of migrated cells expressed as mean ± SD of number of migrated cells per field. *$p<0.05$ vs. 6 hours CTRL; **$p<0.001$ vs. 24 hours CTRL.

The expression of Proliferating Cell Nuclear Antigen (PCNA), a marker of cell proliferation, and Ras-related C3 botulinum toxin substrate 1 (Rac1), a known marker for cell migration, was analysed with both the free and blocked receptor for PTN and SDF-1. The analysis on the blocked receptor group, in which cells were treated with blockers specific for PTN receptor, RPTP β/ζ, and SDF-1 receptor, CXCR4, has been performed to evaluate if the

effects of the two proteins was limited to the interaction with their own specific receptor. As shown in **Figure 2.5A**, after 24 hours, SDF-1 (1.33±0.08) significantly increases the expression of PCNA compared to CTRL (1.15±0.06; p<0.01); however, after 3 days, SDF-1 treatment significantly inhibited PCNA expression (0.51±0.05) with respect to CTRL (0.95±0.08; p<0.001) and PTN (1.16±0.04; p<0.001) (**Figure 2.5B**). With the blocked receptors, after 24 hours of incubation the treatment with PTN can significantly increase the expression of PCNA (1.72±0.13) compared to CTRL (1.07±0.09; p<0.001) and SDF-1 (1.17±0.07; p<0.001). After 3 days, PTN treatment again improved the expression of PCNA (1.51±0.09) compared to CTRL (0.84±0.08; p<0.001) and SDF-1 (0.50±0.06; p<0.001). Still, the treatment with SDF-1 significantly inhibited the expression of PCNA with respects to CTRL (p<0.01) (**Figure 2.5C**).

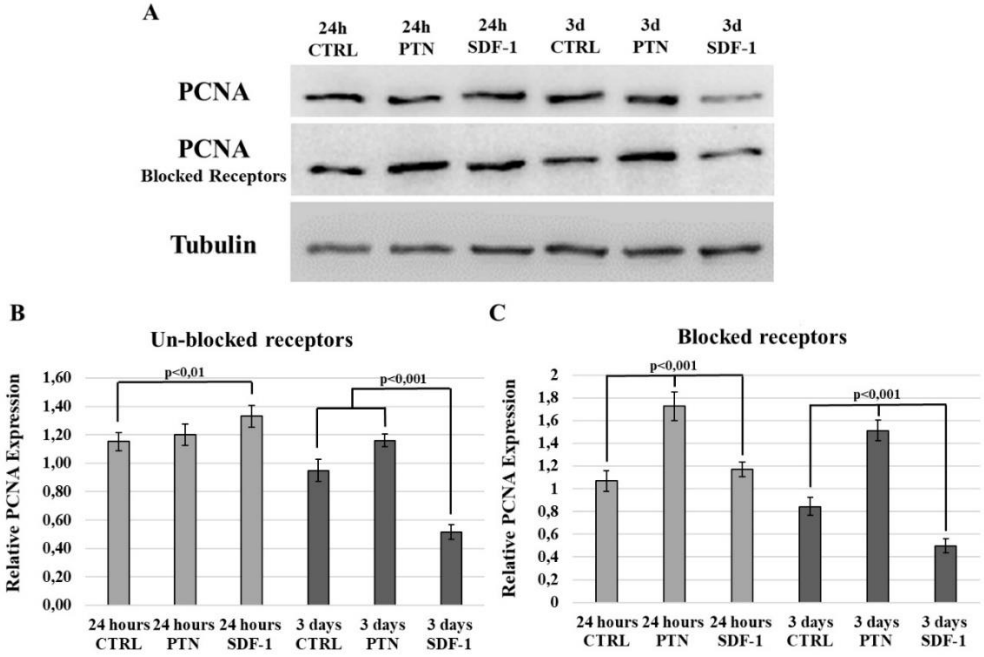

Figure 2.5*: Western Blot Analysis for PCNA on EA.hy926***. A)** Cells were treated with: C-D-MEM (CTRL), C-D-MEM containing 50ng/ml PTN (PTN) and C-D-MEM containing 50ng/ml SDF-1 (SDF-1). Lysates were collected after 24 hours (24h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **B**) Densitometric analysis showing the absolute quantification for PCNA expression with un-blocked receptors for PTN and SDF-1. **C**) Densitometric analysis showing the absolute quantification for PCNA expression with the blocked receptors for PTN and SDF-1.

The Western Blot analysis for the expression of Rac-1 (**Figure 2.6A**) showed that, after 24 hours of incubation with the free receptors, PTN (0.81±0.13) and SDF-1 (0.82±0.12) were able to significantly increase the expression of Rac1 compared to the CTRL (0.54±9;

p<0.05). Moreover, after 3 days of incubation with free receptors, Rac-1 was significantly higher in PTN (1.63±0.16) group compared to SDF-1 (0.77±0.09; p<0.05) (**Figure 2.6B**). With the blocked receptors, after 24 hours of incubation no significant difference between the treatments was observed. However, after 3 days of incubation, the treatment with PTN (1.54±0.10), along with CTRL (1.22±0.08), were able to significantly increase the expression of Rac-1 compared to the SDF-1 group (0.90±0.11; p<0.001) (**Figure 2.6C**).

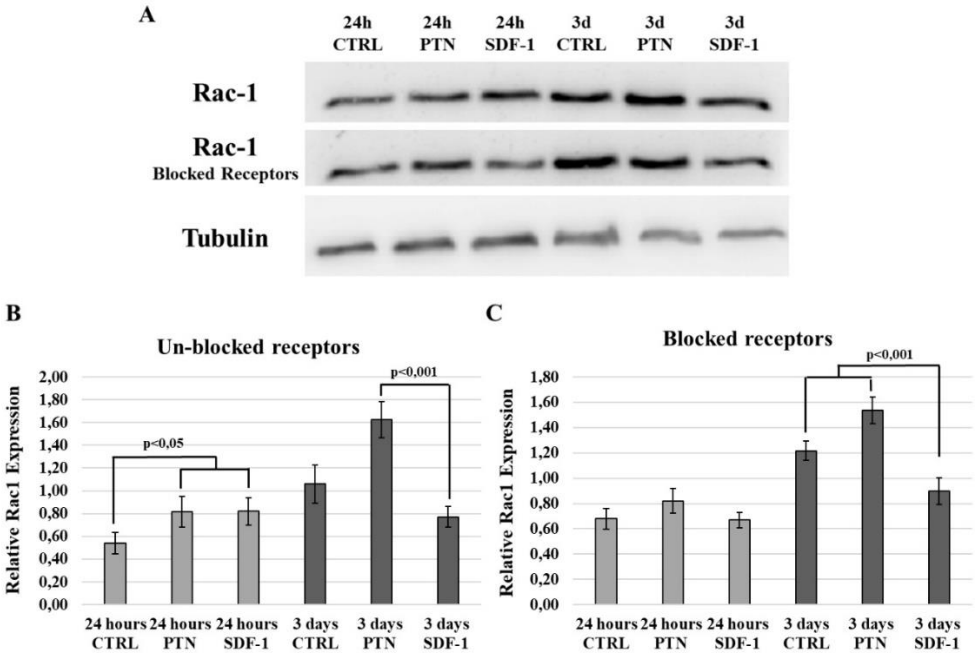

Figure 2.6*: Western Blot Analysis for Rac-1 on EA.hy926.* **A)** Cell were treated with: C-D-MEM (CTRL), C-D-MEM containing 50ng/ml PTN (PTN) and C-D-MEM containing 50ng/ml SDF-1 (SDF-1). Lysates were collected after 24 hours (24h) and 3 days (3d). Data were normalized over tubulin, used as loading control. **B**) Densitometric analysis showing the absolute quantification for Rac-1 expression with un-blocked receptors for PTN and SDF-1. **C**) Densitometric analysis showing the absolute quantification for Rac-1 expression with the blocked receptors for PTN and SDF-1.

As the data show, with the free receptors, the treatment with PTN was able to significantly increase the expression levels of both PCNA and Rac1 compared to SDF-1 and the CTRL conditions. The PCNA expression analysis with the unblocked receptor, as shown, does not shows significative difference in between the CTRL and PTN condition, suggesting that the significant increase observed with the MTT assay is due to an amelioration of cells viability rather than an increase in cells proliferation. However, the Western Blot analysis has been conducted over a 3-days period, while the MTT analysis has been conducted on a longer 7 days period. Thus, we can't conclude if PTN, on longer times, is able to induce a proliferation

of the treated ECs. However, of interest, is to note how SDF-1, compared to PTN, seems to downregulate PCNA expression, suggesting that the use of PTN could be preferred to SDF-1 for vascular grafts enrichment. Concerning Rac1 expression with the un-blocked receptor, the results obtained confirm the one obtained by the Wound Healing and Transwell migration assays. Interestingly, the blockage of the receptors seems to not affect the effects of PTN on the expression of PCNA and Rac-1, resulting in a significant increase of their expression compared to the effects exerted by SDF-1 with its receptor blocked. In this study, the interaction of PTN with RPTP β/ζ has been studied: despite the fact that it is not the only receptor that is able to bind to PTN [277, 278], RPTP β/ζ is known to be the main receptor for PTN [84, 86, 276]. Moreover, has been suggested that for PTN to exert his effects through certain receptors, such as Anaplastic Lymphoma Kinase (ALK), a previous interaction between PTN and RPTP β/ζ is necessary [279]. However, as shown by our results, even when RPTP β/ζ is blocked, PTN is still able to affects the expression of the two marker proteins studied. Thus, the effect obtained with the blocked RPTP β/ζ could be explained by the interaction of PTN with other receptors, resulting in an increased expression of the two markers and conferring an added value to the use of PTN for vascular grafts enrichment.

2.6 Conclusions

In conclusion, due to the effects exerted on viability, migration and repair ability of ECs, PTN could be an interesting molecule for vascular grafts enrichment. The comparison of the effects of PTN with the one exerted by SDF-1, along with the effects of PTN on the expression of PCNA and Rac-1 suggesting that PTN can exerts his beneficial effects on endothelial cells even when its primary receptor RPTP β/ζ is blocked, can open the path to the use of PTN for vascular grafts enrichments applications.

Chapter 3: Collagen gels for controlled release of Pleiotrophin: Potential for vascular applications

Francesco Copes[1,2], Pascale Chevallier[2], Caroline Loy[2], Daniele Pezzoli[2], Francesca Boccafoschi[1,2*], Diego Mantovani[2*]

[1] Laboratory of Human Anatomy, Department of Health Sciences, University of Piemonte Orientale, Novara, Italy

[2] Laboratory for Biomaterials and Bioengineering, Canada Research Chair Tier I for the Innovation in Surgery, Department of Min-Met-Materials Engineering & CHU de Quebec Research Center, Laval University, Quebec City, Quebec, Canada.

This chapter has been submitted for publication to Frontiers in Bioengineering and Biotechnology in the Biomaterials specialty.

3.1 Résumé

Une ré-endothélialisation rapide, associée à l'inhibition de l'hyperplasie néo-intimale, est cruciale pour réduire l'échec des substituts utilisés lors de pontage vasculaire. Ainsi, au cours des dernières années, l'ajout de différentes molécules capables d'accélérer le processus de ré-endothélialisation ont été proposées dans la littérature. Cependant, les essais cliniques basés sur l'administration de facteurs angiogéniques ont été pour la plupart décevants, soulignant ainsi la nécessité d'étudier un plus large éventail de facteurs angiogéniques. Ce travail propose un nouveau système de libération de médicament basé sur un hydrogel de collagène de type I, chargé de pléiotrophine (PTN), cytokine connue pour ses effets pro- L'héparine, en raison de sa capacité à séquestrer, à protéger et à libérer les facteurs de croissance, a été utilisée pour mieux contrôler la libération de la molécule d'intérêt, la PTN. Les performances de ce système et les effets de la libération de la PTN sur les cellules endothéliales (CE) et cellules musculaires lisses (CML) ont été évalués. Une caractérisation structurelle (tests mécaniques et analyses par immunofluorescence des fibres de collagène) a été réalisée sur les gels afin de déterminer si l'héparine a une quelconque influence sur les propriétés du gel ainsi obtenu. Le relargage de la PTN à partir des différentes formulations de gel a été ensuite quantifié par dosage ELISA, spécifique à la PTN. La viabilité cellulaire a été aussi évaluée par Alamar Blue en faisant des tests directs, cellules ensemencées directement sur les gels, et par des tests indirects. Dans ce cas, les cellules sont incubées avec du surnageant, contenant la PTN libérée qui était contenue au préalable dans les gels. Les effets des différentes compositions de gels sur la migration des CE et des SMC ont été évalués à l'aide d'un essai de migration Transwell. L'hémocompatibilité du gel a été contrôlée via un test de coagulation / hémolyse. Les analyses structurelles ont montré que l'héparine ne modifiait pas le comportement structural des gels de collagène. La quantification ELISA a démontré que l'héparine induisait une libération constante de PTN au fil du temps par rapport aux autres conditions testées. Les tests de viabilité directs et indirects ont montré une augmentation de la viabilité des CE alors qu'aucun effet n'a été noté sur les CML. Les résultats de la migration cellulaire ont aussi démontré que les gels modifiés par l'héparine / PTN augmentaient significativement la migration des CE et réduisaient ceux des CML. Enfin, l'héparine a considérablement augmenté l'hémocompatibilité des gels de collagène. En conclusion, le collagène modifié par l'héparine, pour contrôler le relargage de la PTN, proposé ici semble

prometteur et présente une valeur ajoutée pour la médecine vasculaire, car pourrait être capable d'améliorer la performance biologique et l'intégration des substituts vasculaires.
Mots-clés: collagène, pléiotrophine, héparine, cellules endothéliales, cellules musculaires lisses

3.2 Abstract

A fast re-endothelialization, along with the inhibition of neointima hyperplasia, are crucial to reduce the failure of vascular bypass grafts. Implants modifications with molecules capable of speeding up the re-endothelialization process have been proposed over the last years. However, clinical trials of angiogenic factor delivery have been mostly disappointing, underscoring the need to investigate a wider array of angiogenic factors. In this work, a drug release system based on a type I collagen hydrogel has been proposed for the controlled release of Pleiotrophin (PTN), a cytokine known for its pro-angiogenetic effects. Heparin, in virtue of its ability to sequester, protect and release growth factors, has been used to better control the release of PTN. Performances of the PTN drug delivery system on endothelial (ECs) and smooth muscle cells (SMCs) have been investigated. Structural characterization (mechanical tests and immunofluorescent analyses of the collagen fibers) was performed on the gels to assess if heparin caused changes in their mechanical behavior. The release of PTN from the different gel formulations has been analyzed using a PTN-specific ELISA assay. Cell viability was evaluated with the Alamar Blue Cell Viability Assay on cells directly seeded on the gels (direct test) and on cells incubated with supernatant, containing the released PTN, obtained from the gels (indirect test). The effects of the different gels on the migration of both ECs and SMCs have been evaluated using a Transwell migration assay. Hemocompatibility of the gel has been assessed with a clotting/hemolysis test. Structural analyses showed that heparin did not change the structural behavior of the collagen gels. ELISA quantification demonstrated that heparin induced a constant release of PTN over time compared to other conditions. Both direct and indirect viability assays showed an increase in ECs viability while no effects were noted on SMCs. Cell migration results evidenced that the heparin/PTN-modified gels significantly increased ECs migration and decreased the SMCs one. Finally, heparin significantly increased the hemocompatibility of the collagen gels. In conclusion, the PTN-heparin-modified collagen here proposed can represent an added value for vascular medicine, able to ameliorate the biological performance and integration of vascular grafts.

Keywords: collagen, pleiotrophin, heparin, endothelial cells, smooth muscle cells

3.3 Introduction

Every year is estimated that around 17.9 million of people die of cardiovascular diseases, mainly heart attacks and strokes, making them the first cause of death in the world [1]. Most of the time the root of these diseases can be found in atherosclerosis, a progressive pathology in which a plaque made of lipids, cholesterol, foamy cells, cellular debris and calcium builds up in the walls of the arteries [10]. Overtime, this plaque hardens and narrows the lumen of the affected artery, reducing the blood flow and ultimately leading to the aforementioned conditions. Despite the advances in pharmacological treatment and minimally-invasive surgical treatment, vascular bypass surgery remains the treatment of choice for atherosclerosis [18]. The gold standard is the use of autologous vessels, such as saphenous vein, internal mammary arteries and radial artery [280]. However, these options are not always available because of patients' condition. For these reasons, over the last 50 years surgeons reverted to the use of synthetic graft, such as polyethylene terephthalate (Dacron) or expanded polytetrafluoroethylene (ePTFE) based substitutes, instead of autologous vessels [23]. Despite being widely used in the clinical practice, the use of this substitute is still hampered by a high rate of graft failure, especially for small diameter vessels (Ø<6 mm) [20]. The main reasons of the grafts failure are intra-graft thrombosis, occurring in the first month after the implantation, and intimal hyperplasia, arising in the chronic phase especially at the anastomotic site [281]. Incomplete healing process of the graft's, especially the lack of endothelialization, are the main causes of these two outcomes. The formation of a functional endothelial cells (ECs) layer is of crucial importance to avoid complications and to obtain an optimal integration of the implanted graft. Modification of the luminal surface with pro-endothelialization factors has been proposed over the years to help and increase ECs adhesion and proliferation, both alone [256, 257, 282] and in tandem with extracellular matrix (ECM) proteins [168, 247]. However, clinical trials of pro-endothelialization enrichment of vascular grafts have been mostly unsatisfactory, thus the need to investigate new approaches [71] for this application. In this work, a release system based on a type I collagen hydrogel [50] has been proposed for the controlled release of pleiotrophin (PTN) [277], a 168 amino acids secreted cytokine known for its involvement in different cellular processes like cell growth and cell motility and for the beneficial effects exerted on the cardiovascular system [86, 104]. Moreover, PTN has been recently shown to be able to exert potent pro-angiogenic factor on

ECs compared to already used pro-endothelialization factors [283]. Type I collagen is widely chosen as a biomaterial for medical applications due to its ease of extraction, weak antigenicity, robust biocompatibility, and its ability to be physically and chemically modified for a variety of applications [116-118]. Due to its favorable properties, collagen-based matrices have been thoroughly investigated as a releasing system for therapeutic drug delivery applications [50, 126, 129, 132]. Drug delivery systems have been widely studied in the last years. These systems utilize specific non-covalent interactions to stabilize drugs and immobilize them within a biocompatible matrix, thus protecting their biological activity and slowing their diffusion from the matrix. One of such drug delivery systems are heparin-based delivery systems [144]. Heparin is a polysaccharide made up of repeating disaccharides [145] best known for its anticoagulant properties, but has also been shown to promote cell adhesion, inhibit smooth muscle cell proliferation and to moderate inflammation [146]. Moreover, heparin is also known to sequester, stabilize and protect growth factors and cytokines and has been widely used in conjunction with different scaffolds to enhance their retention ability [153]. In virtue of this favorable properties and in light of its high binding affinity for PTN [72], heparin has been used to better control the release of PTN from the collagen gel. The biological performances of the PTN-based drug delivery system have been investigated on both ECs and smooth muscle cells (SMCs).

3.4 Materials and Methods

3.4.1 Cell Isolation and Culture

Human umbilical vein endothelial cells (HUVECs) and human umbilical artery smooth muscle cells (HUASMCs) were used in this study. Cells were isolated from human umbilical cord samples obtained from normal term pregnancies. Written informed consent was obtained from all mother donors according to the Declaration of Helsinki. All experiments were performed in compliance with the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans and institutional CHU de Quebec - Laval University guidelines. The protocol was approved by the Ethics Committee of the CHU de Quebec Research Centre (CER #S11-03-168). Briefly, umbilical cord samples, approximately 15 cm in length, were collected in phosphate-buffered saline solution (PBS, Fisher Scientific, Fair Lawn, NJ, USA) supplemented with 5% penicillin/streptomycin (P/S,

Gibco, Invitrogen Corporation, Burlington, ON, Canada), solution to avoid any contamination and maintained at 4 °C until processing.

As previously described [284], for HUVECs isolation, veins were rinsed with PBS, filled with 10× trypsin–EDTA solution (Gibco, Invitrogen Corporation, Burlington, ON, Canada), and incubated for 15 min at 37 °C, after which the trypsin–EDTA solution containing the HUVECs was collected. PBS was added to wash the lumen, collected along with the previous solution, and centrifuged at 1000 rpm for 5 min. Thereafter, the supernatant was removed and the cells resuspended in M199 culture medium (Gibco, Invitrogen Corporation, Burlington, ON, Canada) with 5% fetal bovine serum (FBS, Gibco, Invitrogen Corporation, Burlington, ON, Canada), 1% P/S (Gibco, Invitrogen Corporation, Burlington, ON, Canada), 2 ng/ml fibroblast growth factor (FGF, Life Sciences, Grand Island, NY, USA), 1 ng/ml endothelial growth factor (EGF, Life Sciences, Grand Island, NY, USA), 1 µg/ml ascorbic acid (Sigma Aldrich, Oakville, ON, Canada), 1 µg/ml hydrocortisone (Sigma Aldrich, Oakville, ON, Canada) and seeded in a 75 cm^2 flask (Corning, Oneonta, NY, USA). This medium, that will be referred to as complete HUVECs M199 culture medium (HUVEC-M199), has been used in the experiments along with a basic version containing P/S (P/S-M199) only. Culture medium was changed after 24 h and then every 48 h until confluence was reached. ECs were characterized using a rabbit primary antibody against von Willebrand factor (VWF, Abcam, Ab6994, dilution 1/100, Toronto, ON, Canada, data not shown). The cells were then maintained in culture at 37 ° C in a saturated atmosphere at 5% CO_2. When 85% - 90% of confluence was reached, cells were then enzymatically detached from the plate (0.05% trypsin, Gibco, Invitrogen Corporation, Burlington, ON, Canada) and then reseeded at a ratio of 1:3 or used for experiments. For the experiment here reported, cells have been used at passage 5 and 6. For the HUASMCs, once all the associated connective tissues were carefully removed, arteries were cut open longitudinally. The intima layer, composed of endothelial cells, was carefully scraped off and the arteries were then cut in smaller pieces using a scalpel. The pieces were then placed in Petri dishes in presence of M199 culture medium (Gibco, Invitrogen Corporation, Burlington, ON, Canada) additioned with 5% fetal bovine serum (FBS, Gibco, Invitrogen Corporation, Burlington, ON, Canada), 1% penicillin/streptomycin (P/S, Gibco, Invitrogen Corporation, Burlington, ON, Canada), 2 ng/ml fibroblast growth factor (FGF, Life Sciences, Grand Island, NY, USA), 1 ng/ml

endothelial growth factor (EGF, Life Sciences, Grand Island, NY, USA), 1 µg/ml ascorbic acid (Sigma Aldrich, Oakville, ON, Canada), 1 µg/ml hydrocortisone (Sigma Aldrich, Oakville, ON, Canada) and 5 µg/mL of human insulin solution (Santa Cruz Biotechnology, Dallas, TX, USA). This medium, that will be referred to as complete HUASMCs M199 culture medium (HUASMC-M199), has been used in our experiments along with P/S-M199). After two weeks, SMCs from the explants had migrated and colonized the surface of the Petri dishes. Once the artery pieces have been removed, cells were expanded in HUASMC-M199. Culture medium was changed every 48 h until confluence. SMCs were identified by immunostaining for smooth muscle-α-actin (SM-α-actin) and calponin (Ab7817, dilution 1/200 and Ab46794, dilution 1/200, Abcam, Toronto, ON, Canada) (data not shown,). Again, cells were maintained in culture at 37 ° C in a saturated atmosphere at 5% CO_2. When 85% - 90% of confluence was reached, cells were enzymatically detached from the plate (0.05% trypsin, Gibco, Invitrogen Corporation, Burlington, ON, Canada) and then reseeded at a ratio of 1:3 or used for experiments. For the reported experiments, cells have been used at passage 7.

3.4.2 Collagen Gels preparation

Type I collagen was extracted from rat tails tendons and subsequently solubilized in 0.02 N acetic acid, as previously reported [34, 174], to obtain a final collagen concentration of 4 g/L. For the preparation of the collagen gel, the collagen solution has been mixed with a buffer solution containing Dulbecco's modified Eagle medium (DMEM, Gibco, Invitrogen Corporation, Burlington, ON, Canada, 1.1X), NaOH (15 mM), and HEPES (20 mM) in deionized water to adjust the pH of the final solution and to initiate the polymerization process. P/S-M199 was then added to complete the basic composition of the control collagen gel (CTRL Gel). For the heparin-modified collagen gels, heparin sodium salt (Sigma Aldrich, Oakville, ON, Canada) has been added to the M199 portion of the collagen gel mix to obtain a final concentration of 10 µg/ml (H10 Gel). Heparin concentration have been chosen based on gelification analysis results of 3 different concentrations obtained in the preliminary steps of the study (data not shown). For the PTN-modified collagen gels, recombinant human PTN (Sigma Aldrich, Oakville, ON, Canada) has been added to the M199 portion of the collagen gel mix to obtain a final concentration of 150 ng/ml (P150 Gel). For the heparin-PTN-modified collagen gels, both heparin and PTN have been added to the M199 portion of the

collagen gel mix to obtain the aforementioned concentration (H/P Gel). The concentrations used for the heparin and PTN have been chosen following a dose-response curve obtained in the preliminary steps of the study (data not shown). All the blends for the different experimental condition have been carefully mixed and 500 µl of the different solutions were poured into 24 wells culture plates and let gelify at room temperature (RT) for 1 hour. Once jellified, collagen gels have been used for the subsequent experiments.

3.4.3 Unconfined stress/relaxation compression mechanical tests

Stress/relaxation unconfined compression tests were performed on CTRL gel and H10 gels to evaluate possible changes in the mechanical properties due to the addition of heparin to the gel mix. Briefly, CTRL and H10 gels were prepared and after 24 hours they have been placed in the chamber of a MACH-1 Mechanical Testing System (Biomomentum Inc., Laval, QC, Canada). Tests were performed in a bath containing PBS 1X at room temperature. The relaxation test consisted of compressing the sample according to parameter presented in **Table 3.1**. The relaxation time was defined in order to consider the viscoelastic behavior of the collagen gels and to reach a steady value for the load (equilibrium stress). The stress was recorded as a function of time.

Table 3.1: Parameter used for the stress/relaxation test performed on the Collagen gels.

Parameter	Value
Ramp Amplitude (mm)	5% of initial sample thickness
Ramp Velocity (mm/s)	5% of initial sample thickness
Number of Ramp	5
Fixed Relaxation Time (s)	1500 s

Following the stress/relaxation, the data obtained have been analyzed using MATLAB software (MathWorks, Natick, MA, USA) considering equilibrium strains and using the linear portion of the stress-strain curve at 15% of strain to obtain the equilibrium elastic modulus of the different gel formulations.

3.4.4 Immunofluorescence

For immunofluorescence, CTRL and H10 gels have been prepared as already mentioned. After 24 hours, gels have been incubated in PBS 1X with 3% of bovine serum albumin (BSA, Sigma Aldrich, Oakville, ON, Canada) for 10 minutes. Then gels have been incubated with

mouse primary antibody for collagen type 1 (1: 1000; Novus Biological, Oakville, ON, Canada) for 2 hours at 37°C. Following, gels were incubated with an Alexa Fluor® 488 goat anti-mouse secondary antibody (Life Technologies, Sigma Aldrich, Oakville, ON, Canada) for 2 hours at room temperature under agitation. Afterwards, gels have been rinsed three time with PBS 1X with 0.01% Tween 20 and have been kept overnight at 4°C before being placed on fluorescent microscope slides. Images at a magnification of 20X have been collected using an Olympus BX51 Fluorescence Microscope (Olympus Canada Inc., Toronto, ON, Canada).

3.4.5 Conditioned Medium Collection

After gelification, 600 µl of P/S-M199 has been added to each experimental condition. After 1, 3 and 7 days of incubation, medium has been completely removed and collected for subsequent experiment. For the ELISA quantification, additional time points at 10 and 14 days were added. At each time point, 600 µl of fresh P/S-M199 medium has been added to the gels.

3.4.6 ELISA quantification

For the quantification of the amount of PTN released by the different collagen gels preparation, an enzyme-linked immunosorbent assay (ELISA) was applied. Examination of PTN was done by RayBio® Human Pleiotrophin ELISA kit (RayBiotech, Norcross, Georgia, USA). The assay was performed according to the protocol provided by the manufacturer. Absorbance at a wavelength of 450nm was recorded using a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, California, USA).

3.4.7 Indirect Viability assay

The effect of the released PTN on cells viability have been analyzed using an indirect viability assay performed on both HUVECs and HUASMCs. Briefly, cells have been seeded at a concentration of 20000 cells/cm^2 in 96 well culture plates. After 24 hours of incubation with HUVEC-M199 or HUASMC-M199, depending on the cell type used, at 37 °C in a saturated atmosphere at 5% CO_2 to allow the adhesion of the cells, media has been removed and cells have been incubated for 24 hours in presence of the different conditioned media collected as previously described. Cells cultivated in P/S-M199 medium have been used as a positive control (CTRL Cell). After the treatment, the conditioned media have been removed and cells have been incubated with a resazurin solution for 4 hours. After the incubation, the

highly fluorescent resorufin product obtained by the reduction of the resazurin was collected and fluorescence intensity at a 545 nm_{ex}/590 nm_{em} wavelength was measured with a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, California, USA). Fluorescence intensity is proportional to cell viability. Data has been normalized towards the CTRL Cell condition.

3.4.8 Direct Viability Assay

The effect of the addition of heparin and PTN to the CTRL gel formulation on cells viability has been analyzed using a direct viability assay performed using both HUVECs and HUASMCs. Briefly, the following collagen gels formulations have been prepared: 1) CTRL gel; 2) H10 gel; 3) P150 gel and 4) H/P gel. After gelification, cells have been seeded in HUVEC-M199 or HUASMC-M199, according to the cell type, at a concentration of 20000 cells/cm^2 onto the different gels and incubated at 37 °C in a saturated atmosphere at 5% CO_2. Cells cultivated on culture polystyrene in HUVEC-M199 or HUASMC-M199 medium have been used as a positive control (CTRL Cell). After 1, 3 and 7 days 2, media has been removed and cells have been incubated for 6 with a resazurin solution. After the incubation, the resorufin product obtained was collected and fluorescence intensity at a 545 nm_{ex}/590 nm_{em} wavelength was measured with a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, California, USA). Fluorescence intensity is proportional to cell viability.

3.4.9 Migration Assay

To test the effects of the released PTN on the migration of HUVECs, the transwell migration assay was used. 7500 cells were seeded in the upper compartment of 24 well-format transwell with 8µm pores (Corning, Amsterdam, the Netherlands) in 250 µL of HUVEC-M199 or HUASMC-M199, according to the cell type. In the lower compartment, the following collagen gels formulations have been prepared: 1) CTRL gel; 2) H10 gel; 3) P150 gel and 4) H/P gel and 600 µl P/S-M199 have been added. Cells were incubated at 37°C in a saturated atmosphere at 5% CO_2 for 24 hours. After the incubation, cells on both faces of the insert membranes were fixed by incubation with formaldehyde 3.7% for 20 minutes at room temperature. Then, cells were stained with 1% Crystal Violet for 20 minutes at room temperature. Once stained, cells on the upper side of the porous membranes were gently removed using a cotton swab. The transwell inserts were then placed under a phase-contrast

microscope and images of different fields (n=5) were collected at 20X magnification. To assess the migration rate for each condition, stained cells were counted.

3.4.10 Hemocompatibility Assay

To study the hemocompatibility of the different collagen gel formulations, the hemoglobin free methodology was used [285]. Briefly, the following collagen gels formulations have been prepared: 1) CTRL gel; 2) H10 gel; 3) P150 gel and 4) H/P gel. After 24 hours, 100 ml of citrated blood were deposited onto the surfaces of the different collagen gels and 20 µl of 0.1 M $CaCl_2$ (Sigma Aldrich, Oakville, Canada) were immediately added to inhibit the anti-coagulant effect of the citrate. Samples were incubated at 37°C in a saturated atmosphere at 5% CO_2 and after 10, 25 and 50 minutes, 2 ml of distilled water were added to each sample. The erythrocyte not entrapped in a blood clot were hemolyzed. One minute later, the obtained solution was removed and placed into a 96 well plate. The free hemoglobin molecules released in water following hemolysis were measured by reading the absorbance at a 540 nm wavelength by means of a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, California, USA). The higher is the absorbance recorded, the higher is the amount of free hemoglobin, therefor the higher is the hemocompatibility. The test was performed, with blood from different donors used for each experiment. The maximum amount of hemoglobin (Max Hemoglobin) was obtained by immediately hemolyzed after the citrate inhibition. Data where normalized towards the Max Hemoglobin value.

3.4.11 Statistical Analysis

For each experiment, a n=5 replicates for each condition has been used. Each of the experiments were performed 3 independent times. For the hemocompatibility test, blood from 3 different donors was used for each experiment. The data shown are means ± standard deviation (SD). Statistical significance of the presented results was calculated using ANOVA non-parametric Kruskal-Wallis method through the software InStat ™ (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ or less were considered significant.

3.5 Results

3.5.1 Mechanical and Structural characterization

The stress/relaxation unconfined compression tests were performed on CTRL gels and on the H10 gels to evaluate if the addition of heparin to the gel mix caused any changes in the mechanical properties of the gels. The equilibrium elastic modulus of the two gels composition has been analyzed: no significant differences in the equilibrium elastic modulus of the two gel compositions were detected. The immunofluorescence performed on the gels confirmed the results obtain by the mechanical characterization. In fact, no visible differences were noted in the arrangement of the collagen fibers in both the CTRL gels and the Hep 10 µg/ml gels (**Figure 3.1**).

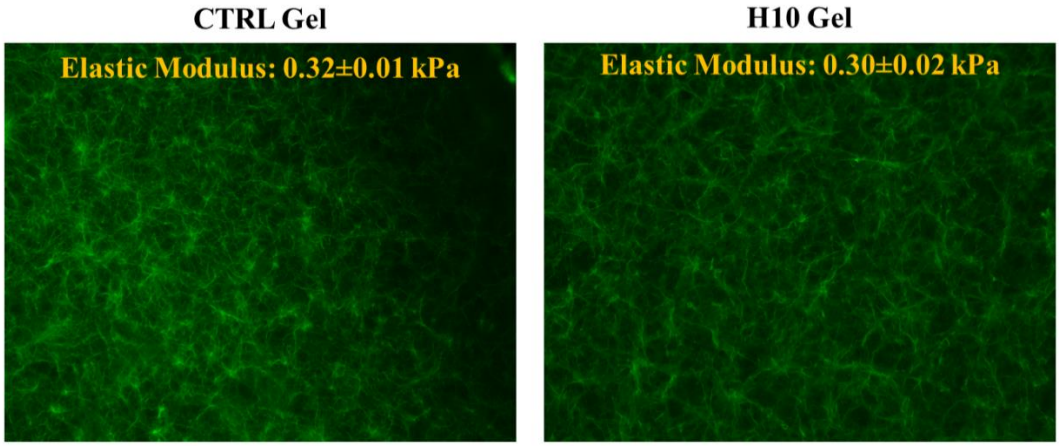

Figure 3.1*: Mechanical and Structural Characterization.* The images show the immunofluorescent staining of the type 1 collagen fibers (green color) in the two gel formulations tested: CTRL and Hep 10µg/ml gels. Images were taken after 24 hours at a 20X magnification. In yellow are reported the values of the Equilibrium Elastic Modulus for the two gels formulation.

3.5.2 Released PTN quantification

The amount of PTN released over a 10-days period, along with the kinetic of its release, has been analyzed by means of a PTN-specific ELISA quantification assay (**Figure 3.2**).

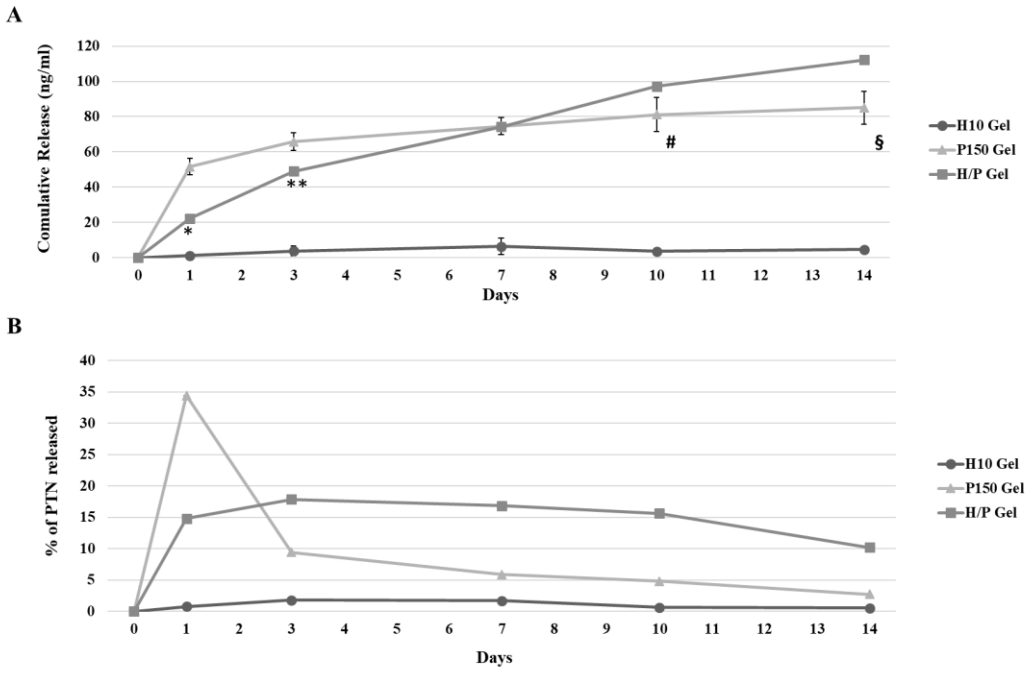

Figure 3.2*: PTN ELISA Quantification.* The graphic shows the results for the quantification of PTN release by the Hep 10 µg/ml (H10), PTN 150 ng/ml (P150) and Hep 10 µg/ml PTN 150 ng/ml (H/P) collagen gels after 1, 3, 7 and 10 days of incubation. **A)** The graphic shows the mean cumulative release ± SD measured at each time point. *p<0.001 vs. 1 Day P150; **p<0.01 vs. 3 Days P150; # p<0.01 vs. 10 Days P150. **B**) The graphic shows the % of released PTN ± SD measured at each time point.

The results of the ELISA quantification have been used to analyses the cumulative released of PTN over 14 days, as shown in **Figure 3.2A**. For P150, after 1 day, the concentration of PTN found in the collected conditioned medium was 51.6 ± 4.7 ng/ml. After 3 days, the total PTN concentration detected was 65.8 ± 4.9 ng/ml and after 7 days the concentration was 74.6 ± 4.8 ng/ml. At 10 days, the cumulative released PTN was 81.2 ± 9.5 ng/ml wile at 14 days it reached 85.0 ± 9.2 ng/ml. A similar cumulative release was observed for the H/P gel, but the concentrations of PTN measured at the 1 and 3-days time points were significantly lower compared to the P150 condition (Day 1: 22.2 ± 1.5 ng/ml, p<0.001 vs. P150 gel; Day 3: 49.0 ± 2.1 ng/ml, p<0,01vs. P150 gel). At 7 days, the cumulative release for the H/P gel, 74.3 ± 2.3 ng/ml, was almost the same as for the P150. However, at Day 10 the cumulative released PTN was higher compared to the P150 gel (97.3 ± 1.5 ng/ml, p<0.01 vs. P150 gel). This trend continued until Day 14, with the cumulative release of H/P (112.3 ± 0.1 ng/ml) being higher than the P150 condition (p<0.01 vs. P150 gel). **Figure 3.2B** shows the release kinetic of the different gel formulation expressed as % of released PTN at each time point studied. It is possible to observe how the PTN 150 ng/ml gel released a high amount of PTN at the first

day while, during the following time points, the amount of released PTN decreased drastically. On the contrary, the amount of PTN released by the H/P gel was constant over time.

3.5.3 Indirect viability test

Indirect viability tests have been performed on ECs and SMCs to evaluate if the released PTN present in the collected conditioned medium was able to exert any effect on the viability of the treated cells. Regarding the ECs (**Figure 3.3A**), viability test has shown that after 1 day of incubation, the conditioned medium collected after 1 day from the H/P gel condition $(2.23 \pm 0.17)E^8$ was able to significantly increase the viability compared to the CTRL Cell $(1.71 \pm 0.06)E^8$ ($p<0.01$), the CTRL Gel $(1.77 \pm 0.33)E^8$ ($p<0.01$) and the H10 gel $(1.76 \pm 0.30)E^8$ ($p<0.01$) conditions. For the 3 days conditioned media, the P150 gel condition $(2.09 \pm 0.22)E^8$ was able to significantly increase the ECs viability against th CTRL Gel $(1.62 \pm 0.24)E^8$ ($p<0.01$). Again, the H/P gel $(2.29 \pm 0.17)E^8$ was able to significantly increase the viability of the HUVECs compared to CTRL Cell ($p<0.001$) and CTRL gel ($p<0.001$). With the 7 days conditioned media, both P150 gel $(2.17 \pm 0.18)E^8$ and H/P gel (2.46 ± 0.15) conditions were able to significantly increase cells viability compared to the CTRL Cell ($p<0.05$ vs. P150 gel, $p<0.001$ vs. H/P gel) and CTRL Gel conditions $(1.76 \pm 0.11)E^8$ ($p<0.05$ vs. P150 gel and $p<0.001$ vs. H/P gel).

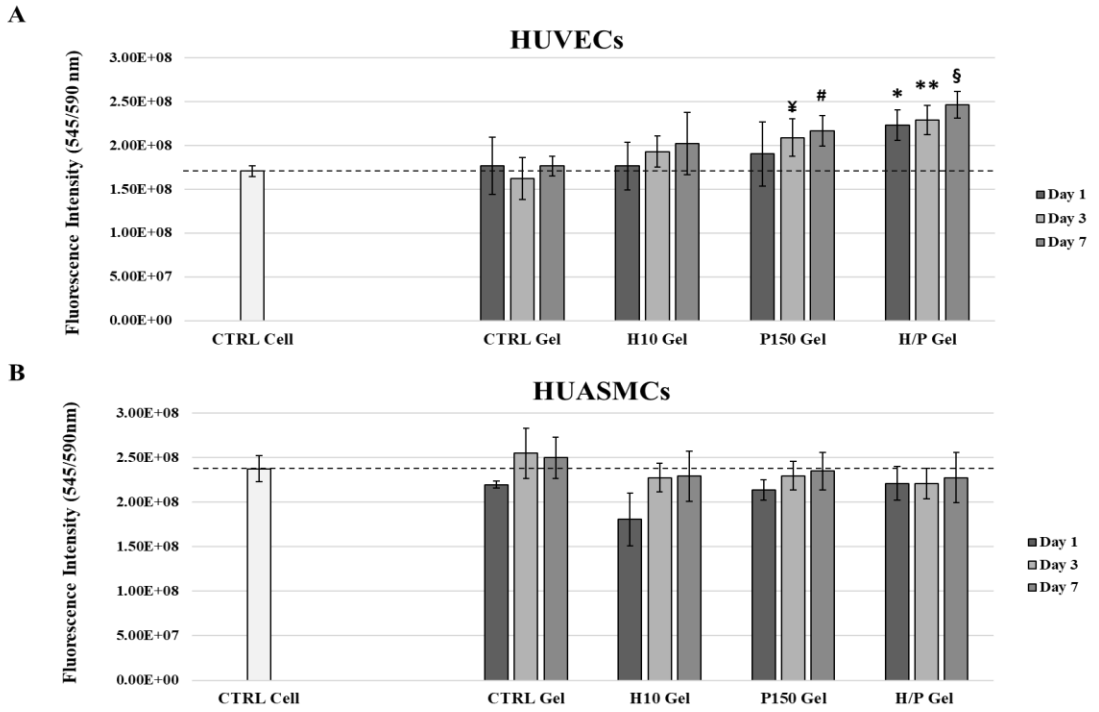

Figure 3.3*: Indirect Viability Assay.* HUVECs and HUASMCs were treated with conditioned medium collected after 1, 3 and 7 days of incubation with the following collagen gel conditions: control collagen gel (CTRL Gel); collagen gel with 10 µg/ml of heparin (H10 gel); collagen gel with 150 ng/ml of PTN (P150 gel); collagen gel containing 10µg/ml of heparin and 150 ng/ml of PTN (H/P gel). Cell viability was measured after 24 hours by means of a resazurin salt solution assay. **A)** The graphic shows the relative viability ± SD recorded from HUVECs treated with the different experimental conditions. * $p<0.01$ vs. Day 1 CTRL Cell, CTRL Gel and H10 gel; ** $p<0.001$ vs. Day 3 CTRL Cell and CTRL Gel; ¥ $p<0.01$ vs. Day 3 CTRL Gel; # $p<0.05$ vs. Day 7 CTRL Cell and CTRL Gel; § $p<0.001$ vs. Day 7 CTRL Cell and CTRL Gel. **B**) The graphic shows the mean fluorescence ± SD recorded from HUASMCs treated with the different conditions.

The indirect viability assay performed on SMCs has shown that, regardless of the time point at which the conditioned media were collected from the different collagen gel conditions, no significant change was observed in between the CTRL Cell and CTRL Gel conditions and the modified collagen gels (**Figure 3.3B**).

3.5.4 Direct Viability Assay

Direct viability tests have been performed on ECs and SMCs directly seeded on the different collagen gel conditions to evaluate the direct effects of the PTN present in the gels. After 1 day of incubation, HUVECs seeded on the P150 gels $(1.90 \pm 0.05)E^8$ showed a significant increased viability compared to the CTRL Cell $(1.15 \pm 0.11)E^8$ ($p<0.,01$) and to cells seeded on the H10 gels $(1.08 \pm 0.05)E^8$ ($p<0.01$). After 3 days of incubation, both P150 gels $(2.61 \pm 0.17)E^8$ an H/P gels $(2.22 \pm 0.11)E^8$ were able to significantly increase the viability of the

seeded HUVECs compared to the CTRL Cell $(1.89 \pm 0.20)E^8$ ($p<0.001$ vs. P150 gel and $p<0.01$ vs. H/P gel), CTRL gel $(1.96 \pm 0.11)E^8$ ($p<0.001$ vs. P150 gel and $p<0.01$ vs. H/P gel) and the H10 gel $(1.15 \pm 0.05)E^8$ ($p<0,001$ vs. P150 gel and $p<0.01$vs. H/P gel). Finally, both P150 gels $(2.86 \pm 0.33)E^8$ an H/P gels $(2.86 \pm 0.11)E^8$ were able to significantly increase the viability of the seeded huvec compared to the CTRL Cell $(2.30 \pm 0.22)E^8$ ($p<0.001$ vs. P150 gels and H/P gel), CTRL gels $(2.19 \pm 0.15)E^8$ ($p<0.001$ vs. P150 gels and H/P gel) and H10 gels $(1.57 \pm 0.14)E^8$ ($p<0.001$ vs. P150 gels and H/P gels) (**Figure 3.4A**).

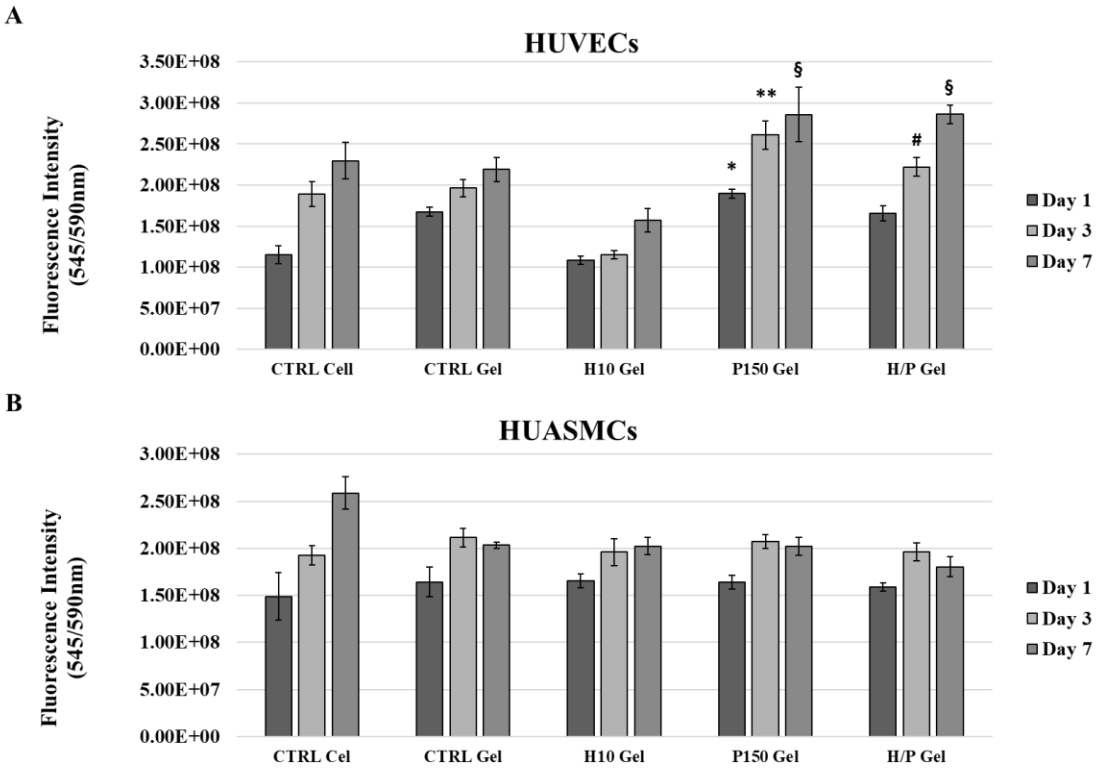

Figure 3.4*:* *Direct Viability Assay.* HUVECs and HUASMCs were directly seeded on the following collagen gel conditions: control collagen gel (CTRL Gel); collagen gel with 10µg/ml of heparin (H10 gel); collagen gel with 150ng/ml of PTN (P150 gel); collagen gel containing 10µg/ml of heparin and 150ng/ml of PTN (H/P gel). Cell viability was measured after 1, 2 and 7 days by means of a resazurin salt solution assay. **A)** The graphic shows the mean fluorescence ± SD recorded from HUVECs treated with the different experimental conditions. * $p<0.01$ vs. Day 1 CTRL Cell and H10 gel; ** $p<0.001$ vs. Day 3 CTRL Cell, CTRL Gel and H10 gel; # $p<0.01$ vs. Day 3 CTRL Cell, CTRL Gel and H10 gel; § $p<0.01$ vs. Day 7 CTRL Cell, CTRL Gel and H10 gel. **B)** The graphic shows the mean fluorescence ± SD recorded from HUASMCs treated with the different experimental conditions.

Regarding the direct viability tests performed on the SMCs, as for the indirect tests, no significant difference was shown between the different experimental conditions (**Figure 3.4B**).

3.5.5 Migration Assay

EC migration was analyzed using the Transwell migration Assay. After 24 hours of incubation, the H/P gel was able to induce a significant higher migration (1.90 ± 0.44) compared to the CTRL gel (1.00 ± 0.41; p<0.001) and the H10 gel (1.14 ± 0.32; p<0.001) (**Figure 3.5A**).

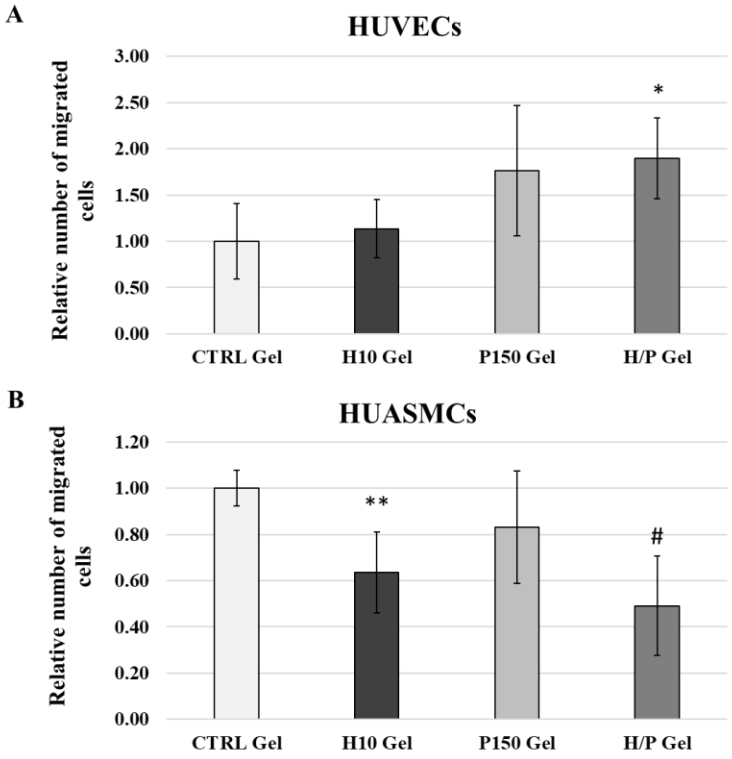

Figure 3.5*: Transwell Migration Assay***. A)** Quantitative analysis of migrated HUVECs expressed as mean ± SD of number of migrated cells per field. Results have been normalized against the CTRL Gel condition. *p<0.01 vs. CTRL gel and H10 gel. **B**) Quantitative analysis of migrated HUASMCs expressed as mean ± SD of number of migrated cells per field. Results have been normalized against the CTRL Gel condition. **p<0.01 vs. CTRL gel; # p<0.001 vs. CTRL gel and P150 gel.

Concerning the SMCs migration, the Transwell Assay (**Figure 3.5B**) showed that after 24 hours of incubation, the H10 gel were able to significantly inhibit the HUASMCs migration (0.64 ± 0.18) compared to the CTRL gel (p<0.01). Moreover, in presence of the H/P gel the migration of the HUASMCs (0.49 ± 0.21) was also significantly lower if compared to the CTRL gel (p<0.001) and the P150 gel (0.83 ± 0.24; p<0.001).

3.5.6 Hemocompatibility Assay

The hemocompatibility of the different collagen gel formulation has been tested using the hemoglobin free methodology. Absorbance at a wave length specific for hemoglobin

(540nm) was measured after the blood was incubated for 10, 25 and 50 minutes with the gels (**Figure 3.6**). After 10 minutes, both the gel formulation containing heparin, the H10 gel (0.39 ± 0.03) and the H/P gel (0.43 ± 0.06) where able to significantly increase the amount of free hemoglobin, hence the hemocompatibility, compared to both the CTRL gel (0.22 ± 0.01; $p<0.05$ vs. H/P gel) and P150 gel (0.14 ± 0.01; $p<0.001$ vs. H10 gel and H/P gel). The same behavior was observed after 25 minutes of incubation: both the H10 gel (0.45 ± 0.11) and the H/P gel (0.37 ± 0.09) were able to significantly increase the hemocompatibility compared to the CTRL gel (0.15 ± 0.07; $p<0.001$ vs. H10 gel and H/P gel) and P150 gel (0.12 ± 0.02; $p<0.001$ vs. H10 gel and H/P gel). Again, after 50 minutes of incubation, the H10 gel (0.24 ± 0.08) and the H/P gel (0.29 ± 0.11) significantly increase the measured free hemoglobin compared to the CTRL gel (0.08 ± 0.01; $p<0.001$ vs. H10 gel and H/P gel) and the P150 gel (0.08 ± 0.01; $p<0.001$ vs. H10 gel and H/P gel).

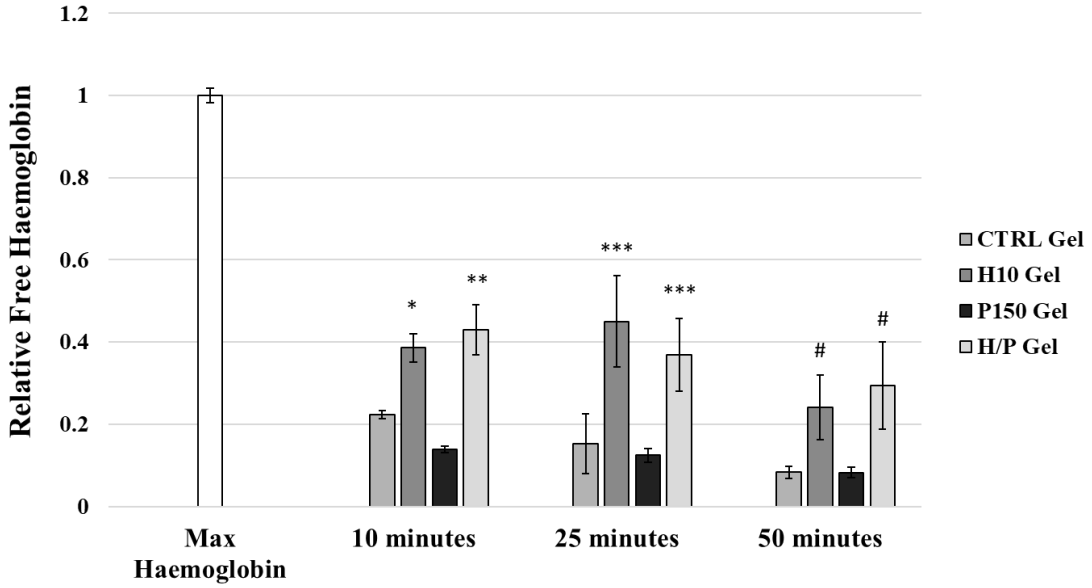

Figure 3.6*:* *Hemocompatibility Test.* Whole human blood was put in contact with the following collagen gel conditions: control collagen gel (CTRL Gel); collagen gel with 10µg/ml of heparin (H10 gel); collagen gel with 150ng/ml of PTN (P150 gel); collagen gel containing 10µg/ml of heparin and 150ng/ml of PTN (H/P gel). Blood was incubated with the gels for 10, 25 and 50 minutes. At each time point blood was solubilized and absorbance was recorded at 540nm. The graphic shows the relative free hemoglobin ± SD. * $p<0.001$ vs. 10 minutes P150 gel; ** $p<0.05$ vs. 10 minutes CTRL gel and $p<0.001$ vs. 10 minutes P150 gel; ***$p<0.001$ vs. 25 minutes CTRL gel and P150 gel; # $p<0.001$ vs. 50 minutes CTRL gel and P150 gel.

3.6 Discussion

An effective and fast re-endothelialization is of crucial importance to guarantee the patency of polymeric vascular graft [165]. The formation of confluent endothelial coverage has the

benefit to speed up and improve the integration of the implanted graft, effectively shortening the healing time [22]. Moreover, graft endothelialization has the benefit of limiting the insurgence of adverse processes like in-graft thrombosis and neo-intima hyperplasia [2]. These two conditions, as of today, represent a major concern hampering the performances of synthetic vascular graft in the clinical practice. The modification of the luminal surface of synthetic graft with natural components of the vascular extracellular matrix (ECM), such as collagen, has been already used to provide receptor-ligand binding sites for ECs on the graft surface. Another important factor in promoting ECs adhesion and proliferation on the implanted graft is the use of biological signaling. This represents a crucial point in cell-driven tissue regeneration. However, the concentration of biological molecules must be fine-tuned in order to accomplish the desired effects, thus the need to release these molecules in a controlled way. Therefore, the development of collagen-based drug delivery systems, in which specific non-covalent interactions are used to stabilize small molecule and protein-based drugs, immobilize them within the collagen scaffold and to control their release for biomedical applications, have been widely developed [50]. These systems help in protecting the biological activity of the loaded molecules while slowing their diffusion from collagen scaffolds, providing optimal effects on the targeted vascular cells. The use of heparin to control the release of therapeutic agents from collagen scaffolds has been widely studied, due to its ability to sequester, stabilize and protect growth factors and cytokines [144]. In this study, a drug delivery system for PTN, a known pro-angiogenetic factor, is presented as a potential strategy to induce in-graft migration and proliferation of ECs. The release system is based on a Type 1 collagen hydrogel, chosen for his favorable biological properties. The hydrogel has been further modified with the addition of heparin to help controlling the release of the loaded PTN over time and to confer anticoagulant properties to the system. The mechanical properties of the modified gels have been tested, along with the biological performances. To do so, the different gel compositions have been tested for cell viability and migration on both ECs and SMCs, in order to evaluate their effects on cell behaviors.

The effect of the addition of heparin to collagen gels has been studied for long time [286]. Heparin was reported to alter the structure of the collagen fibers, potentially leading to changes in the mechanical and biological properties of the modified collagen gels [287, 288]. Mechanical tests leading to the equilibrium elastic modulus have shown how the addition of

10 µg/ml of heparin, H10 gels, does not alter in a significant way the mechanical properties of the CTRL gels. Moreover, the immunofluorescence staining for the type 1 collagen fibers shows how the H10 gels presents an unaltered fibers structure, further demonstrating the absence of negative effects of heparin on the collagen gel structure (**Figure 3.1**). The concentration chosen to modify our collagen gel, besides showing the best results in terms of structure preservation compared to other concentration tested (data not shown), is in accordance with pre-existing data present in literature [150].

The release of growth factors and cytokine from heparinized collagen gels is dependent on diffusion from the collagen matrix and the binding affinity to heparin, whereas for simple collagen gels the release rely mainly on the diffusion from the gel [289]. The analysis of the release of PTN from the unmodified (P150 gels) and heparin-modified gels (H/P gels) shed light on how the addition of heparin was able to induce a more controlled, sustained release of PTN over the time period studied. The amount of PTN released during the first week by the H/P gels is significantly lower compared to the P150 gels. Moreover, the amount of PTN released by the H/P gels increase until the fourteenth day, whereas a significant drop in release is notable with the P150 gels. The results here obtained are in accordance with previous observation made with the use of heparin to modulate the release of several growth factor [289, 290], implying a direct effect of heparin in controlling the release of PTN from the collagen gels. Moreover, as shown in **Figure 3.2B**, the addition of heparin to collagen gels resulted effective in decreasing the initial burst of PTN released, as with previously described results where heparin successfully controlled the release of growth factors from heparinized gel scaffolds [291].

PTN is known for is beneficial effects exerted on the cardiovascular system, and especially on ECs. In fact, it has been shown to be a neovasculogenesis inducer [104], a potent pro-angiogenetic factor [105] and to be able to effectively differentiate mononuclear cells into functional ECs [110]. Moreover, as described in our previous work [283], PTN can induce significant effects on viability, migration and repair ability of ECs. The results hereby presented show how the incorporation of PTN in the Heparin-Collagen gel delivery system does not altered the beneficial effects towards the ECs associated to PTN. Regarding the HUVECs directly seeded onto the different collagen gel formulations, the presence of PTN was able to induce a significant increase in cell viability compared to the conditions without

it. Moreover, while in presence of the sole heparin (H10 gel condition) a decrease in ECs viability is appreciable, in the H/P gels no negative effects are detectable, showing a behavior similar to the P150 gels (**Figure 3.4A**), displaying the ability of the added PTN to "mask" the effect of heparin. Those results are in accordance with our previous findings about PTN mitogenic effects on ECs. Regarding the indirect viability tests (**Figure 3.3A**), again PTN was able to significantly increase the treated HUVECs viability compared to the control conditions. Of interest, the released PTN from the H/P gels seems to exert better effects compared to the one obtained from the P150 gels. This difference in efficacity could be explained by the presence of heparin in the H/P gels: heparin has been demonstrated to be able to stabilize and preserve the structure, thus the function of several growth factors and cytokine [144, 292]. The binding of heparin to growth factor has been also demonstrated to be able to increase the efficacy of the bounded factors [293]. Considering the high binding affinity between PTN and heparin, a protective action of the latter on the released PTN could be at the base of the H/P gel released PTN effects towards the treated ECs. The results obtained from the migration assay show a response from the ECs similar to the one obtained for the viability assay. As shown in **Figure 3.5A**, the H/P gels were able to induce a significant increase in the migration of the treated HUVECs compare to control conditions, confirming the beneficial effects of PTN on ECs migration [283, 294]. Interesting, contrary to the H/P gels, the effect exerted by the P150 gels resulted being not significantly higher compared to the control conditions, further validating the hypothesis of a protective/enhancing effect of heparin on PTN activity towards ECs.

Regarding the effects of PTN on the viability and migration of SMCs, the results obtained gave a different picture. In fact, both the indirect and direct cell viability test (**Figure 3.3B** and **3.4B**) show how the presence of PTN does not induce any significant effect on the viability of treated SMCs, contrary to the results presented by Brewster et al. [275], where the treatments with a chimeric PTN fusion protein induced an increase in the viability of SMCs, thus suggesting a non-specificity of PTN for SMCs. The results of the migration assay performed on the HUASMCs (**Figure 3.5B**), show how with the H10 and H/P gels, meaning in presence of heparin, the migration ability of the SMCs was significantly inhibited compared to the CTRL and P150 gels. The inhibitory effects of heparin on SMCs viability, proliferation and migration ability [295, 296] along with their mechanism [146, 297] are well

known. This, along with the results hereby presented, suggests a role for the heparin present in our gel in the inhibitory effects observed on the treated HUASMCs. Moreover, since heparin added to our gels is not immobilized in the collagen matrix, this may account for a portion of the added heparin to be released along with PTN, thus explaining the inhibitory effects observed on SMCs migration and the aforementioned protective/enhancing effect on PTN towards ECs. Altogether, these findings demonstrate how are system could be useful for an application in vascular graft functionalization, showing pro-endothelialization properties and inhibitory effects on SMCs, two of the most sought-after effects for vascular biomaterials.

The effects of heparin as an anticoagulant factor are well known and characterized [298], as is its use in tissue engineering to confer anticoagulation properties [299]. In accordance to the data present in literature, the hemocompatibility of our gels containing heparin, in particular in the H/P gels, was significantly increase compared to the CTRL and P150 gels, suggesting a potential in limiting the formation of thrombosis in an *in vivo* application. These results fall in accordance with the existing literature, where the use of heparin to modify collagen scaffolds to enhance their hemocompatibility properties has already been investigated with promising results [300]. Moreover, the previously described effects of the addition of heparin on the release of PTN and the preservation of its beneficial effects, further support the possible application in vascular medicine of our drug delivery system.

3.7 Conclusions

The obtained results show how the addition of heparin to a type I collagen gel can control over time the release of PTN, without altering the gel properties while limiting the thrombogenicity of the modified gels. The added PTN, moreover, is able to exert beneficial ECs-specific effects on cell viability and migration while not affecting SMCs behavior. In conclusion, the PTN-heparin-modified collagen gels here proposed can represent an added value for their use in vascular medicine, being able to improve the biological performance and integration of vascular grafts.

3.8 Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the CHU de Québec Research Center. Francesco Copes is a recipient

of a PhD scholarship from the NCPRM (www.ncprm.ulaval.ca). The authors would like to thank Lucie Levesque and Charles-Éric Bernier-Cloutier for the scientific support, help and guidance.

Chapter 4: General Discussion

Vascular occlusive diseases, and in particular atherosclerosis, remain the leading cause of death in Western countries. Several strategies have been developed over the years and are currently available for their treatment. Arterial bypass surgery remains the primary therapy for patients with severe vascular occlusive diseases. In bypass surgery, blood is redirected around a section of a blocked or partially blocked artery to restore the blood flow to the affected area. This procedure involves that a healthy blood vessel harvested from the patient vascular bed will be implanted to replace the blocked arteries. As previously stated, autologous vessels represent the privileged choice. However, this approach requires multiple surgical procedures, and the vascular bed of patients needing bypass surgery is often in bad conditions. Therefore, synthetic prostheses have been developed as alternatives to autografts. These implants, mainly made of Dacron and Teflon, are commonly used in the medical practice. Unfortunately, due to low patency especially in small diameter grafts (<6 mm), the use of synthetic vascular is burden by high-rate failure, thus limiting their applications. The main causes of post-implantation failure are in-graft thrombosis and intimal hyperplasia. In-graft thrombosis is the formation of a blood clot inside the implanted vascular substitutes. The main cause is the lack of biocompatibility of the graft, linked to several properties of the implant itself (inherent thrombogenicity, hemodynamic characteristics). The incomplete healing process contribute to the formation of blood clots during the first months after the implantation. Intimal hyperplasia, which is the abnormal migration and proliferation of vascular smooth muscle cells in the vessel lumen (starting from the anastomotic site), is the main cause of post-implantation arterial restenosis. Intima hyperplasia is characterized by the migration of vascular smooth muscle cells (VSMC) from the media to the intima, where they proliferate and deposit extracellular matrix, that greatly contributes to the re-occlusion of the artery. Several events may cause this condition, all of them involving damage or lack of a functional and confluent endothelial layer. This results in the missed production by the endothelial cells of factors capable to inhibit VSMC proliferation.

To overcome all of these complications, the development of tissue-engineered vascular substitutes has emerged as a promising alternative to generate physiologically-relevant substitutes. These vascular substitutes are expected to mimic the complex three-dimensional extracellular matrix (ECM) structure of the native tissues and, thus, their original mechanical

properties. The seeding of these scaffolds with vascular cells will then ensure the suitable biological properties for the optimal integration and functionality of the graft. In the past thirty years, several groups have focused their efforts on the *in vitro* construction of blood vessels from collagen, especially Type 1, as a natural polymer scaffolds. Weinberg and Bell first reported the use of collagen gels as substrates for cells in vascular tissue engineering [125]. After this work, several groups have developed different technique to obtain tissue-engineered blood vessel. Despite the promising results obtained, the mechanical properties of collagen-based products remain inadequate for clinical implantation. To overcome this limit, the use of specialized bioreactors to stimulate collagen-based substitutes have been developed, along with the addition to the scaffold of other ECM components or the use of polymeric mesh as support. The cellular component, especially the presence of vascular smooth muscle cells, has been proven to contribute largely to the increase in the mechanical properties of the grafts. Slight improvements in the mechanical properties of the substitutes, with an increase in both cellular and matrix reorganization, have been achieved. However, they are still not enough to allow the implantation and, thus, the clinical use. Nonetheless, the knowledge gathered on the use of collagen as a biomaterial and strategies developed pursuing the goal of an implantable artificial blood vessel may still find a practical use in vascular medicine, such as provide biomaterials for advances *in vitro* models or for the amelioration of existing vascular graft.

Another strategy to face the high-rate failure of vascular grafts is their enrichment with pro-endothelialization molecule. This approach involves the modification of the luminal surface of the artificial vascular grafts with bio-active molecules, such as growth factors, cytokines, natural polymers, proteins, peptides and genes to improve the endothelialization of the implants. Regarding pro-endothelialization growth factors and cytokines, several molecules, including vascular endothelial growth factor (VEGF) and stromal cell derived factor 1 (SDF-1) among the others, have been tested and have shown to promote angiogenesis or endothelial cells recruitment. The discovery of these effects initially had promising prospects for their use in therapy. However, clinical trials of angiogenic factor delivery have been mostly disappointing, underscoring the need for a wider array of angiogenic factors for this application. Another problem linked to the application of this strategy is that the concentrations of the bioactive molecules must be fine-tuned in order to accomplish the

desired effects on the targeted ECs and to avoid complications or adverse outcomes. Thus, the need to release these molecules in a controlled way results being of crucial importance.

For the all these reasons, the aim of this project was to develop a drug delivery system based on a Type 1 collagen gel for the controlled release of pleiotrophin (PTN), a growth/differentiation cytokine that has been described as a potent pro-angiogenic factor. As a first step, the biological effects of PTN on the viability, migration and repair ability of ECs, both at a cellular and molecular level, have been compared to the one exerted by SDF-1, a chemokine known for its important role in angiogenesis and neovascularization and already used for vascular grafts enrichments. Subsequently, a Type I collagen gel developed in the Laboratory for Biomaterials and Bioengineering at Laval University (LBB) [34] has been used as a scaffold for the development of a controlled release system for PTN. To increase the binding of PTN to the gel and to prolong its release over time, heparin has been added to the standard gel formulation. Finally, structural analyses have been performed on the heparin-modified collagen gels. Quantification of the released PTN has been analyzed to evaluate the ability of the modified collagen gels to bind and released PTN over time in a controlled way. The effects of the released PTN on the viability and migration of both ECs and SMCs has been evaluated along with the evaluation of the hemocompatibility properties of the modified collagen gels.

This chapter will discuss the work done throughout this thesis and review the important results obtained throughout the different steps of the project. The discussion will focus on the methodologies and characterization techniques developed as well as the challenges encountered. This discussion will also bring together the work presented and propose some future perspectives envisioned for the project.

4.1 Assessment of PTN effects on the viability and migration ability of ECs

4.1.1 Selection of cell lines as ECs model

Before starting the assessment of the biological effects exerted by PTN, one of the choices to face was related to the experimental model to be used. In order to guarantee a higher reproducibility of the data and to ensure a more solid consistency of the results, compared to the one obtainable with the use of a primary cell line, the human umbilical vein endothelial

cell line EA.hy926 has been chosen. This cell line has been established in 1983 and since then has been widely used as *in vitro* model for endothelial cells. These cells demonstrated the highly differentiated functions characteristic of human vascular endothelium, while offering the advantages of immortality, stability through passage number and permits more consistent responses to specific variables and greater reproducibility of data. For these reasons, they were selected for the biological characterization of PTN.

4.1.2 Choice of PTN concentrations

Another important point was the choice of concentration of PTN to be used for the project. For the comparation of the biological effects of PTN and SDF-1, the concentration of PTN has been selected following an experiment to determine a dose-response curve for the protein. In **Figure A.1** of the Annex, the results of the dose-response experiments are presented. They show how the 10ng/ml, despite being able to significantly increase cell viability, was still not able to achieve the results seen with the concentration of 50ng/ml. On the other end, the concentration of 100ng/ml did not induce better effects on the viability of the treated cells compared to the 50ng/ml concentration. For these reasons, we opted for the 50ng/ml concentration. Regarding SDF-1, the concentration has been chosen accordingly to data present in literature [267, 268].

4.1.3 Comparison of the pro-endothelialization effects of PTN compared to SDF-1

Pleiotrophin has been known to have mitogenic, differentiating and angiogenic properties [86]. Through the activation of its receptor, protein tyrosine phosphatase beta/zeta (RPTP β/ζ), PTN can regulate multiple functions including cell adhesion, cell migration, cell proliferation and cytoskeletal stability. PTN has been described as a potent pro-angiogenic factor acting on ECs. Its expression by endothelial cells during healing from ischemic brain injury has been reported and was found to stabilize the formation of tube structures by cultured capillary endothelial cells [105]. Interestingly, recent studies have also showed a PTN-induced transdifferentiation of monocytes into functional EC suggesting a role for PTN in inflammation-mediated neovascularization [108] and its role in the recruitment of endothelial progenitor cells (EPC) during angiogenesis [107]. The hypotheses behind the first part of the project was that PTN could promote fast endothelialization of vascular grafts. The novelty of this research lays in the fact that the effects of PTN has been compared with the

ones exerted by SDF-1, a known pro-endothelialization factor that has been already used to improve/enhance the re-endothelialization of vascular grafts. We showed that, if compared to SDF-1, higher effects on the viability and migration ability of ECs were achieved with the use of PTN. The MTT Viability Assay and both the Wound Healing and Transwell Migration assay demonstrated the superiority of PTN on SDF-1 in promoting the viability and migration of the treated EA.hy926. Moreover, the results obtained by the analysis of two molecular markers, PCNA for proliferation and Rac-1 for migration, further demonstrated the beneficial effects exerted by PTN. Interestingly, the analysis of the expression of PCNA and Rac-1 suggests that PTN is able to exert these beneficial effects on endothelial cells even if its primary receptor, while RPTP β/ζ, is blocked or not available. This suggests that PTN can have more possibility to beneficially influence ECs compared to other pro-endothelialization factors. Thus, the results of this research can shed light on the properties of this cytokine, and on its possible application for vascular grafts endothelialization.

4.2 Development of a DDS for the controlled release of PTN based on type I collagen

4.2.1 Addition of Heparin to the collagen gel

The protein structure of PTN has been analyzed with heteronuclear nuclear magnetic resonance (NMR) [77]. The analysis revealed that PTN contains two β-sheet domains closely related to the thrombospondin type I repeat (TSR) domain, which mediate different cell/extracellular matrix and cell/cell interactions. PTN, through these sites, can bind to heparin with high affinity [85]. In fact, additional studies identified the binding sites of heparin primarily within the β-sheet domains of the protein [301]. To enhance the binding of PTN to the collagen gels, we decided to add heparin. The addition of heparin to biomaterials for the controlled delivery of protein-based drugs, such as growth factors and cytokines has already been reported. These systems utilize the specific non-covalent interactions between heparin and the target molecule to stabilize immobilize it within a biomaterial matrix, thus protecting its biological activity, while slowing the diffusion from the matrix, thus mimicking the interaction naturally occurring with native ECM proteoglycans [144].

In this project, 3 different concentration of heparin (0,1; 0,25 and 0,5mg/ml) were added to the standard collagen gel recipe used in the LBB. After the addition of heparin, the collagen

gel mixes were poured in the wells of a 24-well plate then left to gelify for 1 hour at room temperature (RT). While the control gels gelified normally after 1 hour. However, the gels containing the three concentration of heparin did not. Even after 2 and 24 hours, still no gelification was observed with the heparin-containing gel mixes, that stayed in a liquid state. Two possible causes were thought for the non-gelification of the heparin-modified collagen: 1) changes in the pH of the gel mix; 2) too high concentration of heparin in the mix. Following pH analyses on the different mixes (**Figure A.2** of the Annex) that show no significant differences in the pH of the different gel preparations, the concentration of heparin was identified as the cause of the non-gelification of the gels. For the following experiments, the concentrations of heparin used were lowered to 10, 25 and 50µg/ml. As for the previous gels, gels were poured in a 24-well plate and left to gelify at RT for 1 hour. After 30 minutes of incubation, both the control and 10µg/ml heparin gels have gelified, while the 25 and 50µg/ml have not. After 1 hour of incubation, the 25µg/ml heparin gels have correctly gelified. Again, the 50µg/ml heparin gel did not. Even after 2 and 24 hours of incubation, the gelification never happened. Considering the results of these preliminary experiments, we decided to work with the heparin concentration of 10µg/ml.

4.2.2 Choice of cell lines as ECs model

For the development of the collagen-based PTN release system, the choice for the cells to use in the experiments fell on the use of two primary cell lines, the human umbilical vein endothelial cells (HUVECs) and human umbilical artery smooth muscle cells (HUASMCs), both of them isolated in the LBB following an established ethical protocol [284]. This time, primary lines were chosen to perform the biological characterization experiments to better mimic *in vitro* the condition of heterogeneity found in the medical application of this kind of devices. Once established the effects of PTN in a more stable model, as done in the first part of the project, in C***hapter 3*** the effect of the system was investigated in an advanced model. While retaining the intrinsic simplicity of a 2-D cell culture model, this model was able to show a higher degree of complexity, thus a higher noteworthiness of the obtained results.

4.2.3 Choice of PTN concentrations

As for the first part of the project, a dose-response curve has been repeated also for the second part. The test was performed anew since, as previously described, the cellular model for the

ECs changed. Being the HUVECs used in the characterization of the release system primary cells, their response to the PTN treatment may have been different than the one obtained with the EA.hy926 cell line. As shown in **Figure A.3** of the Annex, the concentration of 150ng/ml achieved the best results among the concentration tested, thus the choice to use it in the experiments.

4.2.4 Performances of the heparin-modified collagen-based PTN controlled release system

The aim of the second part of the project was to determine if the addition of Heparin to the standard formulation of a Type 1 collagen gel could affect its retention and release ability towards PTN, without modifying both 1) collagen gels mechanical properties; 2) the biological properties of PTN towards endothelial cells. The obtained results showed how the addition of heparin at the concentration of 10µg/ml to the collagen gel formulation currently used in the LBB, not only does not alter the mechanical properties and the structure of the collagen gel, but induce a controlled release of PTN over time, thus avoiding a burst of release during the first time-points and maintaining a constant release over time. Moreover, heparin seems to help in preserving the beneficial properties of PTN on endothelial cells viability. In fact, the released PTN was found to retain its ability to increase the viability and the migration ability of the treated HUVEC compared to the other conditions. Moreover, also the ECs seeded directly on the modified gels are beneficially influenced by the presence of PTN. Noteworthy, PTN seems to have no influence on the SMC. In fact, the beneficial effects exerted by PTN on HUVECs viability and migration ability were completely absent in presence of the HUASMCs. Therefore, the system developed seems to be specific for ECs and imply a possible role in limiting neointima hyperplasia. These results constitute a genuine novelty. They show that the pro-endothelialization effects of PTN mainly focus on the response of ECs, and not on the effects exerted on SMC. Moreover, hemocompatibility tests showed how the addition of heparin to the collagen gel formulation, both with or without PTN, resulted in an increase of the detected free hemoglobin, meaning a reduction of the thrombogenicity and a better hemocompatibility of the modified collagen gels. All these findings prompt the use of the developed PTN release system in cardiovascular applications for pro-endothelialization purposes.

Conclusion

This project took place in a multidisciplinary atmosphere requiring notions in biology, biochemistry, biomaterials, biomechanics and bioengineering. The research was focused on the development of drug delivery system for the controlled release of Pleiotrophin with the aim of ameliorate the endothelialization performances of synthetic vascular grafts. Starting from a Type 1 collagen gel intended for tissue engineering purposes, and through the modification with heparin to increase the binding/release ability of the scaffold, this project allowed design of collagen gel easy to produce and able to significantly increase the proliferation and migration ability of endothelial cells, without stimulating the same effects in smooth muscle cells. The higher hemocompatibility resulting from the addition of heparin further increase the prospect of its use for vascular graft enrichment.

Different perspectives are possible for these controlled release systems. One of the first steps will be the validation of the effects here reported in more complex model. To do so, the physiologically relevant model of the artery wall developed in the LBB will be used. This model of the arterial wall has characteristics close to those of a natural artery, displaying important mechanical and biological features, such as the complex interactions existing between different vascular cells types. This model could be adapted to the study of pathophysiological processes, such as neo-intima hyperplasia, and for validation studies of pharmacological molecules and medical devices, such as the developed PTN release system. This model will definitely be of great use for the validation of the PTN release system to be an effective strategy for the induction of the endothelialization process and the limitation of smooth muscle cells-related pathologies affecting the human cardiovascular system.

References

1. World Health Organization. Top 10 causes of death http://www.who.int/gho/mortality_burden_disease/causes_death/top_10/en/ [
2. Haruguchi H, Teraoka S. Intimal hyperplasia and hemodynamic factors in arterial bypass and arteriovenous grafts: a review. J Artif Organs. 2003;6(4):227-35.
3. Eble JA, Niland S. The extracellular matrix of blood vessels. Curr Pharm Des. 2009;15(12):1385-400.
4. Michiels C. Endothelial cell functions. J Cell Physiol. 2003;196(3):430-43.
5. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. Pflugers Arch. 2007;454(3):345-59.
6. Deng Q, Huo Y, Luo J. Endothelial mechanosensors: the gatekeepers of vascular homeostasis and adaptation under mechanical stress. Sci China Life Sci. 2014;57(8):755-62.
7. Robertson AMW, P.N. Chapter 8: Mechanobiology of the Arterial Wall. In: Inc. E, editor. Transport in Biological Media: Elsevier Inc.; 2013. p. 306-8.
8. Gingras M, Farand P, Safar ME, Plante GE. Adventitia: the vital wall of conduit arteries. J Am Soc Hypertens. 2009;3(3):166-83.
9. OpenStax. The Cardiovascular System: Blood. In: OpenStax, editor. Anatomy and Physiology. http://cnx.org/content/col11496/latest/2013. p. 785-823.
10. American Heart Association. Atherosclerosis http://www.heart.org/HEARTORG/Conditions/Cholesterol/WhyCholesterolMatters/Atherosclerosis_UCM_305564_Article.jsp#.WPorq4jhDIU [
11. Graham IM, Catapano AL, Wong ND. Current guidelines on prevention with a focus on dyslipidemias. Cardiovasc Diagn Ther. 2017;7(Suppl 1):S4-S10.
12. Palatini P. Heart rate as a risk factor for atherosclerosis and cardiovascular mortality: the effect of antihypertensive drugs. Drugs. 1999;57(5):713-24.
13. Gruntzig AR, Senning A, Siegenthaler WE. Nonoperative dilatation of coronary-artery stenosis: percutaneous transluminal coronary angioplasty. N Engl J Med. 1979;301(2):61-8.
14. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. Can J Cardiol. 2014;30(1):35-45.
15. Arnulf G. [Long-term results of Jean Kunlin's first venous autograft. Apropos of the report of M. J. Descotes et al]. Chirurgie. 1987;113(7-8):751-2.
16. Kannan RY, Salacinski HJ, Butler PE, Hamilton G, Seifalian AM. Current status of prosthetic bypass grafts: a review. J Biomed Mater Res B Appl Biomater. 2005;74(1):570-81.
17. Voorhees AB, Jr., Jaretzki A, 3rd, Blakemore AH. The use of tubes constructed from vinyon "N" cloth in bridging arterial defects. Ann Surg. 1952;135(3):332-6.
18. Chlupac J, Filova E, Bacakova L. Blood vessel replacement: 50 years of development and tissue engineering paradigms in vascular surgery. Physiol Res. 2009;58 Suppl 2:S119-39.
19. Roll S, Muller-Nordhorn J, Keil T, Scholz H, Eidt D, Greiner W, et al. Dacron vs. PTFE as bypass materials in peripheral vascular surgery--systematic review and meta-analysis. BMC Surg. 2008;8:22.

20. Chester JF. The causes of synthetic vascular graft failure. Ann Coll Surg HK. 2002;6(4):97-101.
21. Klinkert P, Post PN, Breslau PJ, van Bockel JH. Saphenous vein versus PTFE for above-knee femoropopliteal bypass. A review of the literature. Eur J Vasc Endovasc Surg. 2004;27(4):357-62.
22. van der Zijpp YJ, Poot AA, Feijen J. Endothelialization of small-diameter vascular prostheses. Arch Physiol Biochem. 2003;111(5):415-27.
23. Ravi S, Chaikof EL. Biomaterials for vascular tissue engineering. Regen Med. 2010;5(1):107-20.
24. Simon-Yarza T, Bataille I, Letourneur D. Cardiovascular Bio-Engineering: Current State of the Art. J Cardiovasc Transl Res. 2017;10(2):180-93.
25. Vara DS, Salacinski HJ, Kannan RY, Bordenave L, Hamilton G, Seifalian AM. Cardiovascular tissue engineering: state of the art. Pathol Biol (Paris). 2005;53(10):599-612.
26. Heyligers JM, Arts CH, Verhagen HJ, de Groot PG, Moll FL. Improving small-diameter vascular grafts: from the application of an endothelial cell lining to the construction of a tissue-engineered blood vessel. Ann Vasc Surg. 2005;19(3):448-56.
27. Nerem RM, Seliktar D. Vascular tissue engineering. Annu Rev Biomed Eng. 2001;3:225-43.
28. Schmidt CE, Baier JM. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. Biomaterials. 2000;21(22):2215-31.
29. Boccafoschi F, Botta M, Fusaro L, Copes F, Ramella M, Cannas M. Decellularized biological matrices: an interesting approach for cardiovascular tissue repair and regeneration. J Tissue Eng Regen Med. 2017;11(5):1648-57.
30. Olausson M, Patil PB, Kuna VK, Chougule P, Hernandez N, Methe K, et al. Transplantation of an allogeneic vein bioengineered with autologous stem cells: a proof-of-concept study. Lancet. 2012;380(9838):230-7.
31. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. FASEB J. 1998;12(1):47-56.
32. Vallieres K, Laterreur V, Tondreau MY, Ruel J, Germain L, Fradette J, et al. Human adipose-derived stromal cells for the production of completely autologous self-assembled tissue-engineered vascular substitutes. Acta Biomater. 2015;24:209-19.
33. Stegemann JP, Kaszuba SN, Rowe SL. Review: advances in vascular tissue engineering using protein-based biomaterials. Tissue Eng. 2007;13(11):2601-13.
34. Boccafoschi F, Rajan N, Habermehl J, Mantovani D. Preparation and characterization of a scaffold for vascular tissue engineering by direct-assembling of collagen and cells in a cylindrical geometry. Macromol Biosci. 2007;7(5):719-26.
35. Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. Circ Res. 2006;98(1):25-35.
36. Aper T, Wilhelmi M, Gebhardt C, Hoeffler K, Benecke N, Hilfiker A, et al. Novel method for the generation of tissue-engineered vascular grafts based on a highly compacted fibrin matrix. Acta Biomater. 2016;29:21-32.
37. Kurane A, Simionescu DT, Vyavahare NR. In vivo cellular repopulation of tubular elastin scaffolds mediated by basic fibroblast growth factor. Biomaterials. 2007;28(18):2830-8.
38. Matsuda T, Kitamura T, Iwata H, Takano H, Akutsu T. A hybrid artificial vascular graft based upon an organ reconstruction model. Significance and design criteria of an artificial basement membrane. ASAIO Trans. 1988;34(3):640-3.

39. L'Heureux N, Germain L, Labbe R, Auger FA. In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study. J Vasc Surg. 1993;17(3):499-509.
40. Girton TS, Oegema TR, Grassl ED, Isenberg BC, Tranquillo RT. Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation. J Biomech Eng. 2000;122(3):216-23.
41. Berglund JD, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular constructs. Biomaterials. 2003;24(7):1241-54.
42. Orban JM, Wilson LB, Kofroth JA, El-Kurdi MS, Maul TM, Vorp DA. Crosslinking of collagen gels by transglutaminase. J Biomed Mater Res A. 2004;68(4):756-62.
43. Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. Am J Physiol Heart Circ Physiol. 2005;288(3):H1451-60.
44. Yao L, Liu J, Andreadis ST. Composite fibrin scaffolds increase mechanical strength and preserve contractility of tissue engineered blood vessels. Pharm Res. 2008;25(5):1212-21.
45. Meghezi S, Seifu DG, Bono N, Unsworth L, Mequanint K, Mantovani D. Engineering 3D Cellularized Collagen Gels for Vascular Tissue Regeneration. J Vis Exp. 2015(100):e52812.
46. Benrashid E, McCoy CC, Youngwirth LM, Kim J, Manson RJ, Otto JC, et al. Tissue engineered vascular grafts: Origins, development, and current strategies for clinical application. Methods. 2016;99:13-9.
47. Seifu DG, Purnama A, Mequanint K, Mantovani D. Small-diameter vascular tissue engineering. Nat Rev Cardiol. 2013;10(7):410-21.
48. Laflamme K, Roberge CJ, Labonte J, Pouliot S, D'Orleans-Juste P, Auger FA, et al. Tissue-engineered human vascular media with a functional endothelin system. Circulation. 2005;111(4):459-64.
49. Noishiki Y, Marat D, Yamane Y, Satoh S, Ma XH, Iwai Y, et al. A collagen coated fabric vascular prosthesis as a punctureable A-V shunt. ASAIO J. 1996;42(5):M687-93.
50. Wallace DG, Rosenblatt J. Collagen gel systems for sustained delivery and tissue engineering. Adv Drug Deliv Rev. 2003;55(12):1631-49.
51. Ren X, Feng Y, Guo J, Wang H, Li Q, Yang J, et al. Surface modification and endothelialization of biomaterials as potential scaffolds for vascular tissue engineering applications. Chem Soc Rev. 2015;44(15):5680-742.
52. Wang Z, Wang H, Zheng W, Zhang J, Zhao Q, Wang S, et al. Highly stable surface modifications of poly(3-caprolactone) (PCL) films by molecular self-assembly to promote cells adhesion and proliferation. Chem Commun (Camb). 2011;47(31):8901-3.
53. Larsen CC, Kligman F, Kottke-Marchant K, Marchant RE. The effect of RGD fluorosurfactant polymer modification of ePTFE on endothelial cell adhesion, growth, and function. Biomaterials. 2006;27(28):4846-55.
54. Kanie K, Narita Y, Zhao Y, Kuwabara F, Satake M, Honda S, et al. Collagen type IV-specific tripeptides for selective adhesion of endothelial and smooth muscle cells. Biotechnol Bioeng. 2012;109(7):1808-16.
55. Kuwabara F, Narita Y, Yamawaki-Ogata A, Kanie K, Kato R, Satake M, et al. Novel small-caliber vascular grafts with trimeric Peptide for acceleration of endothelialization. Ann Thorac Surg. 2012;93(1):156-63; discussion 63.

56. Plouffe BD, Radisic M, Murthy SK. Microfluidic depletion of endothelial cells, smooth muscle cells, and fibroblasts from heterogeneous suspensions. Lab Chip. 2008;8(3):462-72.
57. Wei Y, Ji Y, Xiao L, Lin Q, Ji J. Different complex surfaces of polyethyleneglycol (PEG) and REDV ligand to enhance the endothelial cells selectivity over smooth muscle cells. Colloids Surf B Biointerfaces. 2011;84(2):369-78.
58. Ren T, Yu S, Mao Z, Moya SE, Han L, Gao C. Complementary density gradient of Poly(hydroxyethyl methacrylate) and YIGSR selectively guides migration of endotheliocytes. Biomacromolecules. 2014;15(6):2256-64.
59. Massia SP, Hubbell JA. Human endothelial cell interactions with surface-coupled adhesion peptides on a nonadhesive glass substrate and two polymeric biomaterials. J Biomed Mater Res. 1991;25(2):223-42.
60. Brewster LP, Washington C, Brey EM, Gassman A, Subramanian A, Calceterra J, et al. Construction and characterization of a thrombin-resistant designer FGF-based collagen binding domain angiogen. Biomaterials. 2008;29(3):327-36.
61. Erzurum VZ, Bian JF, Husak VA, Ellinger J, Xue L, Burgess WH, et al. R136K fibroblast growth factor-1 mutant induces heparin-independent migration of endothelial cells through fibrin glue. J Vasc Surg. 2003;37(5):1075-81.
62. Conklin BS, Wu H, Lin PH, Lumsden AB, Chen C. Basic fibroblast growth factor coating and endothelial cell seeding of a decellularized heparin-coated vascular graft. Artif Organs. 2004;28(7):668-75.
63. Bai Y, Leng Y, Yin G, Pu X, Huang Z, Liao X, et al. Effects of combinations of BMP-2 with FGF-2 and/or VEGF on HUVECs angiogenesis in vitro and CAM angiogenesis in vivo. Cell Tissue Res. 2014;356(1):109-21.
64. Zhang H, Jia X, Han F, Zhao J, Zhao Y, Fan Y, et al. Dual-delivery of VEGF and PDGF by double-layered electrospun membranes for blood vessel regeneration. Biomaterials. 2013;34(9):2202-12.
65. Han F, Jia X, Dai D, Yang X, Zhao J, Zhao Y, et al. Performance of a multilayered small-diameter vascular scaffold dual-loaded with VEGF and PDGF. Biomaterials. 2013;34(30):7302-13.
66. Zhao W, Jin K, Li J, Qiu X, Li S. Delivery of stromal cell-derived factor 1alpha for in situ tissue regeneration. J Biol Eng. 2017;11:22.
67. Muylaert DE, van Almen GC, Talacua H, Fledderus JO, Kluin J, Hendrikse SI, et al. Early in-situ cellularization of a supramolecular vascular graft is modified by synthetic stromal cell-derived factor-1alpha derived peptides. Biomaterials. 2016;76:187-95.
68. Shafiq M, Zhang Q, Zhi D, Wang K, Kong D, Kim DH, et al. In Situ Blood Vessel Regeneration Using SP (Substance P) and SDF (Stromal Cell-Derived Factor)-1alpha Peptide Eluting Vascular Grafts. Arterioscler Thromb Vasc Biol. 2018;38(7):e117-e34.
69. Lee KW, Johnson NR, Gao J, Wang Y. Human progenitor cell recruitment via SDF-1alpha coacervate-laden PGS vascular grafts. Biomaterials. 2013;34(38):9877-85.
70. Wang H, Feng Y, Yang J, Guo J, Zhang W. Targeting REDV peptide functionalized polycationic gene carrier for enhancing the transfection and migration capability of human endothelial cells. J Mater Chem B. 2015;3:3379-91.
71. Springer ML. A balancing act: therapeutic approaches for the modulation of angiogenesis. Curr Opin Investig Drugs. 2006;7(3):243-50.
72. Rauvala H. An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. EMBO J. 1989;8(10):2933-41.

73. Merenmies J, Rauvala H. Molecular cloning of the 18-kDa growth-associated protein of developing brain. J Biol Chem. 1990;265(28):16721-4.
74. Li YS, Milner PG, Chauhan AK, Watson MA, Hoffman RM, Kodner CM, et al. Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science. 1990;250(4988):1690-4.
75. Hampton BS, Marshak DR, Burgess WH. Structural and functional characterization of full-length heparin-binding growth associated molecule. Mol Biol Cell. 1992;3(1):85-93.
76. Li YS, Hoffman RM, Le Beau MM, Espinosa R, 3rd, Jenkins NA, Gilbert DJ, et al. Characterization of the human pleiotrophin gene. Promoter region and chromosomal localization. J Biol Chem. 1992;267(36):26011-6.
77. Kilpelainen I, Kaksonen M, Kinnunen T, Avikainen H, Fath M, Linhardt RJ, et al. Heparin-binding growth-associated molecule contains two heparin-binding beta -sheet domains that are homologous to the thrombospondin type I repeat. J Biol Chem. 2000;275(18):13564-70.
78. Deepa SS, Yamada S, Zako M, Goldberger O, Sugahara K. Chondroitin sulfate chains on syndecan-1 and syndecan-4 from normal murine mammary gland epithelial cells are structurally and functionally distinct and cooperate with heparan sulfate chains to bind growth factors. A novel function to control binding of midkine, pleiotrophin, and basic fibroblast growth factor. J Biol Chem. 2004;279(36):37368-76.
79. Koyama-Nasu R, Haruta R, Nasu-Nishimura Y, Taniue K, Katou Y, Shirahige K, et al. The pleiotrophin-ALK axis is required for tumorigenicity of glioblastoma stem cells. Oncogene. 2014;33(17):2236-44.
80. Mikelis C, Sfaelou E, Koutsioumpa M, Kieffer N, Papadimitriou E. Integrin alpha(v)beta(3) is a pleiotrophin receptor required for pleiotrophin-induced endothelial cell migration through receptor protein tyrosine phosphatase beta/zeta. FASEB J. 2009;23(5):1459-69.
81. Koutsioumpa M, Papadimitriou E. Cell surface nucleolin as a target for anti-cancer therapies. Recent Pat Anticancer Drug Discov. 2014;9(2):137-52.
82. Elahouel R, Blanc C, Carpentier G, Frechault S, Cascone I, Destouches D, et al. Pleiotrophin exerts its migration and invasion effect through the neuropilin-1 pathway. Neoplasia. 2015;17(8):613-24.
83. Sorrelle N, Dominguez AT, Brekken RA. From top to bottom: midkine and pleiotrophin as emerging players in immune regulation. J Leukoc Biol. 2017.
84. Papadimitriou E, Pantazaka E, Castana P, Tsalios T, Polyzos A, Beis D. Pleiotrophin and its receptor protein tyrosine phosphatase beta/zeta as regulators of angiogenesis and cancer. Biochim Biophys Acta. 2016;1866(2):252-65.
85. Xu C, Zhu S, Wu M, Han W, Yu Y. Functional receptors and intracellular signal pathways of midkine (MK) and pleiotrophin (PTN). Biol Pharm Bull. 2014;37(4):511-20.
86. Deuel TF, Zhang N, Yeh HJ, Silos-Santiago I, Wang ZY. Pleiotrophin: a cytokine with diverse functions and a novel signaling pathway. Arch Biochem Biophys. 2002;397(2):162-71.
87. Milner PG, Li YS, Hoffman RM, Kodner CM, Siegel NR, Deuel TF. A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. Biochem Biophys Res Commun. 1989;165(3):1096-103.
88. Courty J, Dauchel MC, Caruelle D, Perderiset M, Barritault D. Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. Biochem Biophys Res Commun. 1991;180(1):145-51.

89. Fang W, Hartmann N, Chow DT, Riegel AT, Wellstein A. Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. J Biol Chem. 1992;267(36):25889-97.
90. Laaroubi K, Delbe J, Vacherot F, Desgranges P, Tardieu M, Jaye M, et al. Mitogenic and in vitro angiogenic activity of human recombinant heparin affin regulatory peptide. Growth Factors. 1994;10(2):89-98.
91. Rauvala H, Huttunen HJ, Fages C, Kaksonen M, Kinnunen T, Imai S, et al. Heparin-binding proteins HB-GAM (pleiotrophin) and amphoterin in the regulation of cell motility. Matrix Biol. 2000;19(5):377-87.
92. Kuo MD, Oda Y, Huang JS, Huang SS. Amino acid sequence and characterization of a heparin-binding neurite-promoting factor (p18) from bovine brain. J Biol Chem. 1990;265(31):18749-52.
93. Silos-Santiago I, Yeh HJ, Gurrieri MA, Guillerman RP, Li YS, Wolf J, et al. Localization of pleiotrophin and its mRNA in subpopulations of neurons and their corresponding axonal tracts suggests important roles in neural-glial interactions during development and in maturity. J Neurobiol. 1996;31(3):283-96.
94. Lamprou M, Kaspiris A, Panagiotopoulos E, Giannoudis PV, Papadimitriou E. The role of pleiotrophin in bone repair. Injury. 2014;45(12):1816-23.
95. Imai S, Heino TJ, Hienola A, Kurata K, Buki K, Matsusue Y, et al. Osteocyte-derived HB-GAM (pleiotrophin) is associated with bone formation and mechanical loading. Bone. 2009;44(5):785-94.
96. Husmann I, Soulet L, Gautron J, Martelly I, Barritault D. Growth factors in skeletal muscle regeneration. Cytokine Growth Factor Rev. 1996;7(3):249-58.
97. Soh BS, Song CM, Vallier L, Li P, Choong C, Yeo BH, et al. Pleiotrophin enhances clonal growth and long-term expansion of human embryonic stem cells. Stem Cells. 2007;25(12):3029-37.
98. Himburg HA, Harris JR, Ito T, Daher P, Russell JL, Quarmyne M, et al. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche. Cell Rep. 2012;2(4):964-75.
99. Himburg HA, Yan X, Doan PL, Quarmyne M, Micewicz E, McBride W, et al. Pleiotrophin mediates hematopoietic regeneration via activation of RAS. J Clin Invest. 2014;124(11):4753-8.
100. Chang Y, Zuka M, Perez-Pinera P, Astudillo A, Mortimer J, Berenson JR, et al. Secretion of pleiotrophin stimulates breast cancer progression through remodeling of the tumor microenvironment. Proc Natl Acad Sci U S A. 2007;104(26):10888-93.
101. Zhang N, Zhong R, Perez-Pinera P, Herradon G, Ezquerra L, Wang ZY, et al. Identification of the angiogenesis signaling domain in pleiotrophin defines a mechanism of the angiogenic switch. Biochem Biophys Res Commun. 2006;343(2):653-8.
102. Yeh HJ, He YY, Xu J, Hsu CY, Deuel TF. Upregulation of pleiotrophin gene expression in developing microvasculature, macrophages, and astrocytes after acute ischemic brain injury. J Neurosci. 1998;18(10):3699-707.
103. Souttou B, Raulais D, Vigny M. Pleiotrophin induces angiogenesis: involvement of the phosphoinositide-3 kinase but not the nitric oxide synthase pathways. J Cell Physiol. 2001;187(1):59-64.
104. Christman KL, Fang Q, Kim AJ, Sievers RE, Fok HH, Candia AF, et al. Pleiotrophin induces formation of functional neovasculature in vivo. Biochem Biophys Res Commun. 2005;332(4):1146-52.

105. Besse S, Comte R, Frechault S, Courty J, Joel de L, Delbe J. Pleiotrophin promotes capillary-like sprouting from senescent aortic rings. Cytokine. 2013;62(1):44-7.
106. Koutsioumpa M, Drosou G, Mikelis C, Theochari K, Vourtsis D, Katsoris P, et al. Pleiotrophin expression and role in physiological angiogenesis in vivo: potential involvement of nucleolin. Vasc Cell. 2012;4:4.
107. Heiss C, Wong ML, Block VI, Lao D, Real WM, Yeghiazarians Y, et al. Pleiotrophin induces nitric oxide dependent migration of endothelial progenitor cells. J Cell Physiol. 2008;215(2):366-73.
108. Sharifi BG, Zeng Z, Wang L, Song L, Chen H, Qin M, et al. Pleiotrophin induces transdifferentiation of monocytes into functional endothelial cells. Arterioscler Thromb Vasc Biol. 2006;26(6):1273-80.
109. Chen H, Campbell RA, Chang Y, Li M, Wang CS, Li J, et al. Pleiotrophin produced by multiple myeloma induces transdifferentiation of monocytes into vascular endothelial cells: a novel mechanism of tumor-induced vasculogenesis. Blood. 2009;113(9):1992-2002.
110. Palmieri D, Mura M, Mambrini S, Palombo D. Effects of Pleiotrophin on endothelial and inflammatory cells: Pro-angiogenic and anti-inflammatory properties and potential role for vascular bio-prosthesis endothelialization. Adv Med Sci. 2015;60(2):287-93.
111. Ezquerra L, Herradon G, Nguyen T, Vogt TF, Bronson R, Silos-Santiago I, et al. Pleiotrophin is a major regulator of the catecholamine biosynthesis pathway in mouse aorta. Biochem Biophys Res Commun. 2004;323(2):512-7.
112. Herradon G, Ezquerra L, Nguyen T, Vogt TF, Bronson R, Silos-Santiago I, et al. Pleiotrophin is an important regulator of the renin-angiotensin system in mouse aorta. Biochem Biophys Res Commun. 2004;324(3):1041-7.
113. Parenteau-Bareil R, Gauvin R, Berthod F. Collagen-Based Biomaterials for Tissue Engineering Applications. Materials. 2010;3(3):1863-87.
114. Fraser RD, MacRae TP, Suzuki E. Chain conformation in the collagen molecule. J Mol Biol. 1979;129(3):463-81.
115. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders (second of two parts). N Engl J Med. 1979;301(2):77-85.
116. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm. 2001;221(1-2):1-22.
117. Gelse K, Poschl E, Aigner T. Collagens--structure, function, and biosynthesis. Adv Drug Deliv Rev. 2003;55(12):1531-46.
118. Lynn AK, Yannas IV, Bonfield W. Antigenicity and immunogenicity of collagen. J Biomed Mater Res B Appl Biomater. 2004;71(2):343-54.
119. Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. Nat Methods. 2005;2(2):119-25.
120. Miller JM, Zoll DR, Brown EO. Clinical Observations on Use of an Extruded Collagen Suture. Arch Surg. 1964;88:167-74.
121. Roelofs LA, Kortmann BB, Oosterwijk E, Eggink AJ, Tiemessen DM, Crevels AJ, et al. Tissue engineering of diseased bladder using a collagen scaffold in a bladder exstrophy model. BJU Int. 2014;114(3):447-57.
122. Polo-Corrales L, Latorre-Esteves M, Ramirez-Vick JE. Scaffold design for bone regeneration. J Nanosci Nanotechnol. 2014;14(1):15-56.
123. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res. 1998;16(4):406-13.

124. Gentleman E, Lay AN, Dickerson DA, Nauman EA, Livesay GA, Dee KC. Mechanical characterization of collagen fibers and scaffolds for tissue engineering. Biomaterials. 2003;24(21):3805-13.
125. Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. Science. 1986;231(4736):397-400.
126. Bradley WG, Wilkes GL. Some mechanical property considerations of reconstituted collagen for drug release supports. Biomater Med Devices Artif Organs. 1977;5(2):159-75.
127. Lucas PA, Syftestad GT, Goldberg VM, Caplan AI. Ectopic induction of cartilage and bone by water-soluble proteins from bovine bone using a collagenous delivery vehicle. J Biomed Mater Res. 1989;23(A1 Suppl):23-39.
128. Kaufman HE, Steinemann TL, Lehman E, Thompson HW, Varnell ED, Jacob-LaBarre JT, et al. Collagen-based drug delivery and artificial tears. J Ocul Pharmacol. 1994;10(1):17-27.
129. Chiu LL, Radisic M, Vunjak-Novakovic G. Bioactive scaffolds for engineering vascularized cardiac tissues. Macromol Biosci. 2010;10(11):1286-301.
130. Chan SJ, Love C, Spector M, Cool SM, Nurcombe V, Lo EH. Endogenous regeneration: Engineering growth factors for stroke. Neurochem Int. 2017;107:57-65.
131. Fujisato T, Sajiki T, Liu Q, Ikada Y. Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. Biomaterials. 1996;17(2):155-62.
132. Koch S, Yao C, Grieb G, Prevel P, Noah EM, Steffens GC. Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF. J Mater Sci Mater Med. 2006;17(8):735-41.
133. Schroeder JW, Jr., Rastatter JC, Walner DL. Effect of vascular endothelial growth factor on laryngeal wound healing in rabbits. Otolaryngol Head Neck Surg. 2007;137(3):465-70.
134. Kanematsu A, Yamamoto S, Ozeki M, Noguchi T, Kanatani I, Ogawa O, et al. Collagenous matrices as release carriers of exogenous growth factors. Biomaterials. 2004;25(18):4513-20.
135. Ivester KM, Adams SB, Moore GE, Van Sickle DC, Lescun TB. Gentamicin concentrations in synovial fluid obtained from the tarsocrural joints of horses after implantation of gentamicin-impregnated collagen sponges. Am J Vet Res. 2006;67(9):1519-26.
136. Xu XL, Lou J, Tang T, Ng KW, Zhang J, Yu C, et al. Evaluation of different scaffolds for BMP-2 genetic orthopedic tissue engineering. J Biomed Mater Res B Appl Biomater. 2005;75(2):289-303.
137. Chandler LA, Gu DL, Ma C, Gonzalez AM, Doukas J, Nguyen T, et al. Matrix-enabled gene transfer for cutaneous wound repair. Wound Repair Regen. 2000;8(6):473-9.
138. Shih YR, Chen CN, Tsai SW, Wang YJ, Lee OK. Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. Stem Cells. 2006;24(11):2391-7.
139. Xiang Z, Liao R, Kelly MS, Spector M. Collagen-GAG scaffolds grafted onto myocardial infarcts in a rat model: a delivery vehicle for mesenchymal stem cells. Tissue Eng. 2006;12(9):2467-78.
140. Yu H, Cao B, Feng M, Zhou Q, Sun X, Wu S, et al. Combinated transplantation of neural stem cells and collagen type I promote functional recovery after cerebral ischemia in rats. Anat Rec (Hoboken). 2010;293(5):911-7.

141. Gruber HE, Hoelscher GL, Leslie K, Ingram JA, Hanley EN, Jr. Three-dimensional culture of human disc cells within agarose or a collagen sponge: assessment of proteoglycan production. Biomaterials. 2006;27(3):371-6.
142. Gruber HE, Leslie K, Ingram J, Norton HJ, Hanley EN. Cell-based tissue engineering for the intervertebral disc: in vitro studies of human disc cell gene expression and matrix production within selected cell carriers. Spine J. 2004;4(1):44-55.
143. Langer R. Drug delivery and targeting. Nature. 1998;392(6679 Suppl):5-10.
144. Sakiyama-Elbert SE. Incorporation of heparin into biomaterials. Acta Biomater. 2014;10(4):1581-7.
145. Rabenstein DL. Heparin and heparan sulfate: structure and function. Nat Prod Rep. 2002;19(3):312-31.
146. Young E. The anti-inflammatory effects of heparin and related compounds. Thromb Res. 2008;122(6):743-52.
147. Princz MA, Sheardown H. Heparin-modified dendrimer crosslinked collagen matrices for the delivery of heparin-binding epidermal growth factor. J Biomed Mater Res A. 2012;100(8):1929-37.
148. Jin K, Li B, Lou L, Xu Y, Ye X, Yao K, et al. In vivo vascularization of MSC-loaded porous hydroxyapatite constructs coated with VEGF-functionalized collagen/heparin multilayers. Sci Rep. 2016;6:19871.
149. Sun B, Chen B, Zhao Y, Sun W, Chen K, Zhang J, et al. Crosslinking heparin to collagen scaffolds for the delivery of human platelet-derived growth factor. J Biomed Mater Res B Appl Biomater. 2009;91(1):366-72.
150. Johnson MR, Boerckel JD, Dupont KM, Cool SM, Guldberg RE. Integration of heparin in collagen delivery system for BMP-2 induced bone repair. 56th Annual Meeting of the Orthopedic Research Society2010.
151. Koo KH, Yeo DH, Ahn JM, Kim BS, Kim CS, Im GI. Lumbar posterolateral fusion using heparin-conjugated fibrin for sustained delivery of bone morphogenic protein-2 in a rabbit model. Artif Organs. 2012;36(7):629-34.
152. Janse van Rensburg A, Davies NH, Oosthuysen A, Chokoza C, Zilla P, Bezuidenhout D. Improved vascularization of porous scaffolds through growth factor delivery from heparinized polyethylene glycol hydrogels. Acta Biomater. 2017;49:89-100.
153. Sakiyama-Elbert SE. Drug Delivery via Heparin Conjugates. In: Grainger DW, Kirkpatrick CJ, editors. Comprehensive Biomaterials: Elsevier Inc.; 2011. p. 333-8.
154. Rema RB, Rajendran K, Ragunathan M. Angiogenic efficacy of Heparin on chick chorioallantoic membrane. Vasc Cell. 2012;4(1):8.
155. Fujita M. Heparin and angiogenic therapy. Eur Heart J. 2000;21(4):270-4.
156. Conte MS. Critical appraisal of surgical revascularization for critical limb ischemia. J Vasc Surg. 2013;57(2 Suppl):8S-13S.
157. Weintraub WS, Grau-Sepulveda MV, Weiss JM, O'Brien SM, Peterson ED, Kolm P, et al. Comparative effectiveness of revascularization strategies. N Engl J Med. 2012;366(16):1467-76.
158. Davies MG, Hagen PO. Pathophysiology of vein graft failure: a review. Eur J Vasc Endovasc Surg. 1995;9(1):7-18.
159. Taylor LM, Jr., Edwards JM, Brant B, Phinney ES, Porter JM. Autogenous reversed vein bypass for lower extremity ischemia in patients with absent or inadequate greater saphenous vein. Am J Surg. 1987;153(5):505-10.

160. Losi P, Briganti E, Vaghetti M, Soldani G. [Biomaterials and technologies for vascular grafts: from bench to bedside]. Recenti Prog Med. 2013;104(1):1-9.
161. Dorigo W, Pulli R, Piffaretti G, Castelli P, Griselli F, Dorrucci V, et al. Results from an Italian multicentric registry comparing heparin-bonded ePTFE graft and autologous saphenous vein in below-knee femoro-popliteal bypasses. J Cardiovasc Surg (Torino). 2012;53(2):187-94.
162. Rotmans JI, Heyligers JM, Stroes ES, Pasterkamp G. Endothelial progenitor cell-seeded grafts: rash and risky. Can J Cardiol. 2006;22(13):1113-6.
163. Welch M, Durrans D, Carr HM, Vohra R, Rooney OB, Walker MG. Endothelial cell seeding: a review. Ann Vasc Surg. 1992;6(5):473-84.
164. Griese DP, Ehsan A, Melo LG, Kong D, Zhang L, Mann MJ, et al. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. Circulation. 2003;108(21):2710-5.
165. Bordenave L, Fernandez P, Remy-Zolghadri M, Villars S, Daculsi R, Midy D. In vitro endothelialized ePTFE prostheses: clinical update 20 years after the first realization. Clin Hemorheol Microcirc. 2005;33(3):227-34.
166. Mano JF, Silva GA, Azevedo HS, Malafaya PB, Sousa RA, Silva SS, et al. Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. J R Soc Interface. 2007;4(17):999-1030.
167. Crombez M, Chevallier P, Gaudreault RC, Petitclerc E, Mantovani D, Laroche G. Improving arterial prosthesis neo-endothelialization: application of a proactive VEGF construct onto PTFE surfaces. Biomaterials. 2005;26(35):7402-9.
168. De Visscher G, Mesure L, Meuris B, Ivanova A, Flameng W. Improved endothelialization and reduced thrombosis by coating a synthetic vascular graft with fibronectin and stem cell homing factor SDF-1alpha. Acta Biomater. 2012;8(3):1330-8.
169. Rotmans JI, Heyligers JM, Verhagen HJ, Velema E, Nagtegaal MM, de Kleijn DP, et al. In vivo cell seeding with anti-CD34 antibodies successfully accelerates endothelialization but stimulates intimal hyperplasia in porcine arteriovenous expanded polytetrafluoroethylene grafts. Circulation. 2005;112(1):12-8.
170. Lu KV, Jong KA, Kim GY, Singh J, Dia EQ, Yoshimoto K, et al. Differential induction of glioblastoma migration and growth by two forms of pleiotrophin. J Biol Chem. 2005;280(29):26953-64.
171. Ruiz de Almodovar C, Luttun A, Carmeliet P. An SDF-1 trap for myeloid cells stimulates angiogenesis. Cell. 2006;124(1):18-21.
172. De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, et al. SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. Blood. 2004;104(12):3472-82.
173. Rajan N, Habermehl J, Cote MF, Doillon CJ, Mantovani D. Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications. Nat Protoc. 2006;1(6):2753-8.
174. Habermehl J, Skopinska J, Boccafoschi F, Sionkowska A, Kaczmarek H, Laroche G, et al. Preparation of ready-to-use, stockable and reconstituted collagen. Macromol Biosci. 2005;5(9):821-8.
175. Loy C, Pezzoli D, Candiani G, Mantovani D. A Cost-Effective Culture System for the In Vitro Assembly, Maturation, and Stimulation of Advanced Multilayered Multiculture Tubular Tissue Models. Biotechnol J. 2018;13(1).

176. Boccafoschi F, Habermehl J, Vesentini S, Mantovani D. Biological performances of collagen-based scaffolds for vascular tissue engineering. Biomaterials. 2005;26(35):7410-7.
177. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem. 2009;78:929-58.
178. Schweitzer MH, Suo Z, Avci R, Asara JM, Allen MA, Arce FT, et al. Analyses of soft tissue from Tyrannosaurus rex suggest the presence of protein. Science. 2007;316(5822):277-80.
179. Exposito JY, Cluzel C, Garrone R, Lethias C. Evolution of collagens. Anat Rec. 2002;268(3):302-16.
180. Exposito JY, Valcourt U, Cluzel C, Lethias C. The fibrillar collagen family. Int J Mol Sci. 2010;11(2):407-26.
181. Brazel D, Oberbaumer I, Dieringer H, Babel W, Glanville RW, Deutzmann R, et al. Completion of the amino acid sequence of the alpha 1 chain of human basement membrane collagen (type IV) reveals 21 non-triplet interruptions located within the collagenous domain. Eur J Biochem. 1987;168(3):529-36.
182. Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem. 2002;277(6):4223-31.
183. Bella J, Hulmes DJ. Fibrillar Collagens. Subcell Biochem. 2017;82:457-90.
184. Huzella T, Lengyel J. Orientation de la croissance des cultures de tissus sur la trame fibrillaire artificielle coagulée de la solution de "collagène A" (Nageotte) par les forces de la cristallisation. Compt rend Soc biol. 1932;109:515-8.
185. Ehrmann RL, Gey GO. The growth of cells on a transparent gel of reconstituted rat-tail collagen. J Natl Cancer Inst. 1956;16(6):1375-403.
186. Schmitt FO, Levine L, Drake MP, Rubin AL, Pfahl D, Davison PF. The Antigenicity of Tropocollagen. Proc Natl Acad Sci U S A. 1964;51:493-7.
187. Furthmayr H, Timpl R. Immunochemistry of collagens and procollagens. Int Rev Connect Tissue Res. 1976;7:61-99.
188. Gullberg D, Gehlsen KR, Turner DC, Ahlen K, Zijenah LS, Barnes MJ, et al. Analysis of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 integrins in cell--collagen interactions: identification of conformation dependent alpha 1 beta 1 binding sites in collagen type I. EMBO J. 1992;11(11):3865-73.
189. Smethurst PA, Onley DJ, Jarvis GE, O'Connor MN, Knight CG, Herr AB, et al. Structural basis for the platelet-collagen interaction: the smallest motif within collagen that recognizes and activates platelet Glycoprotein VI contains two glycine-proline-hydroxyproline triplets. J Biol Chem. 2007;282(2):1296-304.
190. Konitsiotis AD, Raynal N, Bihan D, Hohenester E, Farndale RW, Leitinger B. Characterization of high affinity binding motifs for the discoidin domain receptor DDR2 in collagen. J Biol Chem. 2008;283(11):6861-8.
191. Chiang TM, Postlethwaite AE, Beachey EH, Seyer JM, Kang AH. Binding of chemotactic collagen-derived peptides to fibroblasts. The relationship to fibroblast chemotaxis. J Clin Invest. 1978;62(5):916-22.
192. Postlethwaite AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. Proc Natl Acad Sci U S A. 1978;75(2):871-5.
193. Yannas IV, Burke JF, Orgill DP, Skrabut EM. Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. Science. 1982;215(4529):174-6.

194. Rodrigues CV, Serricella P, Linhares AB, Guerdes RM, Borojevic R, Rossi MA, et al. Characterization of a bovine collagen-hydroxyapatite composite scaffold for bone tissue engineering. Biomaterials. 2003;24(27):4987-97.
195. Chen F, Yoo JJ, Atala A. Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. Urology. 1999;54(3):407-10.
196. Chandrakasan G, Torchia DA, Piez KA. Preparation of intact monomeric collagen from rat tail tendon and skin and the structure of the nonhelical ends in solution. J Biol Chem. 1976;251(19):6062-7.
197. Exposito JY, Le Guellec D, Lu Q, Garrone R. Short chain collagens in sponges are encoded by a family of closely related genes. J Biol Chem. 1991;266(32):21923-8.
198. Sugiura H, Yunoki S, Kondo E, Ikoma T, Tanaka J, Yasuda K. In vivo biological responses and bioresorption of tilapia scale collagen as a potential biomaterial. J Biomater Sci Polym Ed. 2009;20(10):1353-68.
199. Johnson KA, Rogers GJ, Roe SC, Howlett CR, Clayton MK, Milthorpe BK, et al. Nitrous acid pretreatment of tendon xenografts cross-linked with glutaraldehyde and sterilized with gamma irradiation. Biomaterials. 1999;20(11):1003-15.
200. Wood A, Ogawa M, Portier RJ, Schexnayder M, Shirley M, Losso JN. Biochemical properties of alligator (Alligator mississippiensis) bone collagen. Comp Biochem Physiol B Biochem Mol Biol. 2008;151(3):246-9.
201. Chattopadhyay S, Raines RT. Review collagen-based biomaterials for wound healing. Biopolymers. 2014;101(8):821-33.
202. Abramo AC, Viola JC. Heterologous collagen matrix sponge: histologic and clinical response to its implantation in third-degree burn injuries. Br J Plast Surg. 1992;45(2):117-22.
203. Fleck CA, Simman R. Modern collagen wound dressings: function and purpose. J Am Col Certif Wound Spec. 2010;2(3):50-4.
204. Lee AR. Enhancing dermal matrix regeneration and biomechanical properties of 2nd degree-burn wounds by EGF-impregnated collagen sponge dressing. Arch Pharm Res. 2005;28(11):1311-6.
205. Sripriya R, Kumar MS, Sehgal PK. Improved collagen bilayer dressing for the controlled release of drugs. J Biomed Mater Res B Appl Biomater. 2004;70(2):389-96.
206. Matassi F, Nistri L, Chicon Paez D, Innocenti M. New biomaterials for bone regeneration. Clin Cases Miner Bone Metab. 2011;8(1):21-4.
207. Zhang D, Wu X, Chen J, Lin K. The development of collagen based composite scaffolds for bone regeneration. Bioact Mater. 2018;3(1):129-38.
208. Kon E, Delcogliano M, Filardo G, Busacca M, Di Martino A, Marcacci M. Novel nano-composite multilayered biomaterial for osteochondral regeneration: a pilot clinical trial. Am J Sports Med. 2011;39(6):1180-90.
209. Stephan EB, Renjen R, Lynch SE, Dziak R. Platelet-derived growth factor enhancement of a mineral-collagen bone substitute. J Periodontol. 2000;71(12):1887-92.
210. Patino MG, Neiders ME, Andreana S, Noble B, Cohen RE. Collagen as an implantable material in medicine and dentistry. J Oral Implantol. 2002;28(5):220-5.
211. Willoughby CE, Batterbury M, Kaye SB. Collagen corneal shields. Surv Ophthalmol. 2002;47(2):174-82.
212. Eshar D, Wyre NR, Schoster JV. Use of collagen shields for treatment of chronic bilateral corneal ulcers in a pet rabbit. J Small Anim Pract. 2011;52(7):380-3.

213. Delarive T, Rossier A, Rossier S, Ravinet E, Shaarawy T, Mermoud A. Aqueous dynamic and histological findings after deep sclerectomy with collagen implant in an animal model. Br J Ophthalmol. 2003;87(11):1340-4.
214. Liu Y, Gan L, Carlsson DJ, Fagerholm P, Lagali N, Watsky MA, et al. A simple, cross-linked collagen tissue substitute for corneal implantation. Invest Ophthalmol Vis Sci. 2006;47(5):1869-75.
215. Nicosia RF, Belser P, Bonanno E, Diven J. Regulation of angiogenesis in vitro by collagen metabolism. In Vitro Cell Dev Biol. 1991;27A(12):961-6.
216. Vernon RB, Lara SL, Drake CJ, Iruela-Arispe ML, Angello JC, Little CD, et al. Organized type I collagen influences endothelial patterns during "spontaneous angiogenesis in vitro": planar cultures as models of vascular development. In Vitro Cell Dev Biol Anim. 1995;31(2):120-31.
217. Abraham GA, Murray J, Billiar K, Sullivan SJ. Evaluation of the porcine intestinal collagen layer as a biomaterial. J Biomed Mater Res. 2000;51(3):442-52.
218. Shen YH, Shoichet MS, Radisic M. Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells. Acta Biomater. 2008;4(3):477-89.
219. Chan EC, Kuo SM, Kong AM, Morrison WA, Dusting GJ, Mitchell GM, et al. Three Dimensional Collagen Scaffold Promotes Intrinsic Vascularisation for Tissue Engineering Applications. PLoS One. 2016;11(2):e0149799.
220. Wahl EA, Schenck TL, Machens HG, Balmayor ER. VEGF released by deferoxamine preconditioned mesenchymal stem cells seeded on collagen-GAG substrates enhances neovascularization. Sci Rep. 2016;6:36879.
221. Cherubino M, Valdatta L, Balzaretti R, Pellegatta I, Rossi F, Protasoni M, et al. Human adipose-derived stem cells promote vascularization of collagen-based scaffolds transplanted into nude mice. Regen Med. 2016;11(3):261-71.
222. Schmidt VJ, Wietbrock JO, Leibig N, Gloe T, Henn D, Hernekamp JF, et al. Collagen-Elastin and Collagen-Glycosaminoglycan Scaffolds Promote Distinct Patterns of Matrix Maturation and Axial Vascularization in Arteriovenous Loop-Based Soft Tissue Flaps. Ann Plast Surg. 2017;79(1):92-100.
223. Bou-Gharios G, Ponticos M, Rajkumar V, Abraham D. Extra-cellular matrix in vascular networks. Cell Prolif. 2004;37(3):207-20.
224. Hirai J, Kanda K, Oka T, Matsuda T. Highly oriented, tubular hybrid vascular tissue for a low pressure circulatory system. ASAIO J. 1994;40(3):M383-8.
225. Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. Ann Biomed Eng. 2000;28(4):351-62.
226. Tranquillo RT, Girton TS, Bromberek BA, Triebes TG, Mooradian DL. Magnetically orientated tissue-equivalent tubes: application to a circumferentially orientated media-equivalent. Biomaterials. 1996;17(3):349-57.
227. Charulatha V, Rajaram A. Influence of different crosslinking treatments on the physical properties of collagen membranes. Biomaterials. 2003;24(5):759-67.
228. Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. Matrix Biol. 2003;22(4):339-50.
229. Stitzel J, Liu J, Lee SJ, Komura M, Berry J, Soker S, et al. Controlled fabrication of a biological vascular substitute. Biomaterials. 2006;27(7):1088-94.

230. Jeong SI, Kim SY, Cho SK, Chong MS, Kim KS, Kim H, et al. Tissue-engineered vascular grafts composed of marine collagen and PLGA fibers using pulsatile perfusion bioreactors. Biomaterials. 2007;28(6):1115-22.
231. He W, Yong T, Teo WE, Ma Z, Ramakrishna S. Fabrication and endothelialization of collagen-blended biodegradable polymer nanofibers: potential vascular graft for blood vessel tissue engineering. Tissue Eng. 2005;11(9-10):1574-88.
232. Cummings CL, Gawlitta D, Nerem RM, Stegemann JP. Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures. Biomaterials. 2004;25(17):3699-706.
233. Koens MJ, Faraj KA, Wismans RG, van der Vliet JA, Krasznai AG, Cuijpers VM, et al. Controlled fabrication of triple layered and molecularly defined collagen/elastin vascular grafts resembling the native blood vessel. Acta Biomater. 2010;6(12):4666-74.
234. Koens MJ, Krasznai AG, Hanssen AE, Hendriks T, Praster R, Daamen WF, et al. Vascular replacement using a layered elastin-collagen vascular graft in a porcine model: one week patency versus one month occlusion. Organogenesis. 2015;11(3):105-21.
235. Berglund JD, Nerem RM, Sambanis A. Incorporation of intact elastin scaffolds in tissue-engineered collagen-based vascular grafts. Tissue Eng. 2004;10(9-10):1526-35.
236. Yates SG, Barros D'Sa AA, Berger K, Fernandez LG, Wood SJ, Rittenhouse EA, et al. The preclotting of porous arterial prostheses. Ann Surg. 1978;188(5):611-22.
237. Humphries AW, Hawk WA, Cuthbertson AM. Arterial prosthesis of collagen-impregnated Dacron tulle. Surgery. 1961;50:947-54.
238. Scott SM, Gaddy LR, Sahmel R, Hoffman H. A collagen coated vascular prosthesis. J Cardiovasc Surg (Torino). 1987;28(5):498-504.
239. Reigel MM, Hollier LH, Pairolero PC, Hallett JW, Jr. Early experience with a new collagen-impregnated aortic graft. Am Surg. 1988;54(3):134-6.
240. Freischlag JA, Moore WS. Clinical experience with a collagen-impregnated knitted Dacron vascular graft. Ann Vasc Surg. 1990;4(5):449-54.
241. Prager MR, Hoblaj T, Nanobashvili J, Sporn E, Polterauer P, Wagner O, et al. Collagen- versus gelatine-coated Dacron versus stretch PTFE bifurcation grafts for aortoiliac occlusive disease: long-term results of a prospective, randomized multicenter trial. Surgery. 2003;134(1):80-5.
242. Jonas RA, Schoen FJ, Ziemer G, Britton L, Castaneda AR. Biological sealants and knitted Dacron conduits: comparison of collagen and fibrin glue pretreatments in circulatory models. Ann Thorac Surg. 1987;44(3):283-90.
243. Suehiro K, Hata T, Yoshitaka H, Tsushima Y, Matsumoto M, Ohtani S, et al. Impact of collagen-coated and gelatine-impregnated woven Dacron branched grafts on the early postoperative period. Jpn J Thorac Cardiovasc Surg. 2003;51(12):641-5.
244. Kobayashi J, Backer CL, Zales VR, Crawford SE, Muster AJ, Mavroudis C. Failure of the Hemashield extension in right ventricle-to-pulmonary artery conduits. Ann Thorac Surg. 1993;56(2):277-81.
245. Guidoin R, Marois Y, Deng X, Chakfe N, Marois M, Roy R, et al. Can collagen impregnated polyester arterial prostheses be recommended as small diameter blood conduits? ASAIO J. 1996;42(6):974-83.
246. Friess W. Collagen--biomaterial for drug delivery. Eur J Pharm Biopharm. 1998;45(2):113-36.

247. Steffens GC, Yao C, Prevel P, Markowicz M, Schenck P, Noah EM, et al. Modulation of angiogenic potential of collagen matrices by covalent incorporation of heparin and loading with vascular endothelial growth factor. Tissue Eng. 2004;10(9-10):1502-9.
248. He Q, Zhao Y, Chen B, Xiao Z, Zhang J, Chen L, et al. Improved cellularization and angiogenesis using collagen scaffolds chemically conjugated with vascular endothelial growth factor. Acta Biomater. 2011;7(3):1084-93.
249. Laiva AL, Raftery RM, Keogh MB, O'Brien FJ. Pro-angiogenic impact of SDF-1alpha gene-activated collagen-based scaffolds in stem cell driven angiogenesis. Int J Pharm. 2018;544(2):372-9.
250. Hao W, Han J, Chu Y, Huang L, Zhuang Y, Sun J, et al. Collagen/Heparin Bi-Affinity Multilayer Modified Collagen Scaffolds for Controlled bFGF Release to Improve Angiogenesis In Vivo. Macromol Biosci. 2018;18(11):e1800086.
251. Wissink MJ, Beernink R, Poot AA, Engbers GH, Beugeling T, van Aken WG, et al. Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices. J Control Release. 2000;64(1-3):103-14.
252. Yeager RA, Taylor LM, Jr., Moneta GL, Edwards JM, Nicoloff AD, McConnell DB, et al. Improved results with conventional management of infrarenal aortic infection. J Vasc Surg. 1999;30(1):76-83.
253. Chervu A, Moore WS, Chvapil M, Henderson T. Efficacy and duration of antistaphylococcal activity comparing three antibiotics bonded to Dacron vascular grafts with a collagen release system. J Vasc Surg. 1991;13(6):897-901.
254. Batt M, Magne JL, Alric P, Muzj A, Ruotolo C, Ljungstrom KG, et al. In situ revascularization with silver-coated polyester grafts to treat aortic infection: early and midterm results. J Vasc Surg. 2003;38(5):983-9.
255. Salacinski HJ, Goldner S, Giudiceandrea A, Hamilton G, Seifalian AM, Edwards A, et al. The mechanical behavior of vascular grafts: a review. J Biomater Appl. 2001;15(3):241-78.
256. Adipurnama I, Yang MC, Ciach T, Butruk-Raszeja B. Surface modification and endothelialization of polyurethane for vascular tissue engineering applications: a review. Biomater Sci. 2016;5(1):22-37.
257. Avci-Adali M, Perle N, Ziemer G, Wendel HP. Current concepts and new developments for autologous in vivo endothelialisation of biomaterials for intravascular applications. Eur Cell Mater. 2011;21:157-76.
258. Liu KK, Dorovini-Zis K. Regulation of CXCL12 and CXCR4 expression by human brain endothelial cells and their role in CD4+ and CD8+ T cell adhesion and transendothelial migration. J Neuroimmunol. 2009;215(1-2):49-64.
259. Wu CC, Chen YC, Chang YC, Wang LW, Lin YC, Chiang YL, et al. Human umbilical vein endothelial cells protect against hypoxic-ischemic damage in neonatal brain via stromal cell-derived factor 1/C-X-C chemokine receptor type 4. Stroke. 2013;44(5):1402-9.
260. Zhou J, Ye X, Wang Z, Liu J, Zhang B, Qiu J, et al. Development of decellularized aortic valvular conduit coated by heparin-SDF-1alpha multilayer. Ann Thorac Surg. 2015;99(2):612-8.
261. Zhang L, Zhu J, Du R, Zhu Z, Zhang J, Han W, et al. Effect of recombinant human SDF-1a on re-endothelialization after sirolimus-eluting stent implantation in rabbit aorta abdominalis. Life Sci. 2011;89(25-26):926-30.

262. Gao Y, Lu Z, Chen C, Cui X, Liu Y, Zheng T, et al. Mesenchymal stem cells and endothelial progenitor cells accelerate intra-aneurysmal tissue organization after treatment with SDF-1alpha-coated coils. Neurol Res. 2016;38(4):333-41.
263. Yu J, Wang A, Tang Z, Henry J, Li-Ping Lee B, Zhu Y, et al. The effect of stromal cell-derived factor-1alpha/heparin coating of biodegradable vascular grafts on the recruitment of both endothelial and smooth muscle progenitor cells for accelerated regeneration. Biomaterials. 2012;33(32):8062-74.
264. Krieger JR, Ogle ME, McFaline-Figueroa J, Segar CE, Temenoff JS, Botchwey EA. Spatially localized recruitment of anti-inflammatory monocytes by SDF-1alpha-releasing hydrogels enhances microvascular network remodeling. Biomaterials. 2016;77:280-90.
265. Rojas-Mayorquín A.E. O-SD. Pleiotrophin. In: Choi S, editor. Encyclopedia of Signaling Molecules 2nd edition: SPRINGER; 2017.
266. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci U S A. 1983;80(12):3734-7.
267. Mirshahi F, Pourtau J, Li H, Muraine M, Trochon V, Legrand E, et al. SDF-1 activity on microvascular endothelial cells: consequences on angiogenesis in in vitro and in vivo models. Thromb Res. 2000;99(6):587-94.
268. Kuhlmann CR, Schaefer CA, Reinhold L, Tillmanns H, Erdogan A. Signalling mechanisms of SDF-induced endothelial cell proliferation and migration. Biochem Biophys Res Commun. 2005;335(4):1107-14.
269. Naito Y, Shinoka T, Duncan D, Hibino N, Solomon D, Cleary M, et al. Vascular tissue engineering: towards the next generation vascular grafts. Adv Drug Deliv Rev. 2011;63(4-5):312-23.
270. Roy H, Bhardwaj S, Yla-Herttuala S. Biology of vascular endothelial growth factors. FEBS Lett. 2006;580(12):2879-87.
271. Aranda E, Owen GI. A semi-quantitative assay to screen for angiogenic compounds and compounds with angiogenic potential using the EA.hy926 endothelial cell line. Biol Res. 2009;42(3):377-89.
272. Bhowmick T, Berk E, Cui X, Muzykantov VR, Muro S. Effect of flow on endothelial endocytosis of nanocarriers targeted to ICAM-1. J Control Release. 2012;157(3):485-92.
273. Walter MN, Kohli N, Khan N, Major T, Fuller H, Wright KT, et al. Human mesenchymal stem cells stimulate EaHy926 endothelial cell migration: combined proteomic and in vitro analysis of the influence of donor-donor variability. J Stem Cells Regen Med. 2015;11(1):18-24.
274. Arutyunyan IV, Fatkhudinov TH, El'chaninov AV, Makarov AV, Kananykhina EY, Usman NY, et al. Effect of Endothelial Cells on Angiogenic Properties of Multipotent Stromal Cells from the Umbilical Cord during Angiogenesis Modeling in the Basement Membrane Matrix. Bull Exp Biol Med. 2016;160(4):575-82.
275. Brewster LP, Brey EM, Tassiopoulos AK, Xue L, Maddox E, Armistead D, et al. Heparin-independent mitogenicity in an endothelial and smooth muscle cell chimeric growth factor (S130K-HBGAM). Am J Surg. 2004;188(5):575-9.
276. Brewster L, Brey EM, Addis M, Xue L, Husak V, Ellinger J, et al. Improving endothelial healing with novel chimeric mitogens. Am J Surg. 2006;192(5):589-93.
277. Rojas-Mayorquín AE, Ortuño-Sahagún D. Pleiotrophin. In: Choi S, editor. Encyclopedia of Signaling Molecules 2nd edition: SPRINGER; 2017.

278. Gonzalez-Castillo C, Ortuno-Sahagun D, Guzman-Brambila C, Pallas M, Rojas-Mayorquin AE. Pleiotrophin as a central nervous system neuromodulator, evidences from the hippocampus. Front Cell Neurosci. 2014;8:443.
279. Perez-Pinera P, Zhang W, Chang Y, Vega JA, Deuel TF. Anaplastic lymphoma kinase is activated through the pleiotrophin/receptor protein-tyrosine phosphatase beta/zeta signaling pathway: an alternative mechanism of receptor tyrosine kinase activation. J Biol Chem. 2007;282(39):28683-90.
280. Martinez-Gonzalez B, Reyes-Hernandez CG, Quiroga-Garza A, Rodriguez-Rodriguez VE, Esparza-Hernandez CN, Elizondo-Omana RE, et al. Conduits Used in Coronary Artery Bypass Grafting: A Review of Morphological Studies. Ann Thorac Cardiovasc Surg. 2017;23(2):55-65.
281. Bush HLJ. Mechanism of Graft Failure. J Vasc Surg. 1989;9(2):392-4.
282. Greisler HP. Growth factor release from vascular grafts. J Control Release. 1996;39(2-3):267-80.
283. Copes F, Ramella M, Fusaro L, Mantovani D, Cannas M, Boccafoschi F. Pleiotrophin: analysis of the endothelialisation potential. Adv Med Sci. (In Press). 2019;65(1).
284. Loy C, Meghezi S, Levesque L, Pezzoli D, Kumra H, Reinhardt D, et al. A planar model of the vessel wall from cellularized-collagen scaffolds: focus on cell-matrix interactions in mono-, bi- and tri-culture models. Biomater Sci. 2016;5(1):153-62.
285. Montano-Machado V, Chevallier P, Mantovani D, Pauthe E. On the potential for fibronectin/phosphorylcholine coatings on PTFE substrates to jointly modulate endothelial cell adhesion and hemocompatibility properties. Biomatter. 2015;5:e979679.
286. Obrink B. The influence of glycosaminoglycans on the formation of fibers from monomeric tropocollagen in vitro. Eur J Biochem. 1973;34(1):129-37.
287. Guidry C, Grinnell F. Heparin modulates the organization of hydrated collagen gels and inhibits gel contraction by fibroblasts. J Cell Biol. 1987;104(4):1097-103.
288. Salchert K, Streller U, Pompe T, Herold N, Grimmer M, Werner C. In vitro reconstitution of fibrillar collagen type I assemblies at reactive polymer surfaces. Biomacromolecules. 2004;5(4):1340-50.
289. Lee KW, Yoon JJ, Lee JH, Kim SY, Jung HJ, Kim SJ, et al. Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. Transplant Proc. 2004;36(8):2464-5.
290. Yang HS, La WG, Cho YM, Shin W, Yeo GD, Kim BS. Comparison between heparin-conjugated fibrin and collagen sponge as bone morphogenetic protein-2 carriers for bone regeneration. Exp Mol Med. 2012;44(5):350-5.
291. Lee H, Chung HJ, Park TG. Perspectives On: Local and Sustained Delivery of Angiogenic Growth Factors. J Bioact Compat Polym. 2007;22(89):25.
292. Nissen NN, Shankar R, Gamelli RL, Singh A, DiPietro LA. Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. Biochem J. 1999;338 (Pt 3):637-42.
293. Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. Mol Cell Biol. 1992;12(1):240-7.
294. Lampropoulou E, Logoviti I, Koutsioumpa M, Hatziapostolou M, Polytarchou C, Skandalis SS, et al. Cyclin-dependent kinase 5 mediates pleiotrophin-induced endothelial cell migration. Sci Rep. 2018;8(1):5893.

295. Castellot JJ, Jr., Cochran DL, Karnovsky MJ. Effect of heparin on vascular smooth muscle cells. I. Cell metabolism. J Cell Physiol. 1985;124(1):21-8.
296. Stewart EM, Liu X, Clark GM, Kapsa RM, Wallace GG. Inhibition of smooth muscle cell adhesion and proliferation on heparin-doped polypyrrole. Acta Biomater. 2012;8(1):194-200.
297. Gilotti AC, Nimlamool W, Pugh R, Slee JB, Barthol TC, Miller EA, et al. Heparin responses in vascular smooth muscle cells involve cGMP-dependent protein kinase (PKG). J Cell Physiol. 2014;229(12):2142-52.
298. Gray E, Hogwood J, Mulloy B. The anticoagulant and antithrombotic mechanisms of heparin. Handb Exp Pharmacol. 2012(207):43-61.
299. Liang Y, Kiick KL. Heparin-functionalized polymeric biomaterials in tissue engineering and drug delivery applications. Acta Biomater. 2014;10(4):1588-600.
300. Keuren JF, Wielders SJ, Driessen A, Verhoeven M, Hendriks M, Lindhout T. Covalently-bound heparin makes collagen thromboresistant. Arterioscler Thromb Vasc Biol. 2004;24(3):613-7.
301. Ori A, Free P, Courty J, Wilkinson MC, Fernig DG. Identification of heparin-binding sites in proteins by selective labeling. Mol Cell Proteomics. 2009;8(10):2256-65.

Annexes

A.1 Pleiotrophin dose/response curve on EA.hy926 cells

The response of the EA.hy926 cells to different concentrations of pleiotrophin (PTN) was analyzed using the MTT Assay. EA.hy926 cells were seeded at a density of 25000 cell/cm^2 in 96 well culture plates (5 wells for each condition). After an overnight incubation at 37°C in a saturated atmosphere at 5% CO_2, cells were treated respectively with: 1) C-D-MEM (D-MEM with 10% FBS, 100U/ml penicillin, 100U/ml streptomycin and 2mM L-glutamine) (CTRL); 2) C-D-MEM enriched with 10ng/ml PTN; 3) C-D-MEM enriched with 50ng/ml PTN and 4) C-D-MEM enriched with 100ng/ml PTN. After 24 hours, 3 and 7 days, cells were incubated with the MTT reagent for 3 hours at 37°C. After the incubation, the formazan product obtained by the reduction of MTT reagent by the way of the mitochondrial activity was solubilized using dimethyl sulfoxide. Than the absorbance at 570 nm was measured with a SpectraCount Absorbance microplate reader (Packard, Connecticut, USA). Absorbance is proportional to cell viability.

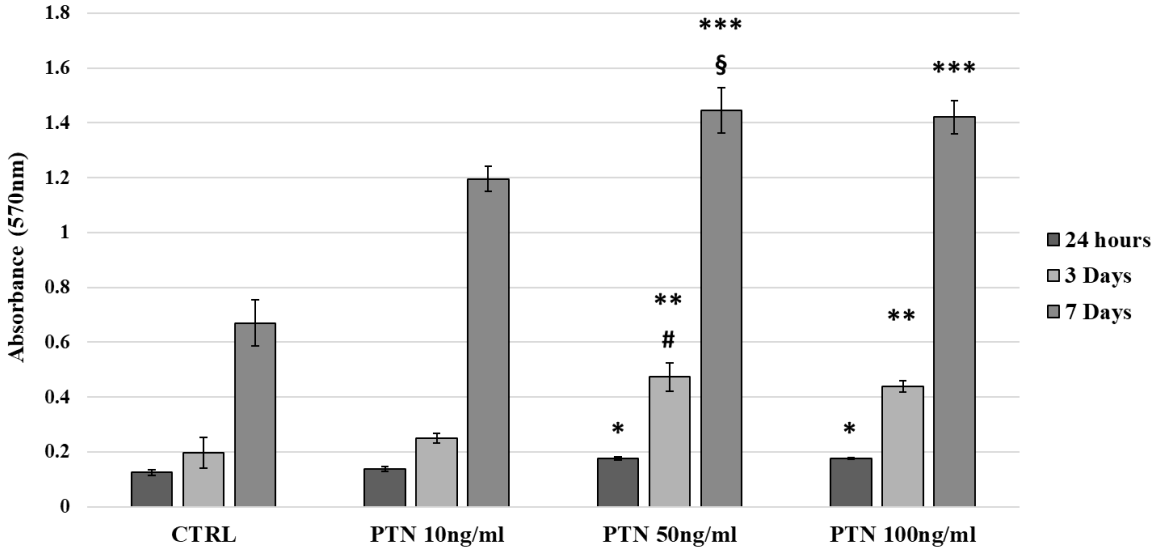

Figure A.1*: Pleiotrophin dose/response curve on EA.hy926 cells.* Ea.hy926 cells were treated up to seven days with: C-D-MEM (CTRL); C-D-MEM enriched with 10 ng/ml PTN (PTN 10ng/ml); C-D-MEM enriched with 50 ng/ml PTN (PTN 50ng/ml), C-D-MEM enriched with 100 ng/ml PTN (PTN 100ng/ml). Cell viability was measured after 24 hours, 3 and 7 days with MTT Assay. The graphic shows the mean absorbance recorded for each condition. * $p<0.001$ vs. 24 hours CTRL and $p<0.05$ vs 24 hours PTN 10nh/ml; ** $p<0.001$ vs. 3 days CTRL; # $p<0.01$ vs. 3 days PTN 10ng/ml; ***$p<0.001$ vs. 7 days CTRL; § $p<0.05$ vs. PTN 10ng/ml.

A.2 Heparin-modified collagen gels pH analysis

The pH of the different gel concentration was measured in order to assess if a possible difference in the pH values was responsible for the non gelification of the collagen gels. Briefly, type I collagen was extracted from rat tails tendons and subsequently solubilized in 0.02 N acetic acid to obtain a final collagen concentration of 4 g/L. For the preparation of the

collagen gel, the collagen solution has been mixed with a buffer solution containing Dulbecco's modified Eagle medium (DMEM), NaOH (15 mM), and HEPES (20 mM) in deionized water to adjust the pH of the final solution and to initiate the polymerization process. M199 culture medium was then added to complete the basic composition of the control collagen gel (CTRL Gel). For the heparin-modified collagen gels, heparin sodium salt has been added to the M199 portion of the collagen gel mix to obtain the final concentrations of 0.1 0.25 and 0.5 mg/ml (H 0.1, H 0.25 and H 0.5 Gels). After mixing, the pH value of each solution was measured using an Accumet® pH meter 25 (Fisher Scientific, Fair Lawn, NJ, USA). Readings were performed in triplicate on three different experiments.

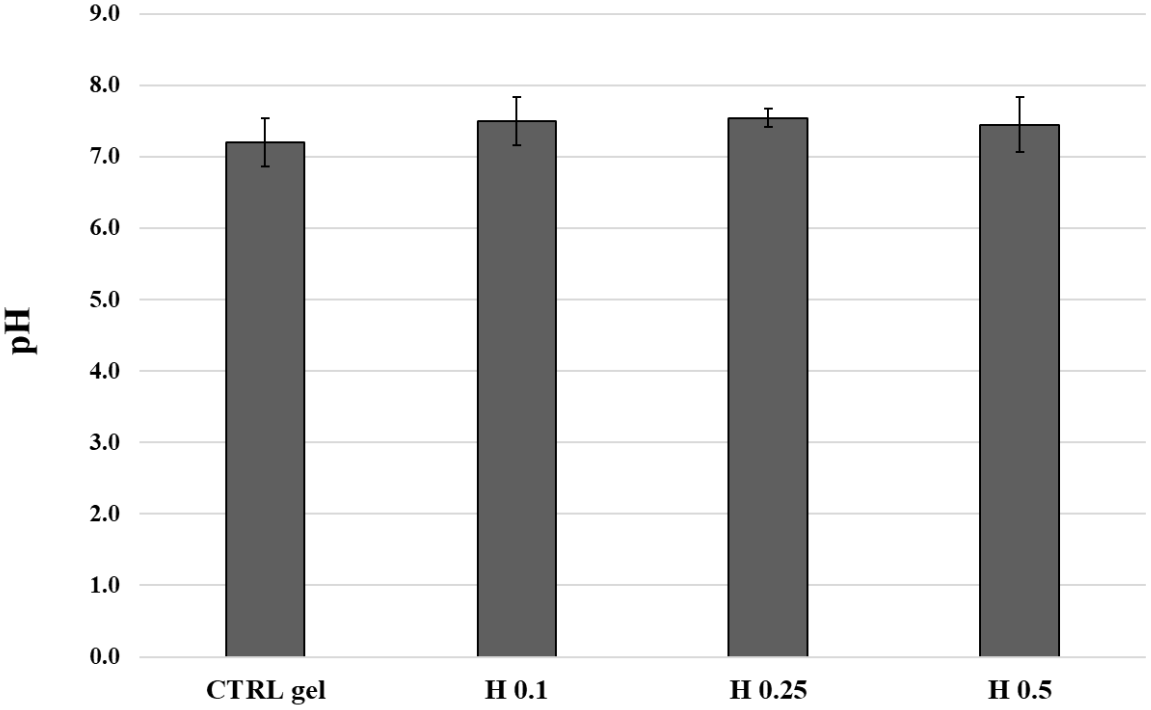

Figure A.2*: pH measurements.* The graphic shows the results of the pH measurements performed on the different collagen gel preparations: control collagen gel (CTRL gel); CTRL gel containing 0.1mg/ml heparin (H 0.1); CTRL gel containing 0.25mg/ml heparin (H 0.25); CTRL gel containing 0.5mg/ml heparin (H 0.5).

As shown in **Figure A.2**, no significant differences were noted among the different conditions.

A.3 Pleiotrophin dose/response curve on HUVECs

The response of HUVECs to different concentrations of PTN was analyzed using a resazurin-based viability assay. Briefly, HUVECs cells were seeded at a density of 20000 cells/cm^2 in 96 well culture plates (5 wells for each condition). After an overnight incubation at 37°C in a saturated atmosphere at 5% CO_2, cells were treated respectively with: 1) HUVEC-M199 (M199 culture medium with 5% FBS, 1100U/ml penicillin, 100U/ml streptomycin, 2ng/ml FGF, 1 ng/ml EGF, 1 µg/ml ascorbic acid and 1 µg/ml hydrocortisone) (CTRL); 2) HUVEC-M199 enriched with 50ng/ml PTN; 3) HUVEC-M199 enriched with 100ng/ml PTN; 4) HUVEC-M199 enriched with 150ng/ml PTN; 5) HUVEC-M199 enriched with 250ng/ml PTN and 6) HUVEC-M199 enriched with 500ng/ml PTN. After 24 hours, 3 and 7 days, cells have been incubated with a resazurin salt solution for 4 hours. After the incubation, the highly

fluorescent resorufin product obtained by the reduction of the resazurin was collected and fluorescence intensity at a 545 nm_{ex}/590 nm_{em} wavelength was measured with a SpectraMax i3x Multi-Mode Plate Reader (Molecular Devices, San Jose, California, USA). Fluorescence intensity is proportional to cell viability.

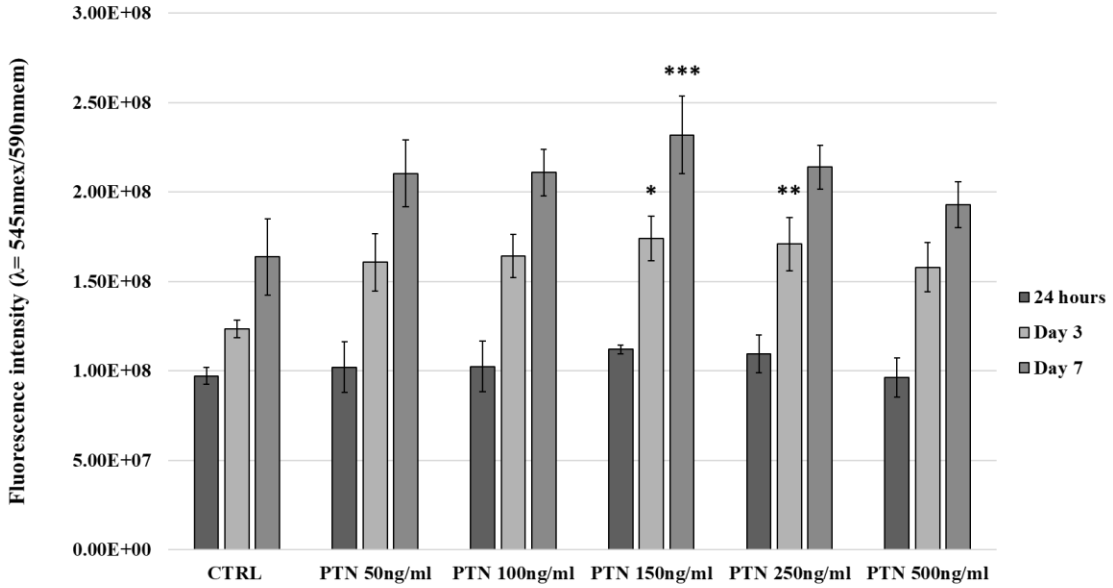

Figure A.1*: Pleiotrophin dose/response curve on HUVECs.* Cells were treated up to seven days with HUVEC-M199 (CTRL); HUVEC-M199 enriched with 50 ng/ml PTN (PTN 50ng/ml); HUVEC-M199 enriched with 100 ng/ml PTN (PTN 100ng/ml), HUVEC-M199 enriched with 150 ng/ml PTN (PTN 150ng/ml), HUVEC-M199 enriched with 250 ng/ml PTN (PTN 250ng/ml), HUVEC-M199 enriched with 500 ng/ml PTN (PTN 500ng/ml). Cell viability was measured after 24 hours, 3 and 7 days with resazurin salt viability assay. The graphic shows the mean fluorescence recorded for each condition. * $p<0.01$ vs. 3 Days CTRL; ** $p<0.05$ vs. 3 Days CTRL; ***$p<0.01$ vs. 7 Days CTRL.