SAPIENZA

UNIVERSITA' DI ROMA

DOTTORATO DI RICERCA IN MEDICINA SPERIMENTALE

XXXII CICLO

"Angiogenic role of extracellular vesicles in human platelet lysate"

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1.1 Regenerative angiogenesis

Angiogenesis, as a complex set of processes, is the result of a balance between stimulating and inhibiting factors, that gives rise to the new formation of capillaries and blood vessels. The growth of normal, stable and functional vasculature is the outcome of the interplay between migration, growth and differentiation of endothelial cells lining the inner walls of blood vessels and angiogenic cytokines and growth factors.[1] The role of the secretome and paracrine effect seems essential for understanding the neoangiogenesis process and are indeed an attractive target for clinical applications[2].

Phases of angiogenesis

Angiogenesis is a stepwise process (Figure 1), activated in response to specific stimuli which can be: low perfusion of a tissue after lesions, inflammation or hypoxia. When these are triggered, the active Endothelial Cells (ECs) release pro- and anti-angiogenic factors like vascular endothelial growth factors (VEGF) and cytokines [3].Consequently, there is a destabilization of pre-existing vessels, leading to an increase in vessel permeability, loss of connections between EC and support cells (pericytes) with the formation of fenestrations, destabilization of the vessel wall and degradation of the basement membrane due to the action of proteolytic enzymes (such as matrix metalloproteases (MMPs) and activators of plasminogen). Fibrin deposition are also formed in the extravasal space. [3]

The proteolytic factors secreted by the EC to degrade the Extra Cellular Matrix (ECM) facilitate the migration of the ECs [100]. The migratory properties of ECs are determined by cellular bonds to matrix molecules and intracellular component responsible for cytoskeletal modulation. Following

the proteolytic degradation of the ECM and migration through the damaged matrix, the ECs are induced to proliferate thanks to the stimuli received by many endogenous angiogenic factors as well as growth factors, such as those belonging to the family of VEGF, mediators of inflammation, enzymes, hormones, cell adhesion molecules e hematopoietic factors [4]. Once the site that needs the formation of new vessels is reached, the ECs stop the mitogenic events in favor of morphological-differentiation, aiming at primitive capillary structures formation (tubulogenesis). In particular, the formation of the new capillary requires the synthesis of the basement membrane with consequent temporary local inhibition of the extracellular proteolysis process to allow the storage and assembly of the components of the new ECM. The vascular genesis is, therefore, the result of continuous cycles of activation and inhibition of extracellular proteases[5]. The processes just described, make the ECs reorganize themselves to form a solid cordon that later develops a lumen.

In order to obtain a functional vascular network the newly formed vessels must be reshaped to form mature vessels, a process known as stabilization. The release of the PDGF factor (Platelet-Derived-Growth Factor) is fundamental for their maturation which is also stimulated by the recruitment of pericytes and of smooth muscle cells that stabilize the morphology of the vessels and prevent the regression of Ecs [6]. Finally, when the neovascularization process reaches completion, a down-regulation of angiogenic factors or a local increase in the concentration of anti-angiogenic factors occurs.

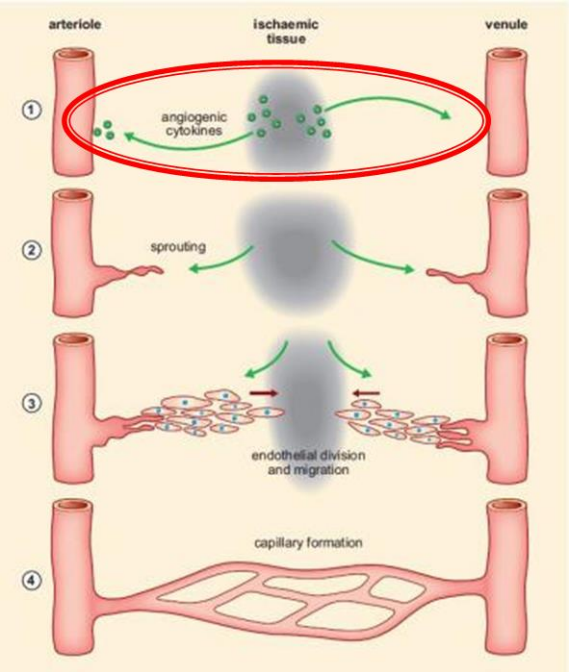

Figure 1 ***Phases of angiogenesis***

Ischaemic regions of a tissue release angiogenic cytokines, including VEGF (vascular endothelial cell growth factor). The cytokines and locally released enzymes cause the breakdown of the vessel walls of arterioles and venules, and sprouting of cells, including pericytes in the vessel wall. Endothelial cells proliferate and migrate out of the vessel into the tissue. They reorganize to form capillaries which interconnect and link to venules, thereby forming a new capillary network

1.1.1 The fine balance between physiological and pathological angiogenesis

Angiogenesis is a complex process involving a variety of cellular subtypes, including endothelial cells, smooth muscle cell precursor, myofibroblast and mesenchymal progenitors. The fine regulation of this process involves a

combination of cell-cell interaction, paracrine-endocrine factors, and mechanosensing. Already in 1794, the Scottish surgeon John Hunter hypothesized for the first time the proportional relationship between the vascular system and the metabolic request both in conditions of well-being and disease. Even though he was not using the specific term "angiogenesis", he has been a precursor of physiological and pathological angiogenesis concept. Indeed angiogenesis is a fundamental physiological process starting from fetal development and continuing actively for all adult life involved in all tissues growth processes[7]. Mechanical signals are at the basis of normal physiological angiogenesis. This can be observed for example with increased muscle activity, where angiogenesis is driven by increased friction on the inside of blood vessels and stretch of vessels caused by the surrounding muscle fibers[8]. Another example is embryonic development where angiogenesis is primarily responsible for fetus maturation. Indeed, during fetal development, the blood vessels are formed *de novo* starting from endothelial precursors (angioblasts) that assemble to form a first primitive vascular network. The newly formed capillaries then begin to differentiate, and new blood vessels are generated starting from the pre-existing ones. Another physiological angiogenetic process in the adult is the establishment and maintainance of the normal structure and function of corpus luteum development. Moreover it was shown how phisical exercise enhances cerebral vascular endothelial growth factor A secretion (VEGFA) and cerebral angiogenesis[9] .

The other side of angiogenesis is instead characterized by the onset of a pathological state where angiogenic regeneration is not sufficient or non-functional to restore normal blood supply or even becomes harmful to the

body. It is important to emphasize that blood vessel density and the degree of angiogenic growth are quantified histologically by enumeration of capillaries in a cross-section of tissue, which increases in the angiogenesis process. Besides, another important functional parameter is the perfusion to the tissue involved, and it is typically expected that these parameters (vascular density and perfusion) change in parallel. However, this is not always the case because an injured tissue may demonstrate both an increase in capillary density and an increase in blood flow but still have under perfused areas. Thus, it is important to consider both the quality of a neovascular network, which depends on its complex 4-dimensional architecture (3 physical dimensions and time), and the functionality of new network works to deliver oxygen to the tissue[10] .The most studied and "dark" side of angiogenesis was already known with the work of Judah Folkman, in1971, who hypothesized the relationship between tumor growth and angiogenesis. This statement opened the doors to numerous modern studies on different cancer types that increase their aggressiveness in relation to the contribution of the capillary network that is established around the neoplastic area [11-12]. If cancer can be described as a pathology where angiogenesis is unhealthily abundant, other diseases related to reduced angiogenesis are recognized in the literature. For example, peripheral artery disease, ischemic stroke, and cardiovascular disease are marked out by a compromised tissue blood flow and reduction of blood supply with limited oxygen transfer and nutrient uptake. In particular Peripheral Arterial Disease (PAD) is a common manifestation of atherosclerosis reducing the arterial flow to both upper and lower extremities, most frequently affecting the lower limbs and the patient frequently experiences limb loss causing impairment in quality of life.

Angiogenesis disease, like an ischemic stroke, occurs when a vessel supplying blood to the brain is obstructed and this causes the death of brain cells with unfortunate consequences. Cardiovascular disease refers to conditions that involve narrowed or blocked blood vessels that can lead to a heart attack, chest pain (angina) or stroke. As we know the muscle, and in particular the myocardium, is a sprayed and rich vessel tissue (made up of 70% muscle fibers, while the remaining 30% is mainly composed of connective tissue and vessels) which allows its complete functionality. When blood supply is blocked due to ischemia, the heart tissue is characterized by a hypoxic situation that makes it fibrotic and non-functional and in extremis put the heart out of order.

The cross-talk between myocardial metabolism and coronary blood flow (CBF) are involved in the pathophysiology of Ischemic heart disease IHD. The exposition to the different cardiovascular risk factors (including smoking, hypertension, hyperlipidemia, renal disease, metabolic syndrome, and diabetes mellitus) and the ischemic condition determine oxidative stress, characterized by an imbalance of oxidant accumulation and antioxidant deficiency. In particular, oxidative stress, through the action on calcium channels, is able to alter the blood flow causing a predisposition to myocardial ischemia [13]. It should be remembered that the above-mentioned disorders related to angiogenesis have in common the etiopathogenesis like traditional risk factors[14] .

1.1.1.2 Oxidative stress and vascular system

Oxidative stress is defined as a condition of oxidant molecule cellular excess compared to antioxidant ones[15]. The presence of oxidants is normally

neutralized by the presence of antioxidant cellular systems which include both enzymatic molecules, such as superoxide dismutase (SOD) and catalase, and nonenzymatic molecules, such as all trans-retinol 2 and ascorbic acid[15] . The activity of these systems and the regulation of redox cell state are crucial for cell function and survival. When produced in low quantity, ROS are involved in several physiological mechanisms regarding the cardiovascular system[15-16]. They stimulate angiogenesis via the vascular endothelial growth factor (VEGF) pathway, and are involved in endothelial cell regeneration, proliferation, and migration. H2O2 is crucial for post-ischemic neovascularization, and they are also involved in the regulation of coronary endothelial-dependent and independent vasodilatation[17]. In pathological conditions, the damage and/or the overload of antioxidant systems make them unable to contrast the production of oxidants. Reactive oxygen species (ROS) play a central role as mediators of oxidative stress and its complications. This term defines several agents among which are both oxygen radicals as hydroxyl (OH-), superoxide (O2--), and peroxile (RO2-) and several nonradical oxygen species as hydrogen peroxide (H2O2). Metabolic cellular activity and environmental factors, such as wrong diet and smoke, contribute to ROS production and therefore oxidative stress which may predispose to several pathological conditions as angiogenic diseases [15-16]. ROS together with inflammatory response and hyperglycaemia play a central role in the initiation and progression of vascular damage, supporting the atherosclerotic process and microvascular dysfunction[18-19] . These mechanisms are the basis of chronic kidney injury, myocardial ischemia, and retinopathy[20], ROS contribute to atherosclerosis, also inducing the worsening of endothelial dysfunction, increasing the expression of adhesion molecules

like intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), and modulating the expression of different growth factors important in the proliferation of vascular smooth muscle cells (VSMCs)[21].

The main source of intravascular ROS is NADPH oxidase (NOX). There are 4 isoforms of this enzyme; NOX1, NOX2, NOX4, and NOX5, which are overexpressed and play a crucial role in the angiogenic process. NOX1 is expressed by endothelial cells. A decreased expression of NOX1 is associated with reduced leucocyte vascular wall adhesion and macrophage recruitment [19]. NOX4 is expressed by endothelial and muscle cells, and it has a protective role for the wall vessel. Also NOX4 is a target for enhancing their reparative function through therapeutic priming to support creation of a pro-reparative microenvironment and effective postischaemic revascularisation[22].The reduction in its expression supports the increase in inflammatory markers production such as IL-1 and MCP-1 and the progression of atherosclerosis[19,23]. Moreover, the reduced expression of NOX4 on smooth muscle cells associates with reduced contractile gene expression and higher production and deposition of collagen[19-24]. NOX5 may alter endothelial nitric oxide synthase (eNOS) activity contributing to endothelial dysfunction[25]. Inside the cell, the most important site of free radical production is mitochondria because they represent the energetic central point of the cell[13].

1.1.2 Current therapies promoting angiogenesis

In the field of angiogenic regeneration, we can identify three different therapies: pharmacological and surgical therapy, cell therapy and cell-free

therapy. In the timeline, the first therapies to populate the world of regenerative medicine are based on drugs. Physicians usually treat heart failure with a combination of medications depending on symptoms. Some of the most commonly used pharmacological therapies are: vasodilator like angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARB), that widens blood vessels, improve blood flow and decrease the workload on the heart; beta-blockers, are competitive antagonists that block the receptor sites for the endogenous catecholamines, adrenaline, and noradrenaline, on adrenergic beta receptors, of the sympathetic nervous system, slowing the heart rate, reducing blood pressure and limiting some of the damage to heart; diuretics treatment to reduce systemic and pulmonary congestion[26]. Although these drugs reduce mortality, they fail to address the underlying loss of cardiomyocytes and vasculature and are intrinsically non-curative. Another negative aspect of the drugs is that they do not suffice when a situation of ischemia is too extensive. The surgical therapies come into play in this arduous situation, characterized by wide ischemia. There are many revascularization techniques like vessel reperfusion, percutaneous coronary intervention (PCI), artery bypass grafting (ABG) or, in extremis, organ transplantation, as is still the case for heart transplantation. Though in extreme cases the surgical technique is the only possible solution and it improved patient survival and quality of life, the clinical complications as anesthesia, intra-cardiac thrombus[27] and organ rejection may occur during a regenerative angiogenesis operation. In addition, surgical therapies cannot stop or reverse the angiogenic deficit and transplant organ shortage, high costs and complex postoperative management limit these strategies. Hence, new healing treatments have appeared at the level of regenerative medicine.

In this scenario, stem cell therapy is emerging and different types of cells are evaluated for their regenerative potential. At present, it is not clear whether such a 'perfect' stem cell exists; what is apparent, however, is that some cell types are more promising than others. Initially, first-generation cell types were introduced, including skeletal myoblasts (SM), mononuclear bone marrow cells (BMMNC), hematopoietic stem cells (HSC), endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC). Despite promising preclinical studies, first-generation approaches have shown heterogeneous clinical outcomes[28]. Variations between studies can be attributed to differences in design (cell preparation, route of delivery, timing, dose, endpoints and follow-up methods). After these smoky and unclear findings on the outcomes of first-generation cell therapy, research has shifted to other cellular models. In order to better match the target organ, second-generation cell therapies propose the use of non-resident stem cells, such as MSCs and pluripotent stem cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), constitute another source for guided tissue differentiation and better match the target organ. Only a few experimental studies compared first-generation and second-generation cell types. They found that cardiac-committed cells displayed an improved therapeutic effect as assessed by improved engraftment, cardiac function, angiogenesis, and scar size. In fact, Cell-based therapies as a very innovative tool still have many obstacles that need to be overcome to ensure effective therapy, ensuring patient safety. It is for this reason that new and more complete studies are needed to make cell therapy available as a standardized "reagent", to be tolerated by the immune system, safe oncologically, ie not create tumors and circumvent societal ethical concerns[29]. Today the last frontier in

regenerative medicine seems to not involve the use of cells but of decellularized materials, small molecules, RNA, growth factors and cytokines. In the intrinsic study of cell-based therapies, know the mechanism underlying the action carried out by the cells is of fundamental importance. The therapeutic effect of stem cells is recognized, in many clinical trials, whether they are first or second generation, but more in-depth studies on the mechanism of action shift attention not to the cell itself but to the performed paracrine effect. Recently, the paracrine effect of transplanted stem cells has been emphasized[30]. An array of cytokines and growth factors are involved in the paracrine effects of MSC, EPCs and ADSCs therapy. Transplanted stem cells can upregulate multiple growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), Ang-1, SDF-1a, endothelial nitric oxide synthase/inducible nitric oxide synthase, pro-angiogenic and antiapoptotic factors such as TGF-β. These factors are helpful in protecting cells from apoptosis and necrosis, promoting angiogenesis, retarding matrix remodeling and augmenting the recruitment of circulating progenitor cells. In addition to the factors just mentioned, the role of extracellular vesicles EVs is gaining ground in new research on the paracrine effect. The main feature that has focused attention on extracellular vesicles (EV) is the function of 'cargo' cellular material from the secreting cells to the surrounding cells[31]. In fact, many of these growth factors and cytokines, do not have signal peptides and their packaging along in membrane-bound vesicles explained, at least partially, the multiple effects throughout the body[32]. EVs can carry a complex content that includes nucleic acids, proteins, lipids, mRNAs and non-coding RNAs such as microRNA (miRNAs) and long non-coding RNAs[33]. These

vescicular proteins and miRNAs are physically intricated and participate in cell-cell communication. For example, exosomes were enriched in several cardio-protective and pro-angiogenic miRNAs, such as miR-132, miR-210, and miR-146[34] and significant association of miR-192, particularly in exosomes, with patients who experienced the development of HF[35]. Another important EVs feature reported by Ibrahim *et al*. was that cell-derived exosomes contain a part of distinctive miRNAs, compared with miRNAs of generating cells, which indicates that miRNA enrichment into exosomes may be through a specifically selected mechanism[36]. Taken together, this evidence makes EV appear as therapeutic candidates in angiogenesis disease and as well as biomarkers of cardiovascular diseases.

1.2 Platelets as key modulators of angiogenesis

Platelets are anucleate cells derived as fragments of bone marrow megakaryocytes that have a half-life of 7–10 days in the blood; in close contact with the endothelial cells, platelets survey the vascular integrity. Although the first role played by platelets was in the coagulation process, where their activation is closely related to thrombus and fibrin clot formation at a site of injury[37], today the importance of platelets seems to affect many more functions. In fact, there are many studies that describe platelets as an actor mediating between the vascular system, hemostasis, and the immune system.

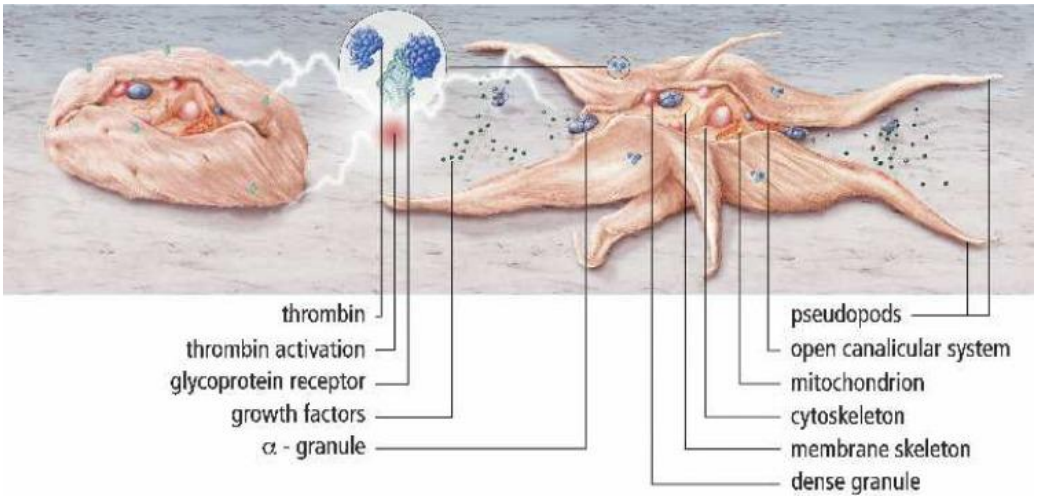

Figure 2 Schematic overview of a resting and activated platelet.
Normally platelets are in a resting, nonactivated state. On activation (e.g., by thrombin), platelets change their shape with the development of pseudopods to promote platelet aggregation and subsequent release of granule content through the open canalicular system (GP, glycoprotein).[38]

Platelets contribute to tissue remodeling and reorganization after injury or inflammatory responses by modulation of angiogenesis and cell apoptosis in steady-state tissue homeostasis or after tissue breakdown. So they became possible protagonists in regenerative medicine. In particular we can define platelets as "angiogenesis modulators" because these contain a variety of factors both pro-angiogenic such as VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), sphingosine-1-phosphate (S1P), angiopoietin-1 (Ang1), and platelet-derived endothelial growth factor (PD-ECGF) and anti-angiogenic such as platelet factor 4 (PF4), thrombospondin 1 (TSP-1), tissue inhibitors of matrix metalloproteinases (TIMPs), Endostatin and Angiostatin.

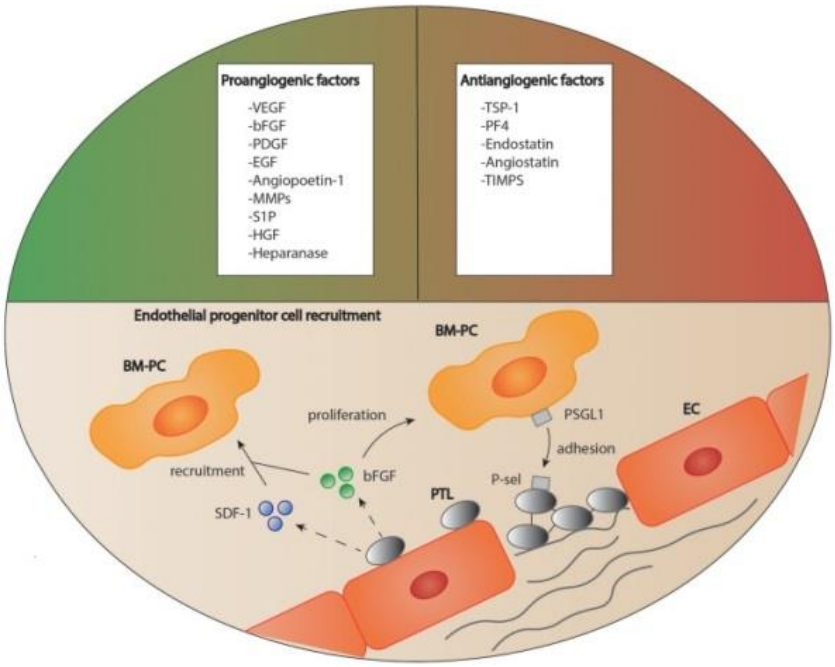

Figure 3 Platelets as regulators of angiogenesis.

Platelets are able to both stimulate and inhibit the process of angiogenesis due to a variety of mediators stored in their granules. Furthermore, platelets are suggested to recruit circulating regenerative cells to the site of vascular lesion supported by soluble mediators[39].

During the dynamic process of angiogenesis, many cells relate to each other, including endothelial progenitor cells (EPCs) and endothelial cells, stromal cells (pericytes) and leukocytes (e.g. neutrophils, macrophages, and natural killer (NK) cells)[40]. In the neogenesis phase, these cellular types co-operate, both in physiological and pathological situations, in a dynamic environment where platelets fulfill a key and active role. In the early stage of angiogenesis, VEGF-platelets source binding endothelial cells through their VEGF receptor 2 and promote proliferation and vessel sprouting[41]. The certain origin of VEGF from platelets has been demonstrated by Arisato et al., who found significant levels of VEGF in the fibrin net after thrombus formation[42]. Recent

antiangiogenic therapies, therefore, intend to block this factor[43] In addition to angiogenic factors, chemoattractant factors such as S1P and PD-ECGF are also essential in formation and guiding of new vessels to recall resident cells and *in vitro* triggered a strong chemotactic response in endothelial cells[44] to enter the migratory process. Matrix metalloproteases (MMP 1, 2, and 9) facilitate the degradation of inhibiting structures, such as the basement membrane and extracellular matrix components. Platelets have been also found to stimulate MMPs released from leukocytes and to secrete several MMPs such as MMP-1, MMP-2, and MMP-14 by themselves, thereby further promoting endothelial cell migration[45]. In the later stages of angiogenesis, the stabilization of the newly formed vessels becomes more important. Thrombin-activated platelets release Ang1, an angiogenic factor that enhances vascular stability and prevents vessel leakage[46]. Moreover, as we have already said in paragraph " The fine balance between physiological and pathological angiogenesis", the dark side of angiogenesis can characterize pathological situations like cancer. And given the clear relationship platelets-angiogenesis, it is known that platelets have a clear link with tumorgenesis. Through angiogenesis-restricting factors, platelets might be able to scavenge angiogenic factors in malignant diseases environment and for instance, in mice, platelet-released TSP-1 has been found to suppress tumor growth[47]. But the amount of proangiogenic factors may have an effect on angiogenesis-related to tumor development[48]. Stressing the importance of platelets in homeostasis in the angiogenic process, new studies are certainly needed to clarify the dual role of anucleate cells and what is the mechanism underlying their action.

1.2.1 Platelet-derived extracellular-vesicles: a novel tool to enhance angiogenesis

Initially described by Wolf in 1967[49] as "platelet dust" in the context of platelet biology, the extracellular vesicles derived from platelet (p-EV) have aroused new interest to understand the role of platelets. Extracellular Vesicles (EV) are small (0.1–1 μm in diameter) plasma membrane and α-granule fragments shed from platelets upon activation. EV formation is known from many cell types, such as endothelial cells and leukocytes, but it is estimated that about 70-90% of EVs in human blood are of platelet origin and CD41-positive[50] . Furthermore, it has been shown that their production after platelet activation is related to pathologies such as arterial thrombosis, atherosclerosis, idiopathic thrombocytopenic purpura, cancer and rheumatoid arthritis[51]. Indeed, subsequent studies have shown their involvement in many processes such as hemostasis, inflammation, coagulation, and angiogenesis. For example in Scott's syndrome, characterized by a defect in a platelet membrane mechanism necessary for blood coagulation, there is a defect in the generation of EVs that contributes to coagulation deficit[52]. Furthermore, the P-EV-angiogenesis link was found in patients with gastric carcinoma who had markedly increased plasma P-EV levels, in positive correlation with levels of pro-angiogenic factors such as VEGF[53]. The fundamental role of EVs in angiogenic pathologies has been observed in angiogenesis and improved post-ischemic revascularization following myocardial ischemia *in vivo*[54] or more recent findings showing comparable responses in a model of cerebral ischemia[55] . As observed in EVs from other parent cells, P-EVs exert their biological effects in multiple ways, such as triggering various intercellular signaling cascades and by participating in transcellular communication by

the transfer of their "cargo" of cytoplasmic components and surface receptors to other cell types. Furthermore, the p-EVs have been characterized by their size. Harry at al. have seen that platelet-derived multivesicular bodies (MVBs) represent a developmental stage in α-granule maturation and, after stimulation of platelets with thrombin receptor agonist peptide SFLLRN (TRAP), are released microvesicles of 100 nm to 1 μm, and exosomes measuring 40 to 100 nm in diameter, similar in size as the internal vesicles in MVBs and α-granules. Microvesicles could be detected by flow cytometry but for the exosomes, probably because of the small size of the latter, using Western blotting and immuno-electron microscopical techniques. Moreover they suggest that the function may be related to vesicular size, in fact, the absence of factor X and prothrombin binding and the low capacity of annexin-V binding to exosomes suggest that the membranes of exosomes have little procoagulant activity. Possibly CD63 on released exosomes serves another specific extracellular function[56] .

1.2.1 Platelet- derived clinical preparation

Blood is an important source of essential therapeutic products that comprise both cellular and protein products that cannot be obtained from other sources. For different surgical occurrences, the blood can be used in its entirety for complete transfusions or its components can be used, such as concentrate of red blood cells (RBCs) or platelets**.** Blood components, such as platelets, are nowadays implicated in many bio-based productions that are used for the clinical treatment of some diseases, depending on their composition. The major platelet products used today are:

a) Platelet-rich fibrin (PRF);

b) Platelet-rich plasma (PRP) and its derivatives: Plasma rich in growth factors (PRGF), Platelet Gel (PG) and Platelet Lysate (PL)

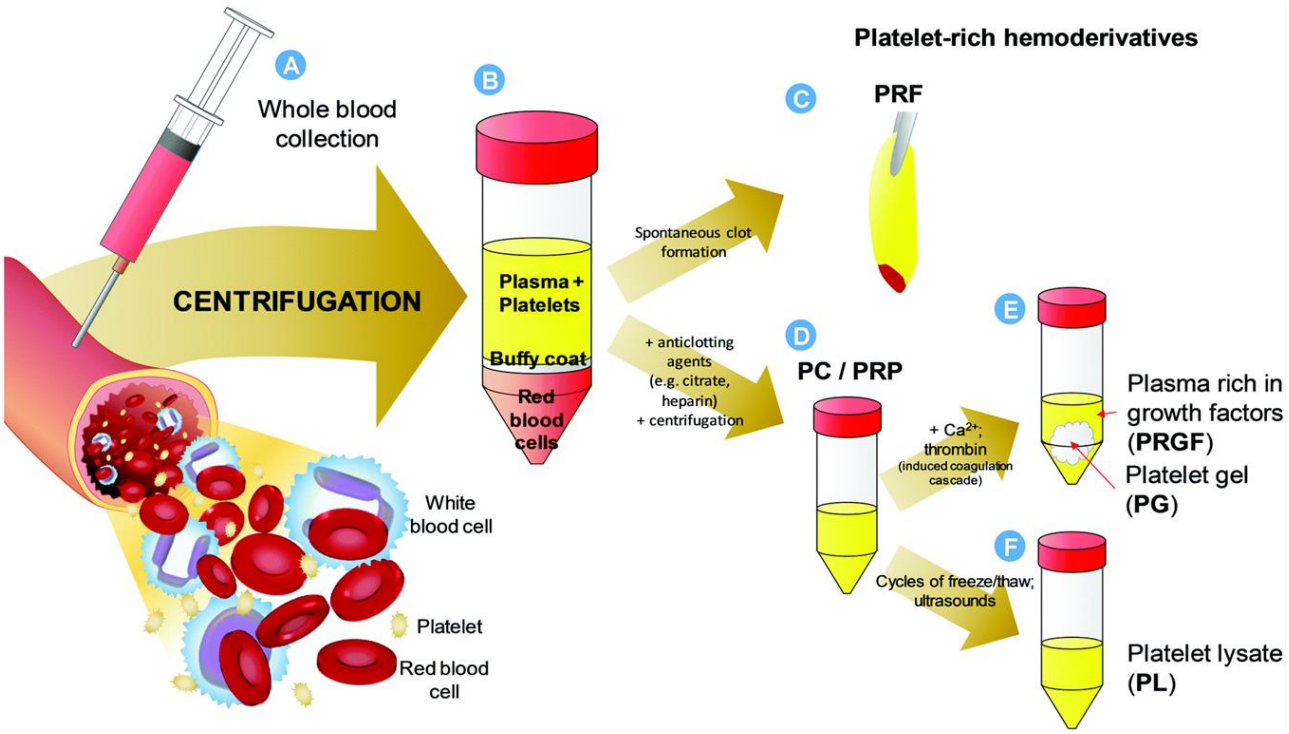

Figure 4

Different preparation of Platelet- derived products

Platelet Rich Fibrin (PRF)

PRF preparation is a simple product as it involves centrifugation of whole blood, without anticoagulation, at nearly 400*g* for 10 min[57]. The absence of an anticoagulant implies the activation in a few minutes of most platelets of the blood sample and the release of the coagulation cascades. Fibrinogen is initially concentrated in the high part of the tube before the circulating

thrombin transforms it into fibrin. A fibrin clot is then obtained in the middle of the tube, just between the red corpuscles at the bottom and acellular plasma at the top. The fibrin will polymerize in a diffuse way in the tube and only a small blood clot without consistency will be obtained. Biochemically PRF consists of an assembly of cytokines, glycanic chains, and structural glycoproteins in the mesh of slowly polymerized fibrin network[58]. Due to the presence of platelets, it also offers stimulation of defense mechanisms along with the regulation of the inflammatory process. Fibrin matrix offers natural guide for angiogenesis and wound healing. It does not require the use of bovine thrombin or any anticoagulants hence it is completely safe whereas a standardized method offers uniform products for clinical applications. As a limitation, it offers a limited amount of volume when compared with PRP. Tissue banking of these products is not feasible due to the short shelf life of PRF and inability to use it as an allogenic graft due to presence of immunogenic cells in the matrix specific to the donor[59].

Platelet-Rich Plasma (PRP)

PRP is a platelet concentrate containing a high level of platelet but minimal amount of natural fibrinogen and plasma. PRP biomaterials documented in the scientific literature vary a lot with regards to their preparation especially centrifugation steps[60]. But in the common protocols for obtaining PRP always have the following phases. PRP is extracted from whole autologous blood through a central vein catheter. The collection of whole blood for PRP preparation requires great care during venipuncture, in order to avoid any activation of platelets at the phlebotomy site. Venipuncture should be clean

without the need to maneuver the vein to maintain the flow. Collection should be with uniform flow and with gentle hands avoiding any activation of platelets. Blood from adult donors can be obtained using a 19–21-gauge needle in a plastic syringe[61]. After getting 400 to 500 ml of autologous blood the anticoagulant is added. Anticoagulant (AC) plays an important role in preventing the coagulation cascade of the collected whole blood because it influences the efficacy of the final platelet product in terms of ratio of anticoagulant, rate of collection, mixing and time between collection of the whole blood and its separation into PRP. The idea is to have minimum aggregation and activation of platelets during anticoagulation of the blood and to harvest maximum naive platelets for therapeutic use. Platelet can undergo alteration during collection, processing, and storage which adversely affect their structure and function as well as the release of growth factors[60]. The AC used are:

1. Sodium citrate at a ratio of nine parts blood to one part anticoagulant is the typical concentration.
2. Heparin inhibits the generation and activity of thrombin via its complex with antithrombin III.
3. Sodium and potassium of EDTA (ethylenediaminetetraacetic acid) are powerful anticoagulants, which act by chelation of calcium molecules in the blood. EDTA also appears to suppress platelet degranulation hence it is not the main AC recommended for platelet biomaterial preparation[62] .

Progressively the basic principle involved in preparing any PRP biomaterial is the selective separation of the liquid and solid components of whole blood

through the technique of centrifugation. Centrifugation technique uses the physical principle described by "Stoke Law", which states that the settling velocity of particles in a liquid environment in response to gravitational forces is approximately proportional to their diameter. Therefore, particles with a larger diameter such as red blood cells (~7 microns in diameter) and WBCs (7–15 microns in diameter) will settle proportionally faster than platelets (~2 microns in diameter) when subjected to a gravitational force.

Centrifugations contribute to the formation of three different colorimetric layers. The least dense layer, which is platelet-poor plasma, is about 45% of the sample; the middle layer consists of RBCs and is about 40% of the sample; and the lower layer is the PRP, which makes up around 15% of the sample. It is also called the buffy coat because of its white or buffy appearance. Buffy-Coat is a layer which is between plasma (above) and red cells (below). This layer is formed when an appropriate amount of centrifugal force (stronger enough to settle all the platelets) to the whole blood resulting settling down of all the platelets and leucocytes above the packed red cells. It's a whitish layer formed on the packed red blood cells (PRBC). Based on number of spins required to prepare platelets we could classify their method of preparation as single spin (primary segregation) and double spin (secondary segregation). After centrifugation layer formation is possible to obtain other preparations from PRP. For example thanks to the addition of calcium chloride ($CaCl_2$) and thrombin, individually or in combination in order to gel the platelet concentrate. It initiates the coagulation process and easy handling. Thought the agitation or mix for 6–10 seconds initiate clotting formation and achieve the Platelet gel (PG)[63]. The liquid part in which the PG floats is called Plasma rich in factors (PRGF) and represents a platelet product used in the clinic.

More specifically, we can say that the potential therapeutic effect of PRP is due to various cytokines, platelet-derived growth factors (PDGF), transforming growth factors β1 (TGF β1), insulin-like growth factors (IGF), platelet factor 4 (PF-4), fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF) which is believed to accelerate the natural healing process and promote cartilage repair for example. These cytokines and growth factors recruit stem cells residing in the lesion site, where they are stimulated to secrete additional growth factors and anti-inflammatory cytokines, causing a greater increase in collagen and matrix synthesis[64]. The PRP has antimicrobial activity for example in wounds or ulcers, in fact the platelets inside the preparation secrete microbicide proteins (PMP) which contain a series of materials that have antibacterial activity, including platelet factor 4, regulated by the activation of proteins normal expressed and secreted T cells, connective tissue activating peptide 3, platelet basic protein, thymosin beta-4, fibrinopeptide A and fibrinopeptide B. PMPs may play a role through the following mechanisms: adhesion to the bacterial membrane, alteration of the permeability of plasma membrane, inhibition of the synthesis of large molecules in the cytoplasm[65]. An example of clinical PRP application is in periodontal defect treatment, root coverage procedures, ridge augmentation grafting, guided bone regeneration, sinus lift grafting, and implant surgery[66-67-68]. The clinical application of PRP also extends to mandibular and maxillary reconstruction (tumor and trauma-related defects)[69], blepharoplasty, dermal fat grafts, and orthopedic surgery[70]. A variant of PRP, Platelet Gel (PG), also contains a variety of growth factors, including PDGF, VEGF, connective tissue growth factor (CTGF) and TGF-β[63]. Many studies have shown that PG can promote the growth of endothelial

cells, improve local microcirculation, promote fibroblast migration, promote skin formation, regulate cell function immune, provide an antibacterial effect and improve the local inflammatory reaction[71-72]. PRP is able to carry cytokines and growth factors on the site, which helps in rapid regeneration in a way that would not occur with fibrin glue due to the presence of platelets. However, its limitations are lack of standardization in PRP preparation protocol, variation in storage time of different platelet concentrations, and life-threatening coagulopathies (for example bovine thrombin can trigger antibodies to clotting factors).

Another product obtained from the modification of the PRP is the Platelet Lysate (PL)

Human PL prepared by the various release strategies has been established as an adequate alternative to the supplement of fetal bovine serum culture medium, allowing an efficient propagation of human cells in animal serum free-conditions for a multiplicity of applications in the advanced somatic cell therapy and tissue engineering. A growing number of studies using platelet-derived products to induce human cell proliferation and differentiation have also discovered a considerable variability in human platelet lysate preparations that limits the comparability of results. The main changes concern the selection of donors, the preparation of the starting material, the possibility of grouping in plasma or additional solution, the implementation of pathogen inactivation and the consideration of ABO blood groups, which can influence applicability[73].

The primary source of the PL is Platelet Concentration (PC) that can be of various derivation. In fact, PC can be prepared either from whole blood units after centrifugation (Buffy coat pooling buffy coats and plasma unit and a

centrifugation step, or PRP method, already explained) or by Single-donor apheresis[74].

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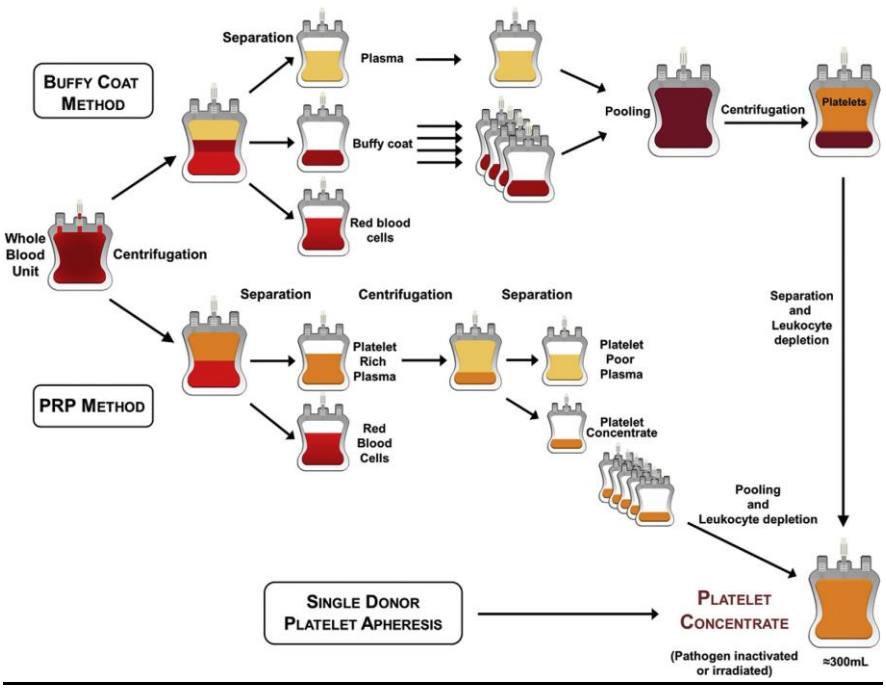


Figure 5 Different modalities of platelet concentrate preparation.

Whole blood-derived platelet concentrates are prepared either by the buffy coat method pooling four buffy coats and one plasma unit and a centrifugation step or by the platelet-rich plasma (PRP) method pooling four to six PRP units. Leukocytes are depleted by filtration. Single-donor platelet concentrates are produced by apheresis. Further processing may include pathogen inactivation or gamma irradiation.

The platelet lysate obtained from the platelet concentrate, defined as a standard blood product of transfusion medicine prepared by apheresis, containing a platelet quantity equal to ten times that present in the PRP.
Starting from PRP or other methods used to induce the release of growth factors and other bioactive molecules of the PL are different. The most used process is characterized by a repetition of freeze/thaw cycles, that was economic and simple to perform. Typically, PRP is shock-frozen at 30 °C or 80 °C and thawed at 37 °C to fragment platelets membrane. The number of cycles so far described in the literature varies from one to five[75-76]. A systematic analysis determining the optimal number and precise conditions of freeze/thaw cycles are still pending.

Another method to achieve PL is sonication, alone or in combination with additional freeze/thaw cycles[77-78]. Sonication for up to 30 min by use of an ultrasound bath, which consisted of a steel tank containing sterile distilled water, with the application of a frequency of 20 kHz was found to be efficient to release the platelet granule content[79].

Also, Solvent/Detergent (S/D) treatment was a double goal to derived PL and inactivation of lipid-enveloped viruses. Viral safety depends on donor selection and screening; thus, there continues to be a small but defined risk of viral transmission comparable with that exhibited by whole blood. Horowitz at al. demonstrated that, treatment with S/D composed by 1% tri(n-butyl)phosphate (TNBP) and 1% Triton X-100 at 30°C for 4 hours, results in a rapid and complete inactivation of many virus-like human

immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and vesicular stomatitis virus (VSV) compared to the untreated[80].

In most procedures, the PL is subjected to centrifugation for removing cell debris and fibrin clots and is kept frozen until use. For processes that do not remove fibrinogen, heparin as an anticoagulant e ideally without a stabilizer has to be added to the medium to avoid subsequent coagulation in the medium during cell culture. All these preparation modes result in PL products that are capable of supporting expansion of MSC from various origins[74-81] . Variations in the capacity to expand and differentiate MSC have been reported, but a systematic comparison to favor one technique is lacking[82] .

Divergent technical sequences can have significant impact on protein content and composition, particularly when thrombin or $CaCl_2$ addition is performed, as this depletes coagulation factors and protein like platelet-derived growth factor-BB (PDGF-BB), transforming growth factor β-1 (TGF-β1), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF)[83].

The biological impact of temperature and duration of liquid storage or repeated freeze/thaw steps of HPL preparations on growth factor and nutrient functionality as well as MSC expansion has not been sufficiently evaluated[61-84].

1.2.2.1 PL in vitro and in vivo applications

Even if the preparation methods of the LP are different, they can be recognized, in the different preparations, enormous biological properties. For

example, in vitro application, PL as an alternative method to the use of FBS in cultures of various cell lines such as dendritic cells, fibroblasts and especially in mesenchymal stem cells (MSC)[85]. In particular, the MSCs seems to be the cells that best responds to the administration of LP. In fact, it has been shown that MSCs (derive from bone marrow, adipose tissue, periodontal etc.) enhance their property of differentiating in the three different lineages (chondrogenic, adipogenic and osteogenic) when they are in cultures with 10% LP media. Furthermore, the morphology, phenotype, and differentiation capacity of MSCs were similar whether expanded in PLs or FBS. However, the proliferative capacity of MSCs expanded in PL appeared superior, while those expanded in FBS were more suitable for preventing/treating all activity-related immune complications[86]. For example, in Adipose tissue-MSC expansion, with maintenance of the immunophenotype and proliferative and differentiation capacities up to passage 10[87]. Another important MSCs capacity that PL can sustain optimally is clonogenicity. The ability to form clones improves both numerically and qualitatively (given by the size of the clone diameter and the number of cells composing it) in MSC-Adipose tissue[76]. PL can be used as medium supplements to expand and support the immunomodulatory capacity of bone marrow MSCs that maintained their immunosuppressive activity, and cell karyotypes showed no genetic alterations[79-74].

Following the excellent results obtained in vitro, recent studies are shifting their attention to the clinical use of LP to be born at various and innovative applications.

PL, in a therapeutic field, was injected intra-articulary[88-89] to improve the thickness of the knee cartilage in Osteoarthritis treatment. The absence of

white blood cells and platelet membranes in PL is the probable reason behind these findings.

Instead, PL contains, which can affect bone growth, cell migration, adhesion, and angiogenesis[90] can induce vertical bone regeneration and promoting bone formation[91]. Also in Ocular disease, a human clinical study [92], found that effective in restoring corneal damage by decreasing Langerhans cells density, promoting corneal, and regenerating nerve cells[93]-[94]. It is used in the dermatological-aesthetic clinic through topical injections for skin rejuvenation[95] and also for the treatment of human alopecia[96] with an increase of follicular and hair growth in the PL-treated regions. Another example, in the repair of wounds healing to severe ulcers, PL as a tool that contributes to release of wound area with a thin layer of epidermis well organized and formed[97].

Surgery periodontal and dentistry use of PL has found excellent results in maxillofacial surgery and as an adjuvant in the application of titanium implants e after tooth extraction it proved useful to prevent alveolar osteitis and improve hemostasis [98]-[99]. In the field of plastic surgery, PL is particularly useful for soft tissue repair, in which the positive effects of growth factors are particularly marked, reducing the risk of infection and length of hospital stay[100].

PL is effective in applications such as cell culture and regenerative medicine however, its preparation needs to be standardized to reach the optimum standard of growth factors[101].

1.2.3 Mesengen ™: a platelet-lysate preparation patent in "Sapienza" University

Mesengen™, (Italian Patent n. RM2011A000500 PCT /IB2012/055062) was born from the collaboration between the Sapienza University of Rome e Futura Stem Cells, is a lysate inactivated platelet derivation human that can be used as an adjuvant for cultivation, growth and/or in vitro and ex vivo expansion of:

- Human mesenchymal stem cells derived from marrow bone and/or adipose tissue.
- Human dendritic cells derived from monocytes.
- Human fibroblasts.
- Stabilized cell lines of hematopoietic derivation.

Until today, these cells were cultivated with media added with serums of animal origin that rendered them difficult to analyze due to the presence of non-human components and unusable for clinical purposes for potential risk of transmission of pathogens or induction of allergic reactions.

The platelet lysate (LP) is a valid alternative for the growth of such cells because it contains numerous growth factors, in uniform concentration if not superior to animal serums, in charge of cell development and being of human origin, in potential clinical use. Furthermore, due to its low plasma content, <15%, it is adverse to developing allergic reactions. Deriving from multiple donors of platelet (about 12), the PL could be a harbinger of agents transmissible but Mesengen ™ is an inactivated platelet lysate, i.e. free of nature contaminants:

- Viral.

- Microbial.
- Fungal.
- Parasitic.

The inactivation of these agents takes place with a quick, simple method effective and economical.

It is subjected to a qualification process that before product releasability requires a sterility test on each lot, an immunometric assay of four growth factors pilot like beta-FGF, TGF-beta, PDGF-AB and VEGF to make Mesengen PL a standardized product.

2. AIM

Despite their well-acknowledged role in hemostasis and thrombosis, platelets have been also described as main regulators of angiogenesis, defined as the ability to induce endothelial proliferation, sprouting and organization into functional tubules from a pre-existing vasculature [102-104]. Angiogenesis is a key process for tissue regeneration and for determining the outcome of vascular insults or wound healing. The ability of platelets to enhance angiogenesis stems from their intrinsic physiological role to interact with endothelium during vascular damage in order to preserve integrity and vessel homeostasis. Platelets exhibit a unique secretory profile of multiple combined factors with a dual pro- and antiangiogenic role. Among them, we could list growth factors, cytokines, microRNAs, small soluble molecules and proteins including those related to cytoskeleton, adhesion, inflammation and extracellular matrix interaction [105-108]. This balanced combination of mediators is mainly contained in defined vehicles of small plasma membranes, named extracellular vesicles (EV) that have a heterogeneous size (exosomes, microparticles, microvesicles) and composition. [109,110].). Platelets represent the most abundant source of EV of different dimensions in the systemic circulation[56,111,112] [113]. Extravesicles have been reported to mirror the haemostatic properties of platelets [114] [114], by exerting both anti- and procoagulant effects depending on the subpopulation of EV involved[56,115,116]. EV of platelet origin have been demonstrated to regulate angiogenesis when released at the site of endothelial sprouts[117] and secretion of VEGF[118] or by transferring proliferative and survival biological information to the endothelium [118-121]. Similarly to their parental cells, EV would also act indirectly in a paracrine fashion and intercellular network through cargos of

cytokines and decoy proteins locally released in the microenvironment. For instance, endothelial progenitors cells-mediated angiogenesis [102,104] and tissue engraftment is enhanced by EV of platelets origin trough the activation of specific targets or after transferring of specific soluble platelet receptors and activation of integrins on the endothelial surface [38,118,122]. Based on these studies, evidence that platelets and platelet-derived biological products such as EV can trigger angiogenic programs in endothelial cells, has encouraged a better understanding of their potential therapeutic tool for those regenerative-based applications where the restoration or the enhancement of angiogenesis represents the clinical goal to achieve.

Accordingly, parallel to the investigations regarding the key involvement of platelets in regulating angiogenesis, it has been clearly demonstrated that platelet-derived clinical preparations also (i.e. platelet-rich plasma, and gel) are similarly able to boost and reflect the angiogenic properties of platelets. Particular focus has been given to platelet lysate (PL), considered the gold preparation concentrate derived from platelets, and whose clinical efficacy is currently considered superior to other platelet-derived formulations [123]. The plethora of highly concentrated factors and soluble molecules contained in this hemoderivate is currently considered the main responsible of the angiogenic capacity of PL. However, we could conceive PL preparations as a primary source of angiogenic EV, as when PL is manufactured, platelets are repeatedly lysed and therefore allowing the availability of vesicles and granules in the preparations. So far, the vast majority of studies have only limited to explore the effects of vesicles of cell origin (i.e. from MSC, fibroblasts, lymphocytes) after treatment with PL or EV released by intact

and activated platelets. The wide range of modalities by which PL-derived EV might regulate angiogenesis still needs to be fully addressed.

Thus the aim of my study is to confirm that PL based formulation represents a source of bioavailable EV and to question their individual role in relation to PL formulations regarding the ability to mediate angiogenesis in endothelial cells. In addition, we want to demonstrate if PL can be employed as a tool for vascular regeneration and which Molecular mechanisms and biological actions mediated by PL and EV derived PL can contribute to vascular regeneration

3 MATERIALS AND METHODS

3.1 Preparation of the Platelet Lysate MESENGEN

Production of pools of platelet concentrates

The platelet concentrates (PLT) were produced from units of whole blood from suitable voluntary donors, collected in dedicated sterile circuits provided with a quintuple bag (Fresenius Kabi AG, Bad Homburg, Germany). In order to produce PLT from buffy coats, the blood units were processed within six hours after collection. After it was verified that the whole blood withdrawal conformed to requirements (volume withdrawn, absence of visible coagulates, absence of chylosis), the unit underwent centrifugation at 6477 relative centrifugal force (rcf) x g for 12 min.

The following were extracted from each unit using Compomat G4 separators (Fresenius Kabi AG):

- 1 unit of fresh plasma
- 1 unit of buffy coat-depleted red blood cells
- 1 unit of buffy coat (BC) containing the platelet component.

The BC products were stored in an incubator at 22°C under continuous agitation and assembled to produce the pool within 24 h. The pool of 6 units of BC was prepared by connecting the BCs (Composeal, Fresenius Kabi AG) together under sterile conditions in a dedicated production environment, with InterSol saline solution (Fenwal Inc. Lake Zurich, Illinois, with the following composition: Na-citrate 2¾0 318mg, Na2-anhydrous phosphate 305mg, Na Dihydrogen-phosphate $2H_20$ 105mg, Na- Acetate $3H_20$ 442mg,

NaCl 452mg, H_20 lOOmL) and the pool collection bag (BioP-plus single-use kit, Fresenius Kabi AG). The BC and final pool collection bags were identified by means of a specific label with a bar code associated with the pool being produced. At the end of the assembly procedure, the collection bag with the 6 BCs was closed and sealed (Composeal, Fresenius Kabi AG), disconnected from the processing kit and centrifuged at 480 rcf x g for 9 min. At the end of centrifugation, the platelet concentrate was separated from the residual BC using the automatic Compomat G4 separator.

Pathogens inactivation procedure

Two pools of 6 PLT units were subjected to pathogen inactivation by means of a photochemical treatment with psoralens and UV exposure (INTERCEPT Blood System for Platelets, Cerus Corporation, Concord, California USA). During the pathogen inactivation process using INTERCEPT technology, a compound of the psoralen family {Amotosalen hydrochloride) was introduced into the PLT pool; this compound, following to the exposure to UVA radiation, forms a covalent bond with any nucleic acids that may be present in the product and blocks the process of duplication of DNA and RNA helices. For the inactivation procedure, the bag of the pool was sterilely connected to the INTERCEPT kit and the inactivation procedure was started; it takes place in a completely closed system and comprises the following steps:

- introduction, into the PLT pool, of the psoralen Amotosalen (S-59, INTERCEPT Blood System for Platelets, Cerus Corporation, Concord, California USA; product code ΓNT2204B) at a final concentration of 150 μιηοι/L;

- exposure of the PLT pool supplemented with psoralen to UVA radiation 3 J/cm^2
- transfer of the product into a bag containing a specific device (CAD Compound Adsorption Device) for the removal of the psoralen;
- transfer into the final bag for storage of the product "Inactivated PLT Pool" until the use thereof.

The final PLT pool subjected to the inactivation treatment described was identified with a label bearing the barcode associated with the pool at the time of production and the specification "Inactivated". At the end the inactivation procedure, the PLT units were resuspended in InterSol with a residual plasma percentage of about 20-30%. From this step onward, the further operations of production of the PL were carried out in a class C controlled environment classified according to international guidelines for Good Manufacturing Practices (GMP), implemented at the European Community level through the following legislative provisions: EU GMP Directive "Manufacturing of Sterile Medicinal Products",-Annex 1, EU GMP Directive "Manufacture of Biological Medicinal Products for Human Use" - Annex 2, EMEA Guideline on Human Cell Based Medicinal Products - EMEA/CHMP/CPWP/323774/2005.

Production of platelet lysate

Samples were drawn from the 2 PLT pools by inserting a sterile sampling device (Terumo, Tokyo, Japan) into the inlet provided in the bag (sterile "sampling site" of Fresenius Kabi AG collection devices) in order to determine the platelet count and sterility. The platelet counts were carried

out using the ABX Pentra DX 120 hematology analyzer (Horiba ABX, Montpellier, France). The sterility tests were performed with the BacT/ALERT System using media-specific for aerobic and anaerobic bacteria and fungi (bioMerieux SA, Marcy L'Etoile, France). At the end of the complete procedure, the volume of the pool of 6 units was 300 mL on average and platelet content ranged between 2.5 and 3.5 x 10^{11}. The 2 pools of 6 units of inactivated PLT were subsequently combined in a single bag, via a sterile connection (TSCD II, Terumo, Tokyo, Japan), so as to obtain a final pool with a volume of approximately 600 mL, made up of 12 units of inactivated PLT. The overall pool underwent 3 successive cycles of freezing (-80°C; kept at this temperature for 24 hours in a mechanical freezer) and thawing (37°C; thawing in a biological defroster for plasma units (Bloodline, KW, Siena, Italy), temperature maintained for 60 minutes) to ensure breakage of the platelets and the release of growth factors. In order to remove the cellular stroma, the pool was divided into 2 bags and each of the 2 bags was sterilely connected (TSCD II) with a second transfer bag (600 ml, JMS Singapore PTE LTD, Singapore) so as to the remove the supernatant after 45' centrifugation at room temperature and 5313 rcf x g. The residual bag containing the cellular stroma was removed after double sealing and the centrifugation procedure was repeated using the same methods. At the end of preparation, samples were drawn for the final sterility test. In order to be able to use the PL produced according to the present invention in cell cultures for cellular therapies, in addition to aerobic and anaerobic bacteria and fungi, endotoxins were also tested for using the LAL test (Limulus Amebocyte Lysate; International PBI spa, Milan, Italy) with a sensitivity of 0.06 UE/mL. The PL products were identified with a suitable code in written and barcode form

serving to identify the production batch, aliquoted via a sterile connection (TSCD II) into bags (100 ml transfer bags for fresh frozen plasma, Fresenius Kabi AG), with identification of the sub-batches. Each sub-batch unit contained 40-50 mL of PL, equivalent to about 3 doses (to be intended as 3 doses for large-scale culture activities, each dose being sufficient to prepare a clinical-grade culture with a starting volume of 100-150 ml) for the ex vivo expansion of MSC in closed systems - CellSTACK® CORNING® cell culture chambers (MacoPharma, Mouvaux, France; Corning Incorporated, Corning, New York, USA) - if used at a concentration of 10%. Following the same operational scheme, excluding the viral inactivation step, inactivated PL was produced as a non-inactivated control. Evaluation of the content of MSC stimulating factors and the proliferative potential induced by the different preparations of PL on MSC. An immunoenzymatic assay was performed on the various PL products to determine the following factors using samples diluted to a varying degree, from 1:2 to 1:10:

- PDGF-AB (Quantikine Human PDGF-AB, R&D Systems, Minneapolis, Minnesota, USA)
- VEGF (Quantikine Human VEGF, R&D System)
- basic-FGF (Quantikine Human FGF basic, R&D System)
- TGF-Beta (Quantikine Human TGF-Beta 1, R&D System)

3.2 Extraction and characterization of extracellular vesicles

The method of differential centrifugation[124] was used for vesicle extraction by an ultracentrifuge (SORVALL® DISCOVERY ™ 90 SE, HITACHI). In the first

phase of EV extraction, PL was centrifuged twice at different speeds (500g and 2000g for 10 min each) to allow the elimination of possible platelet large debris or components (> 1μm). After that LP was centrifuged 100.000g for 1 hour. It is important to balance the samples perfectly, weighing with a precision balance, in order to avoid breaking them during ultracentrifugation. After an hour the supernatant is removed and the pellet containing the EVs can be used to carry out the subsequent experiments.

EV staining

For EV staining, the pellet was suspended in 100 μm of 1:700 PBS/PHK67 solution. In particular PHK67 Fluorescent Cell Linker Kit (cat.P7333 Sigma-Aldrich) uses proprietary membrane labeling technology to stably incorporate a green fluorescent dye with long aliphatic tails (PHK67) into lipid regions of the vesicles membrane allows FACS detection, in the 492nm green channel, and microscopy FITC detection. Afterward, the vesicles were placed at + 37 ° C for dye incubation. Meanwhile, the Exosome Spin Column MW3000 (ESC) kit has been prepared which enables us to remove the non-incorporated dye and any low molecular weight additives (salts, nucleotides, short oligonucleotides).

The ESC kit contains a spin column with the dehydrated gel that must be compacted and rehydrated by adding 650μl of PBS. Then it is vortexed for a few minutes and hydrated to be kept at room temperature for 5-15 minutes. After removing excess of interstitial fluid from the Spin Column, 100 microliters previous colored EVs/ PHK67 were placed inside it and eluted through centrifuged at 750 g for 2 minutes at room temperature. Finally, about 100 microliters of colored and purified vesicles were obtained.

3.2 Size

To determine the size, the EVs, after having been stained with PHK67, were analyzed to FACS ARIA II Cell Sorter equipped with FACs DiVa software v6.1.1 (Becton Dickinson, San Jose, CA). First, they were used Megamix Beads, a mix of fluorescent beads of varies diameters, to calibrate the cytometer. This bead acquisition according to the following procedure allows the settings of the cytometer to study microparticles within a constant size region and to get reproducible micro particles counts[125]. The diameter of Megamix beads used was 500, 240, 200, 160nm. Then, to define the different classes of dimension, the EV PL-derived were filtered with filters 0.8 µm and 0.22 µm (Minisart Syringe Filter, SARTORIUS Cat16592, 16532). The subsequent facs analysis allowed the identification of different dimensional classes compared with Megamix beads dimensional classes.

3.2.2 Specification of EV expression markers

The flow cytometry technique was used for the characterization of EV expression markers. Using the 100 µl of staining EV obtained from extraction described above, they were diluted 1: 6 with PBS and aliquoted into different FACS tubes for staining. The following antibodies were used: CD31 APC/CY7 (cat 303119), CD154 PE/CY7 (cat 310831), CD63 APC (cat 353007), ANNEXIN V PE (cat556421), CD 41 PE (cat 303705 Biolegend). Therefore using the EVs stained with PKH67 for autofluorescence to set panel FACS, the antibodies were diluted 1:20 in the sample and incubated at 4 ° C for 30 minutes to permit the staining. At the end of incubation, 5 µl of EVs were diluted 1:100 with PBS and acquired on a FACs ARIA II set with a minimal threshold (i.e. 200) on FITC channel and no thresholds on forward and side

scatter. The acquisition field was calibrated using Megamix-Plus SSC beads (Biocytex, Marseille, France). Samples were acquired for 120 sec at a minimal flow rate. To avoid carryover, fluidic was extensively washed with 0.1 µm filtered, distilled water before each sample acquisition. Results were analyzed using Flowing software v2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

3.2.3 Electron microscope and ultrastructure

To analyze the electron density and ultrastructure of EV Transmission electron microscopy (TEM) has been used. In particular, EVs pellet was fixed in 2,5% glutaraldehyde (Agar Scientific, Cambridge Road Stansted Essex, UK)/Phosphate Buffer; after fixation, we incubated at 4°C for 1 h. Samples placed onto formvar carbon-coated electron microscopy grids (400 mesh) were stained with Uranyless 22409 (Electron Microscopy Sciences) in water for 20 s. For observation and imaging, the grids were observed using a Transmission Electron Microscope (Zeiss EM10) operating at 80 KV while images were acquired using a digital camera (AMT CCD, Deben UK Ltd, Suffolk, UK).

3.3 Cell culture model: endothelium cell of human umbilical cord veins (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were cultured and expanded (2500 cells/cm^2)[126] in complete medium EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza Cat #: CC-3162). Culture system containing EBM™-2 Basal Medium (CC-3156 Lonza) and EGM™-2 SingleQuots™ Supplements (CC-4176Lonza) required for growth of Endothelial Cells. Cell morphology and growth were monitored by light

microscopy and assessed by Trypan Blue (Sigma, St. Louis, USA). The culture was expanded until passage 3-4 and when the cells were 80% confluent, after starvation over-night, are being stimulated for 24h as follows: EBM™-2 Basal Medium, to negative control; EGM™-2 Endothelial Cell Growth Medium, to positive control; 10% PL in EBM™-2; filtered 0,8 µm 10% PL in EBM™-2; filtered 0,22 µm 10% PL in EBM™-2. The aforesaid filters (0,8 µm and 0,22 µm Minisart Syringe Filter, SARTORIUS Cat16592, 16537, 16532) allow eliminating, from 10% PL solution, all vesicles of a dimension greater than the diameter of filter used. Furthermore, to confirm the functionality of the PL-EVs, the following conditions have been added in some experiments: 10% EV and 10% EV filtered 0,22 µm. These conditions were obtained by the ultracentrifugation technique. To normoxia condition HUVECs passage 3-4 were incubated in 5% CO_2 atmosphere and 37°C.

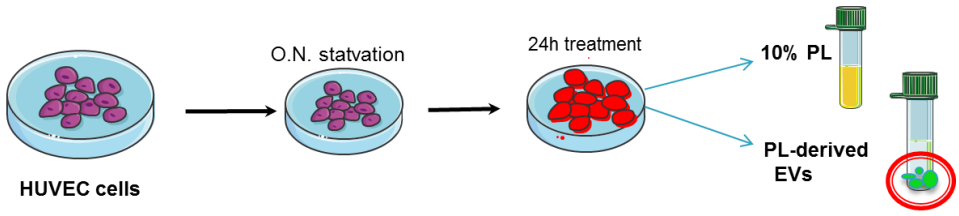


Figure 6 In Vitro Experimental plan

The design of the experimental plan consists of HUVEC conditioned with EBM 10% of PL or EBM 10% of PL-derived EV for 24h. The conditioned media are filtered (0,8 µm and 0,22 µm) before use to satisfy the experimental conditions.

3.4 Hypoxic stimulus

To hypoxia condition, HUVECs, at the same passage in normoxia, were cultivated in a gaseous mixture composed by 1% O2, 5% CO_2 and 94% N_2 through a hood/incubator (Baker Ruskinn Bugbox Plus) at 37°C for 24h.

3.5 Immunofluorescence

EV staining also allowed us to observe their presence and qualitative interaction with HUVEC at a fluorescence microscope. In fact, after ultracentrifugation and staining, 10% EV and 10% EV filtered 0,8 μm and 0,22 μm were placed on HUVEC, over-night, in EBM™-2 Basal Medium for 24h. The same ultracentrifugation and staining technique were used to obtain EV from the EBM™-2 Basal Medium, to negative and EGM™-2 Endothelial Cell Growth Medium, to positive control. After 24h the conditioned medium was removed, then HUVEC was washed and fixed with 4% paraformaldehyde for 10 min at 4 ° C. Each image was taken in the bright field to observe the cellular morphology of HUVEC and in FITC mode for observing EVs at 4X magnification using a Nikon Eclipse Ti.

3.6 Migration and Proliferation rate

To analyze the migration, the HUVEC were cultivated and after starvation overnight, they were plated in Boyden Cell Cultured Insert 12 well format, 8 μm pore (Falcon cat. 353182). 5×10^4 cells/wells in EBM-2 medium were plated in the upper side of the insert. Instead, in the underlying part, the conditions described in the paragraph 3.1.4 have been used, briefly EBM™-2 ,EGM™-2 , 10% PL, filtered 0,8 μm 10% PL, and filtered 0,22 μm 10% PL. At

the end of 24h, the inner side of the insert was wiped with a wet swab, in order to remove the cells and the outer side of the insert gently rinsed with PBS and stained with 1:20 Giemsa (Sigma, St. Louis, USA) for 15 min, rinsed again, and then allowed to dry[127]. Migrated cells were counted (five fields per chamber) under a light microscope(Nikon Eclipse Ti). Experiments were repeated three times in duplicate, both in normoxia and in hypoxia condition. In order to exclude any potential proliferation in 24h, HUVEC was also seeded in the same conditions and were analyzed by MTS assay, allow the protocol of kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay, cat G3582PROMEGA).

3.7 In vitro angiogenesis test

Matrigel assay

HUVEC were trypsinized and suspended in six different media EBM™-2 ,EGM™-2 , 10% PL, filtered 0,8 μm 10% PL, filtered 0,45 μm 10% PL and filtered 0,22 μm 10% PL. In addition, to testing the functionality of EVs, the same angiogenesis test was performed satisfying the conditions already described in paragraph '3.1.3 Extraction and characterization of extracellular vesicles' and in specifically EBM™-2, 10% PL, filtered 0,22 μm 10% PL and 10% EV, filtered 0,22 μm 10% EV. For each condition, HUVEC were plated at a density of 1.5×10^4cells/well in triplicate in 96-well plates coated with 50 µL of growth factor-reduced Matrigel (Corning™ Matrigel™ Growth Factor Reduced cat 356231). Multi-well was incubated for 24 h at 37°C before photomicroscopy. After incubation cells were fixed using 2,5% (v/v) glutaraldehyde in PBS, add to media. Each image from each well was taken at 4× magnification using a Nikon Eclipse Ti.

3.8 Enzyme-linked immuno-absorbing dosages

3.8.1 Determination of Nitric Oxide (NO)

NO production in HUVEC supernatants was evaluated by measurement of nitrite, a stable end product of NO. Nitrite was determined by a colorimetric assay with Griess reagent. One-hundred microliter of supernatant reacted with an equal volume of Griess reagent (one part of 1% sulfanilamide dissolved in 0.5 M HCl and one part of 0.1% N-(1-napthyl)-ethylenediamine dihydrochloride dissolved in distilled water) in 96-well plate under reduced light at room temperature during 10 minutes. The absorbance was measured with a microplate reader at 545 nm using a calibration curve of sodium nitrite standards (0–100 µM).

3.8.2 Determination of hydrogen peroxide (H2O2)

H_2O_2 was quantitatively measured by The DetectX® Hydrogen Peroxide (H_2O_2) Colorimetric Detection Kit (cat K034-H1 Arbor Assays). A hydrogen peroxide standard, diluted in EBM, is provided to generate a standard curve. HUVEC supernatants conditioned EBM, EGM-2, LP10% and LP 10% filtrate 0,8 and 0,22 µm are mixed with a colorless Colorimetric Substrate and the reaction initiated by addition of horseradish peroxidase. The reaction is incubated at room temperature for 15 minutes. The HRP reacts with the substrate in the presence of hydrogen peroxide to convert the colorless substrate into a pink-colored product. The pink product is read at 560 nm. Increasing levels of H_2O_2 cause a linear increase in the production of the pink product. The H_2O_2 levels were expressed as μmol/l. Intra-assay and interassay coefficients of variation were 2.1% and 3.7%, respectively.

3.9 Real-time PCR

3.9.1 Total RNA extraction

The total RNA of HUVECs was extracted starting from 1-3x10^5 cells, in conditions described above, using the miRNeasy MicroKit (Quiagen, cat 217084) following the protocol provided by the kit. The plated cells were lysed by adding 500µl of Lysis Reagent containing β-Mercaptoethanol (QIAzol® cat. 1023537) to which 100 µl of chloroform was then added and shaking vigorously for 15s. Cell lysates were placed inside the columns provided by the kit and centrifuged at 12000 rpm for 15 minutes. To the aqueous phase, 1.5 volumes of 100% ethanol were added and 700 µl sample was pipetted into an RNeasy MinElute spin column and centrifuged 800g for 15s at room temperature. After the elimination of the supernatant, 500µl of washing solution was added inside the columns and centrifuged for 15s at 8000 g. After 3 washes, the columns were centrifuged at 8000g for 2 minutes. To elute the RNA, 14 µl of Rnase-free water was added directly to the center of spin-column membrane. The samples were centrifuged for 2 minutes at full speed to elue the RNA. The extracted RNA was spectrophotometrically quantified at 260-280 nm and stored at -80 ° C.

3.9.2 Reverse transcription

The "The High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems) was used for the RNA reverse transcription following the protocol indicated by the company. A retro transcription mix solution containing the following reagents was prepared

REAGENTS	VOLUME
10X RT Buffer	2,0 µl
25X dNTP Mix(100 mM)	0,8 µl
10XRT Random Primers	2,0 µl
MultiScribe™ Reverse Transcriptase	1,0 µl
H_2O nuclease Free	4,2 µl
total Volume	10 µl

Table 3.1. Amount of reagents in the retro-transcription reaction

10 µl of this solution was added in 1µg of RNA diluted in 9 µl of H_2O Nuclease-free (Qiagen, Hilden, Germany). Retrotscription was performed using (ThermoMixer 5436, Eppendorf, Italy) under the following conditions:

	STEP 1	STEP 2	STEP 3
TEMPERATUR °C	25	37	85
TIME IN MINUTES	10	120	5

Table 3.2 Retro-transcription protocol

The cDNA obtained was stored at -20 ° C.

3.9.3 Real Time-PCR

The cDNA produced by the previous reaction was diluted in 20 µl of H2O of which 0.4 µl of template cDNA was used in a final reaction volume of 12.5 µl. The reaction was performed using the SensiMix SYBR Hi-ROX kit (Bioline, London, UK) which allows

the quantification of newly synthesized DNA strands using SYBR Green: a non-specific fluorescent dye that reveals the amplification of any DNA strand. The samples were run on the 7900HT Fast Real-Time PCR System platform (Applied Biosystems, Cheshire, UK) through the SDS 2.1.1 program (Applied Biosystems, Cheshire, UK) for 40 cycles, according to the protocol:

	STEP 1	**STEP 2**	**STEP 3**
TEMPERATUR(°C)	95	60	72
TIME (sec)	15	10	30

Table3.3: Amplification protocol

The panel of the primers used and their sequence are shown in table 4. The final concentration of the primers is 200nM.

NOX4 forward	AACCAAGGGCCAGAGTATCA
NOX4 reverse	GGATAAGGCTGCAGTTGAGG
GAPDH forward	ACAGTCAGCCGCATCTTC
GAPDH reverse	GCCCAATACGACCAAATCC

Table 3.4: primer sequences

All the primer pairs were previously tested in order to guarantee optimal efficiency and to confirm the specificity of the primers, the analysis of all the denaturation curves was performed at the end of the PCR. The denaturation curves provide an indication of the purity of the reaction allowing to verify the presence of a single and specific amplification product. Each reaction was performed in triplicate, using GAPDH as a housekeeping gene. For the relative quantization the ΔΔCt method was used which consists in applying the formula:

$$2\text{-}(\Delta Ct,r - \Delta Ct,cb) = 2\text{-}\Delta\Delta Ct$$

Ct= threshold cycle value is a point on the curve where the amount of fluorescence begins to increase rapidly, usually some mean square deviations above the baseline. A comparison of the Ct values between multiple reactions allows calculating the concentration of the nucleic acid to be quantified.

Total RNA was isolated (RNeasy kit, Qiagen) and cDNA obtained and amplified by the SensiMix SYBR Hi-ROX kit (Bioline, London, UK). Templates were amplified by the 7900HT Fast Real-Time PCR System (Applied Biosystems, Cheshire, UK) for 40 cycles according to the following protocol: 95 ° C for 15 seconds, 60 ° C for 10 seconds, 72 ° C for 30 seconds. Primer sequences used are: GAPDH forward reverse and NOX4 forward reverse. GAPDH was considered as housekeeping. The reaction products were analyzed by SDS 2.1.1 Software (Applied Biosystems, Cheshire, UK).

	STEP 1	**STEP 2**	**STEP 3**
TEMPERATURE(°C)	95	60	72

TIME (sec)	15	10	30

3.10 Facs Analysis

The HUVEC at a confluence of 80%, after starvation and 24h treatments both in normoxia and in hypoxia as described in section 2.3 were trypsinized and centrifuged 330g for 5min. the pellet was resuspended in PBS/FACS (PBS + 2% FBS).About 6×10^5 HUVEC were stained for 30 min with VCAM also known as CD106 and ICAM also called CD 54 primary antibodies directly conjugated Pe/cy7 and APC respectively (Biolegend Cat 305809. and Cat. 353115). As per protocol indicated by the company both antibodies were used at 1:20 dilution of cells. Cells were then washed and Flow cytometry was performed using FACSAria II (B&D) and data were acquired and analyzed by FlowJo software.

3.11 small RNAseq of PL

PL samples (1ml) were centrifuged at 16000 g in order to remove debris, platelets or cells, and RNA was extracted from 200 µl of the fluid, using the Qiagen miRNeasy kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions with the necessary modifications. Syn-cel-miR-39 spike in synthetic RNA (Qiagen) was added to monitor extraction efficiency. Purified RNA was then concentrated to the appropriate amount, run on a Bioanalyser for QC and used for library preparation. The miRNA levels were quantified using small RNAseq, on a HiSeq Illumina platform at CRG genomics core facility, Barcelona. The library was generated using the

NEBNext multiplex Small RNA kit (New England Biolabs). The samples were run on one lane of Illumina HiSeq sequencer. Bioinformatic analysis was performed by the same service institute. Briefly, after QC, the adapters were trimmed and the sequences aligned to the whole genome using Short Stack mapper, and a number of reads were counted within either coding or non-coding genes. Genes with less than 10 read counts were excluded from the analysis.

3.12 3D-bioprinting experiments

3.12.1 3D bioprinting process

The 3D bioprinting experiment was performed by Rizzi's laboratory (National Institute of Molecular Genetics (INGM)) using a handmade 3D mechanical extrusion microfluidic printer. It consists of three mechanical axes that allow the extrusion system to be moved along the Cartesian axes. The printer is associated with infusion pumps that are managed through software that permits to generate complex constructs of different sizes. The extrusion system is equipped with a coaxial needle, which simultaneously extrudes the bioink and calcium chloride ($CaCl_{2)}$, which causes its gelification.

The bioprinting process consists of three phases:

1. Sample blueprint generation. The blueprint is a computer-assisted design used to guide the placement of the formulated bioink into predetermined geometries that can mimic the organ histo-architecture.

2. Bioink preparation. The bioink is made up of an aqueous solution of one or more light-curing polymerizable biopolymers in which viable cells are

resuspended. This solution is extruded through the inner needle of the coaxial system. At the end of the nozzle, a second solution of $CaCl_2$, flushed through the outer needle of the coaxial system, induces the solidification of the bioink in the form of a hydrogel simultaneously to its deposition. The result of this sudden gelation, coupled with the motorized 3D bio-printer system, allows the deposition of micro-fibre hydrogels in which living cells remain encapsulated.

3. Biomaterial crosslinking. At the end of the printing process, the sample is further cross-linked with UV light in order to allow the crosslink of PF polymeric chains.

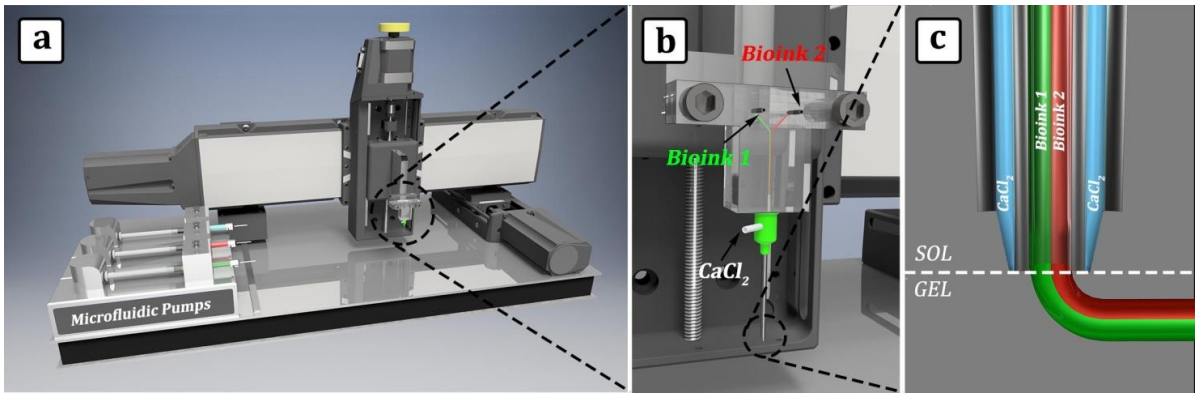


Figure 7 a-c.

a) 3D Bioprinter, b) microfluidic chip bearing a Y channels junction for bioinks, c) needle-coaxial system

3.12.2 Bioink formulation and 3D-bioprinted constructs generation

Gelatin (type A3 from porcin skin) methacrylamide (GelMa) was used as hydrogel to perform tridimensional bioprinted culture and was prepared by reaction of gelatin with methacrylic anhydride.

Human umbilical vein endothelial cells (HUVECs) purchased from Invitrogen were employed to obtain the final bioink. The cells were cultured in EBM2 complete medium (Lonza) at 37°C and 5% CO2, and used to perform 3D culture experiments when 80% confluency was reached.

HUVECs were trypsinized, centrifuged and resuspended in a freshly prepared bioink stock solution at a final concentration of $10x10^6$ cell/ml. 1mg/ml of Irgacure 2959 was added to the bioink to act as radical photoinitiator. GelMA and alginate (ALG), employed for the formulation of the bioink, played different roles in the 3D bioprinting process. ALG was used only as temporary material template to allow a precise deposition of the hydrogel fibers loaded with cells through a custom-built co-axial printing head, delivering simultaneously the bioink and a cross-linking solution of $CaCl_2$. GelMa, after UV-light exposition, generated a covalently cross-linked matrix in which embedded cells can spread, proliferate and differentiate.

Bioink, loaded with HUVECs, and $CaCl_2$ (0.3 M), were loaded in 2ml sterile Hamilton glass syringes, which were placed on microfluidic pumps. For all experiments, 10 layers-thick constructs have been generated with layers perpendicular to each other and 50 μm distance between hydrogel fibers in the X-Y plane. The final result of the bioprinting process with these features was a printed parallelepiped scaffold characterized by a dimension of 8×8×1mm3. Each fiber was printed in a continuous manner with the previous one, to guarantee structural stability of the 3D bioprinted constructs and,

thus, to favour the surface contact among the aligned fibers. After the 3D bioprinting process, all scaffolds were collected using a sterile spatula and placed in a 60mm dish. Then, biomaterial crosslinking was performed by exposing the scaffolds to UV light at low light intensity (365 nm, 4-5 mW/cm2) for 5 min. The bioprinted samples were washed with 25mM HEPES buffer containing 2mM EDTA for 2 min and then cultured in growth medium to remove as much ALG as possible.

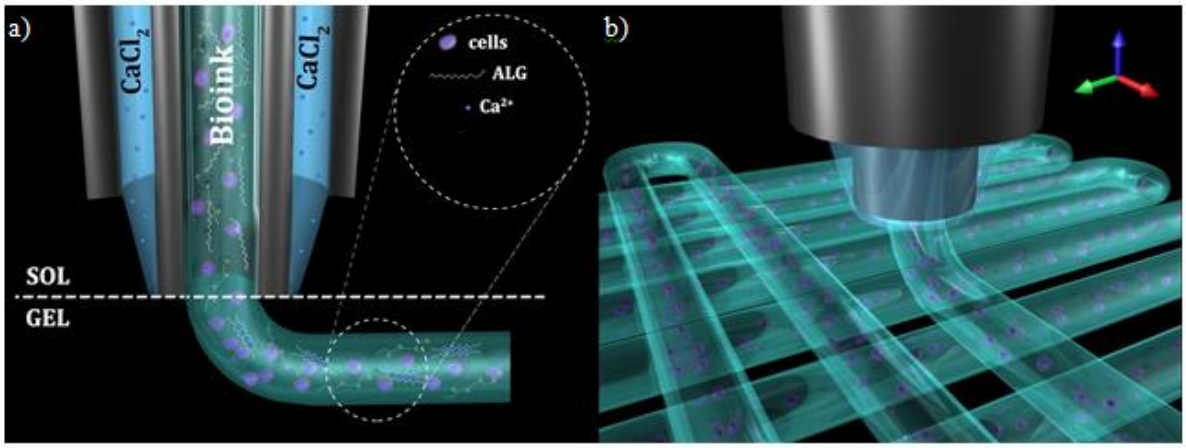


Figure8. 3D bioprinter operation scheme.

a) The coaxial nozzles system, loaded with bioink and calcium chloride solution: at the interface between the inner and the outer coaxial nozzle takes place bioink gelation, b) deposition of overlapped layers of printing fibers

3.12.3 Platelet lysate (PL) treatment and immunofluorescence analysis

The effect of platelet lysate was assessed in vitro on 3D bioprinted construct. In 3D culture, HUVECs were starved over-night and subdivided in 5 experimental groups: (1) EBM2, (2) EGM2, (3) PL10%, (4) PL10% 0.8µm, (5) PL10% 0.22µm. After 48 hours, samples were collected for histological analysis. Bioprinted constructs were washed with PBS for 10 min and fixed in

4% PFA for 2 h at room temperature (RT). Then, the samples were washed twice with PBS 1X for 1h and permeabilized in 0,3% TRITON X-100 (Sigma) diluted in PBS for 20 min at RT to gain access to the intracellular antigens. Thereafter, samples were incubated in a blocking solution containing 5% Bovine Serum Albumin (BSA, Sigma) diluted in PBS for 30 min to saturate the non-specific sites and to further permeabilize. The following primary antibodies were incubated in 0,5% BSA solution at 4°C o.n and used at 1:100 dilution: anti-CD31 (α-CD31, Abcam cat Ab24590) and anti-Von Willebrand Factor (vWF, Abcam cat 11713). Successively, the constructs were incubated with the appropriate secondary fluorophore-conjugated antibody. Nuclei were detected with DAPI (4',6-Diamidino-2-Phenylindole, Life Technology). Laser scanning confocal microscopy using a TCS SP5 microscope (Leica Microsystem) was employed to image the labeled constructs. Analyses were performed in sequential scanning mode to rule out cross bleeding between channels. The HUVEC angiogenic ability on 3D culture was evaluated quantifying the percentage of endothelial area (CD31/vWF+) inside the fibers using ImageJ software.

3.13 STATISTICS

The data obtained in each experiment were performed for a minimum of three experiments to ten and were presented using graphs and dot plots.
Statistical analyses were performed using the GraphPad Prism 5 scientific 2D graphing and statistics software available for Windows. For the t-test (queue2;type2) was used to compare the individual data. Values of $p \leq 0.05$

were considered statistically significant. Results are expressed as mean ± standard deviation.

4.RESULTS

Firstly, we asked whether human PL may contain EV. To this aim, PL-derived EVs were isolated according to methodological standardized guidelines by high-speed ultracentrifugation [128], then labelled by a green fluorescent dye (CFSE) and analyzed by cytofluorimetry to determine the size distribution by comparison with polystyrene beads[129,130]. Moreover, in order to discriminate the contribution of the different dimensional classes of EV contained within PL, this latter was depleted by employing a selected filtration (0.8 and 0.22μn) prior to FACS analysis. We used beads of defined sizes to set detection thresholds as already reported[131,132]. Our instrument was able to detect beads of 160/200 nm in diameter which is grossly comparable to EV of 300nm, in line with reported observations [133].

Results have shown that unfiltered PL contains EV of heterogenous size with a diameter ranging from <200 to >800 nm (Figure 9). The filtration at 0.8 and 0.22μn was effective in selectively depleting the >800 and the >200nm EV, respectively, therefore progressively enriching the PL-based preparation of increasingly smaller EV (Figure 9A). Extravesicles were also further characterized by evaluating their phenotype and their morphology by cytofluorimetry and transmission electronic microscopy (TEM), respectively. Results (Figure 9B) have shown EV mainly expressed CD41 (50.61%±30.01, glycoprotein IIb, a platelet membrane protein, therefore a source marker),

and exhibited low percentages of Annexin V (1.04%±0.98), CD154 (1.68%±1.39, a marker of immune regulation, expressed on larger vesicles and in the platelet-derived vesicles after myocardial infarction [38,134]), CD31 (0.67%±1.17, endothelial marker [135]), CD63 (3.7%±3.83, the tetraspanin canonical exosomal marker [136]) and also very low expression of double-positive CD41/CD63 (0.76%±0.04).

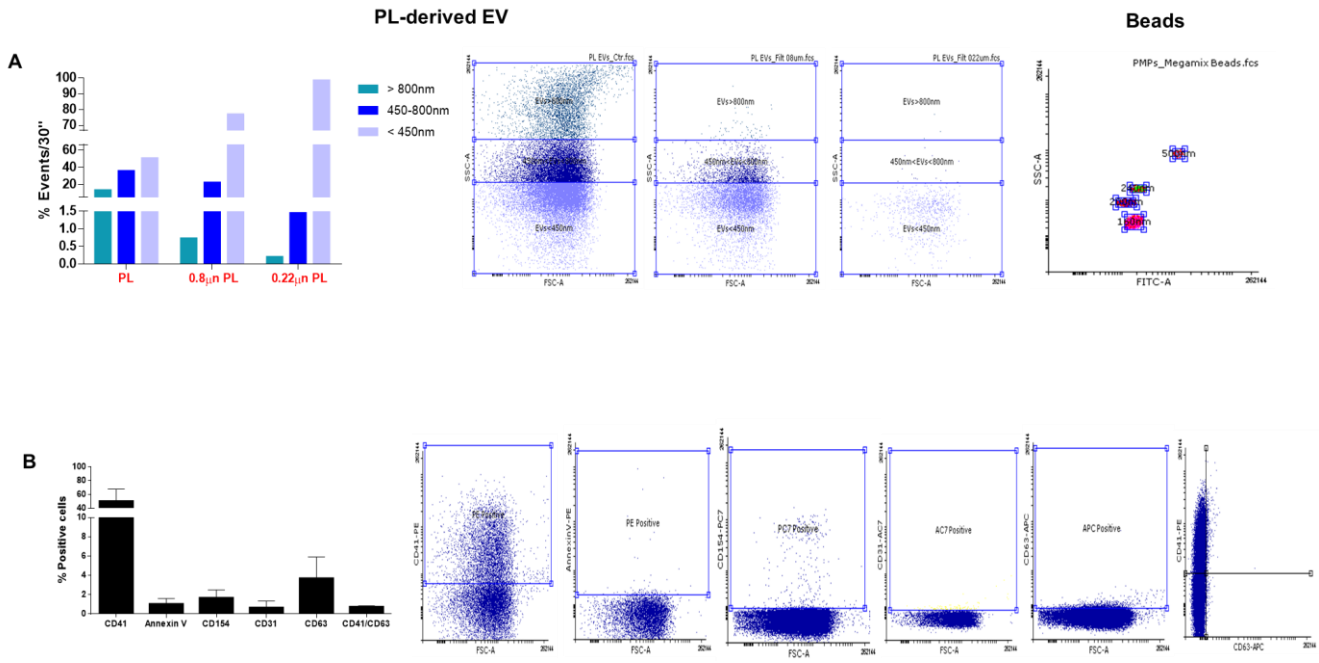


Figure 9 A-B

FACS analysis to characterized PL-derived EV. A) The graph and dot plot show shape and granularity (SSC and FSC axis) of tree different PL-derived EV size in PL, filtered 0,8 and 0.22µm PL conditions. The beads graph used to calibrate FACS EV-detection. B) The graph and dot plot are shown EV CD41+ and low percentages of Annexin V, CD154, CD31, CD63 respect to FSC-a axis. The last right dot plot show a low double positive CD41+/CD63+.

The FACS analysis was qualitatively implemented by the TEM, which has confirmed that PL preparations contain EV with heterogeneous dimensions and roundish morphology (Figure 10A). The TEM also revealed the presence of several <200nm EV harder to detect by flow cytometry. All EV appeared electron dense, suggesting a significant cargo. The ultracentrifugated and labelled PL derived EV (the whole pool of EV in PL or 10% EV correspondent to the percentage of PL routinely employed in cell culture) were uptaken by endothelial cells after 24 hours of treatment as demonstrated in the cytoplasm of HUVEC by the presence of green fluorescent dots (Figure 10 B). Coherently to the depletion by employing the selective filters, green fluorescent EV staining was fainter (Figure10B).

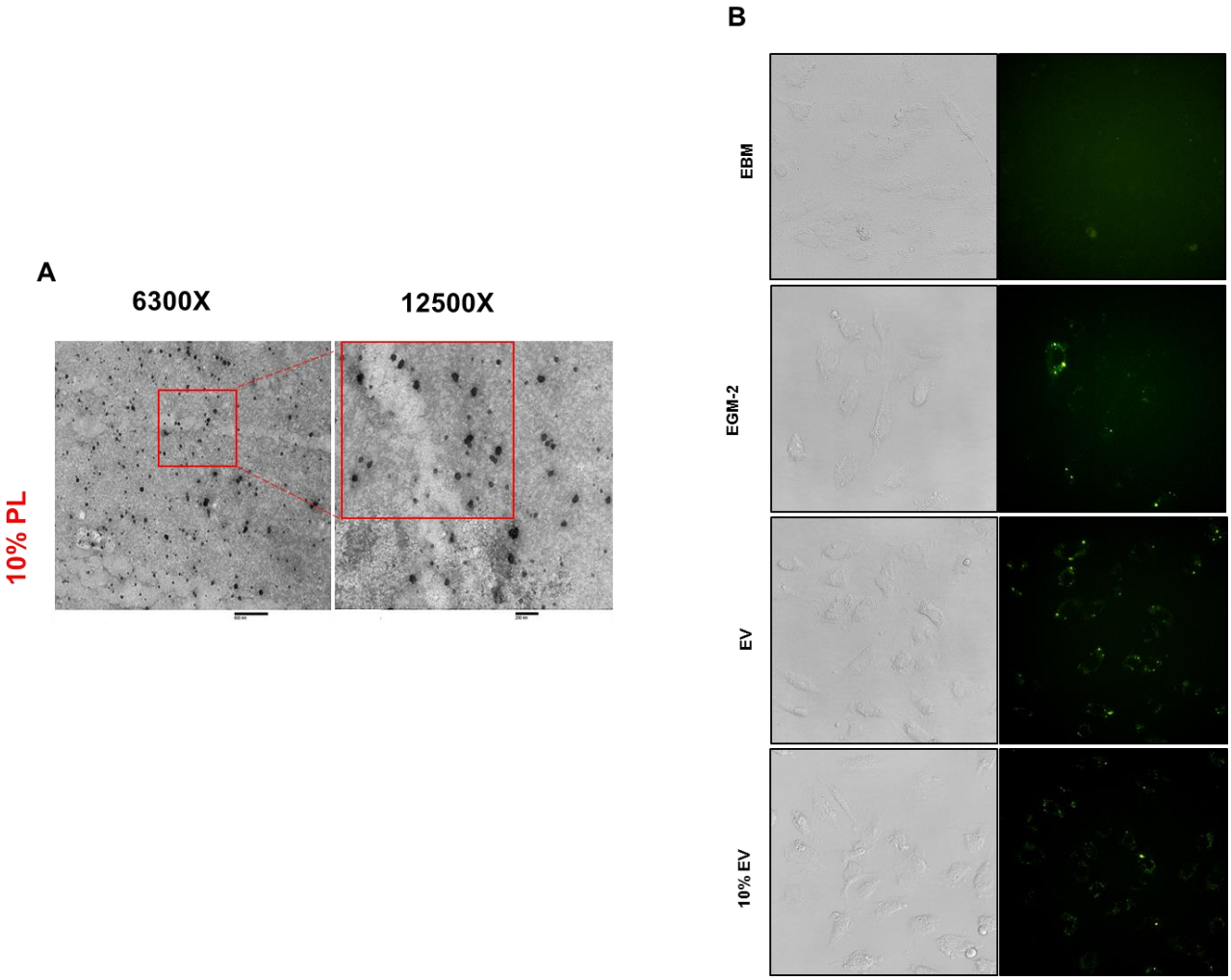


Figure 10 A /B

Qualitative TEM and immunofluorescent analysis. A) The images obtained with TEM show at 6300X and 12500X magnification to 10%PL, the scale bar indicates 200nm. B) The image represents EV-PHK67 staining on cytoplasm of HUVEC. The merge to the bright field and FITC channel (left column) and solo FITC channel in right column. EBM was negative control, EGM-2 contain EV derived from FBS labeling with PHK67. 4X magnification.

Furthermore, two important surface markers of endothelial cells, such as ICAM and VCAM, were cytofluorimetric analyzed to evaluate the reactivity of HUVEC cells to treatment with LP. The results obtained show that ICAM, constitutively expressed by the endothelium[137], is abundantly positive in all conditions (Figure 11A). While VCAM, transmembrane glycoprotein expressed by endothelial cells under stimulation[138], is expressed in a minor percentage and in any case not divergent way among the different conditions of PL (Figure11 B) to indicate that the action of PL on endothelial cell does not act through these endothelial adhesion molecules.

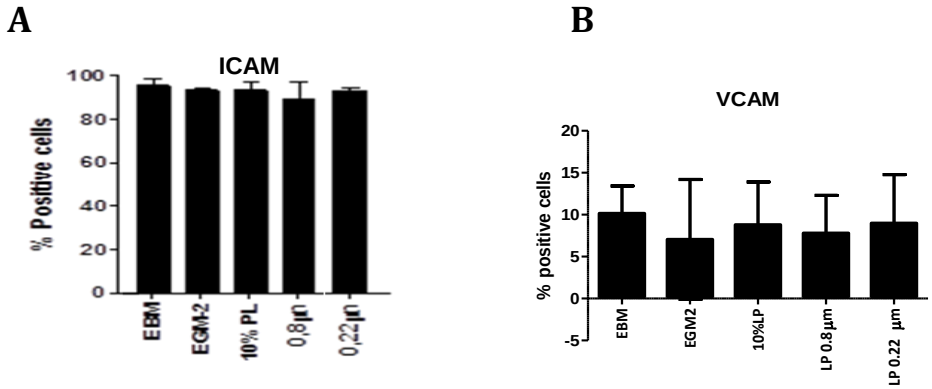


Figure11 A-B

The graphs show that there are not a statistic differences on ICAM and VCAM expression in HUVEC treatment controls (EBM and EGM-2) and 10% LP treatments.

As PL has been largely demonstrated to enhance cell migration, proliferation and exerting angiogenic properties[76,103,127,139,140], we tested the bioactivity of EV in human PL formulations and their role in contributing to these

processes. Results showed that similarly to 10% PL and EGM-2 (complete media used as positive control) the depletion with the selected filters enhanced cell migration compared to serum free media (Figure 12A, EGM-2 p=0.047, 10% PL p=0.041 and 0.8µn 10% PL p=0.047; 0.22µn 10% PL p=0.043 all *vs* EBM). A similar but even more enhanced effect was observed upon hypoxic conditioning (Figure 12B, EGM-2 $p=7.14x10^{-7}$, 10% PL $p=4x10^{-5}$, 0.8µn 10% PL $p=4.54x10^{-5}$, 0.22µn 10% PL $p=2.16x10^{-6}$ all *vs* EBM). The same results have been observed for proliferation, where the selective depletion behaves similarly to 10% PL and EGM-2 compared to negative control both in normoxic and hypoxic conditions (Figure 12C, EGM-2 p=0.047, 10% PL p=0.041 and 0.8µn 10% PL p=0.047; 0.22µn 10% PL p=0.043 all *vs* EBM normoxia; Figure 12D, EGM-2 p=0.037, 10% PL p=0.0091, 0.8µn 10% PL p=0.0177, 0.22µn 10% PL p=0.0128 all *vs* EBM hypoxia).

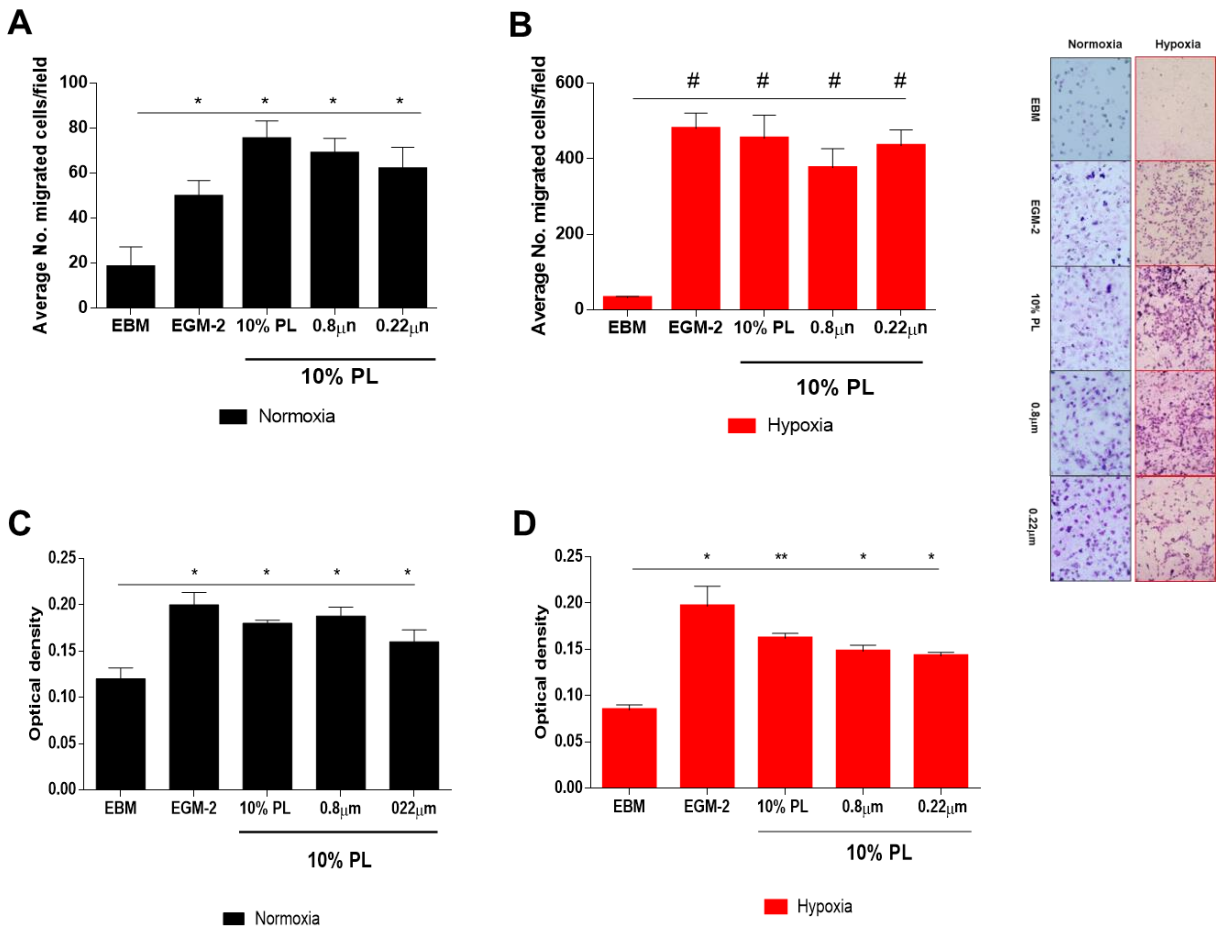


Figure12 A-D Migration and proliferation

Figure 12A-B demonstrates that the migration rate of HUVEC is the same in PL and filtered PL compared to negative control as EBM also in Normoxia (12A EGM-2 p=0.047, 10% PL p=0.041 and 0.8µn 10% PL p=0.047; 0.22µn 10% PL p=0.043 all *vs* EBM*) and Hypoxia condition (12B* EGM-2 $p=7.14x10^{-7}$, 10% PL $p=4x10^{-5}$, 0.8µn 10% PL $p=4.54x10^{-5}$, 0.22µn 10% PL $p=2.16x10^{-6}$ all *vs* EBM*). The images show a representative photo, 4X magnification, of Huvec after the migration process stained in Giemsa.*

Figure C and D The graph show that there are not a significative difference in OD of MTS in PL and filtered PL compared to EBM in normoxia (12C EGM-2 p=0.047, 10% PL p=0.041 and 0.8µn 10% PL p=0.047; 0.22µn 10% PL p=0.043 all *vs* EBM) and hypoxia state (12D EGM-2 p=0.037, 10% PL p=0.0091, 0.8µn 10% PL p=0.0177, 0.22µn 10% PL p=0.0128 all *vs* EBM).

Intriguingly, when HUVEC were subjected to the in vitro angiogenesis functional Matrigel assay, we found that in normoxia the filtration with 0.22µn of 10% PL and therefore the removal of >200nm size EV, significantly decreased the angiogenic ability of endothelial cells respect to 10% PL (Figure 14A-B, $p<0.001$), suggesting the significant angiogenic contribution of a defined set of EV contained in PL. Notably, this phenomenon was reverted by hypoxia, which significantly rescued the impaired angiogenic effect compared to 10% PL (Figure 14A-B, $p>0.05$). To confirm that the >200nm size EV were responsible for the angiogenic effect in PL preparations, we treated HUVEC directly with either the whole pool (10%) or prefiltered (0.22µn of 10%) EV. Results (Figure 14C-D) have shown that the filtered EV significantly decreased the angiogenic effect compared to 10% EV (p=0.04), which was, in turn, able to increase the number of loops in a Matrigel assay respect to serum-free condition (EBM, p=0.015) but still to a lesser extent than 10% PL as expected (p=0.0198). Interestingly, in hypoxic conditions this effect was preserved, showing the same profile found in normoxia (Figure 14C-D, p=0.013 0.22µn EV *vs* 10% EV and EBM *vs* 10% EV p=0.006).

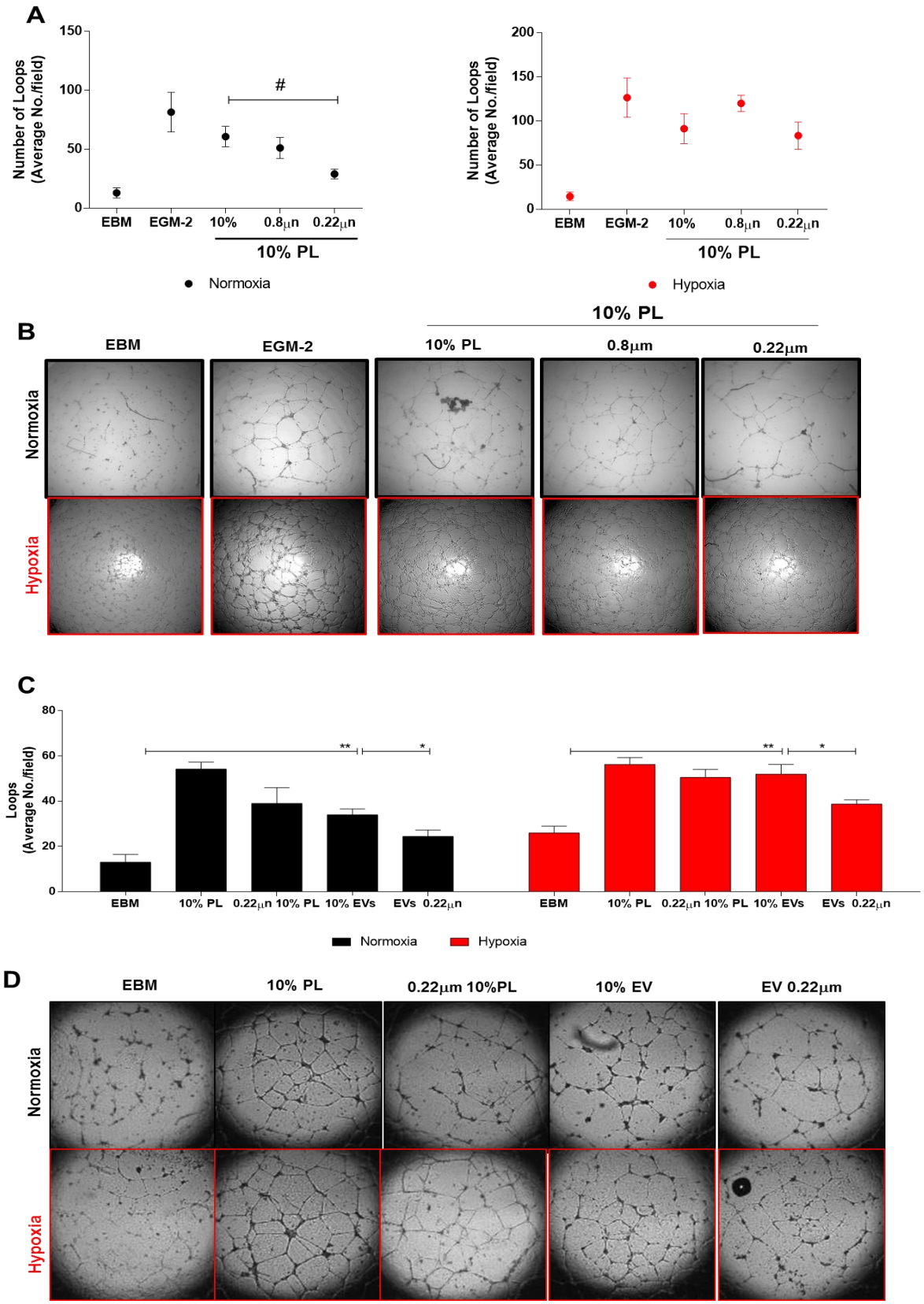


Figure14 A-D In vitro angiogenesis functional Matrigel assay

A) In black normoxia graph there is a significative difference in filtration 0.22µm of 10% PL respect to 10% PL ($p<0.001$). *The differences are absent in the red hypoxia graph(*$p>0.05$*). C) Both in black and red column the filtered EV significantly decreased the angiogenic effect compared to 10% EV. B) and D) Representative image 4X magnification of angiogenic loop formation in Matrigel assay*

To further corroborate this observation, we employed a 3D bioprinting based approach by mixing HUVEC with a bioink and evaluating their intrinsic ability to form vessel-like structures around fibers of hydrogel. The confocal microscopy analysis has shown that endothelial cells are able to fully colonize the fibers only after treatment with EGM-2 (positive control), 10% PL or 0.8µn filtered 10% PL. Coherent to the spatial distribution in the aforementioned conditions, the percentage of the endothelial positive covered area ($CD31^+/vWF^+$), which corresponded to the organization of HUVEC on the 3D tubular structures was higher than EBM (Figure 15A-B, p=0.0146 10% PL, p= EGM-2, p=0.027, 0.8µn 10% PL p=0.0061). Importantly, when HUVEC were conditioned with 0.22µn filtered 10% PL, the endothelial positive area decreased compared to 10% PL, and the tubules-like structure was lost, displaying holes (Figure 15A-B, p=0.0382).

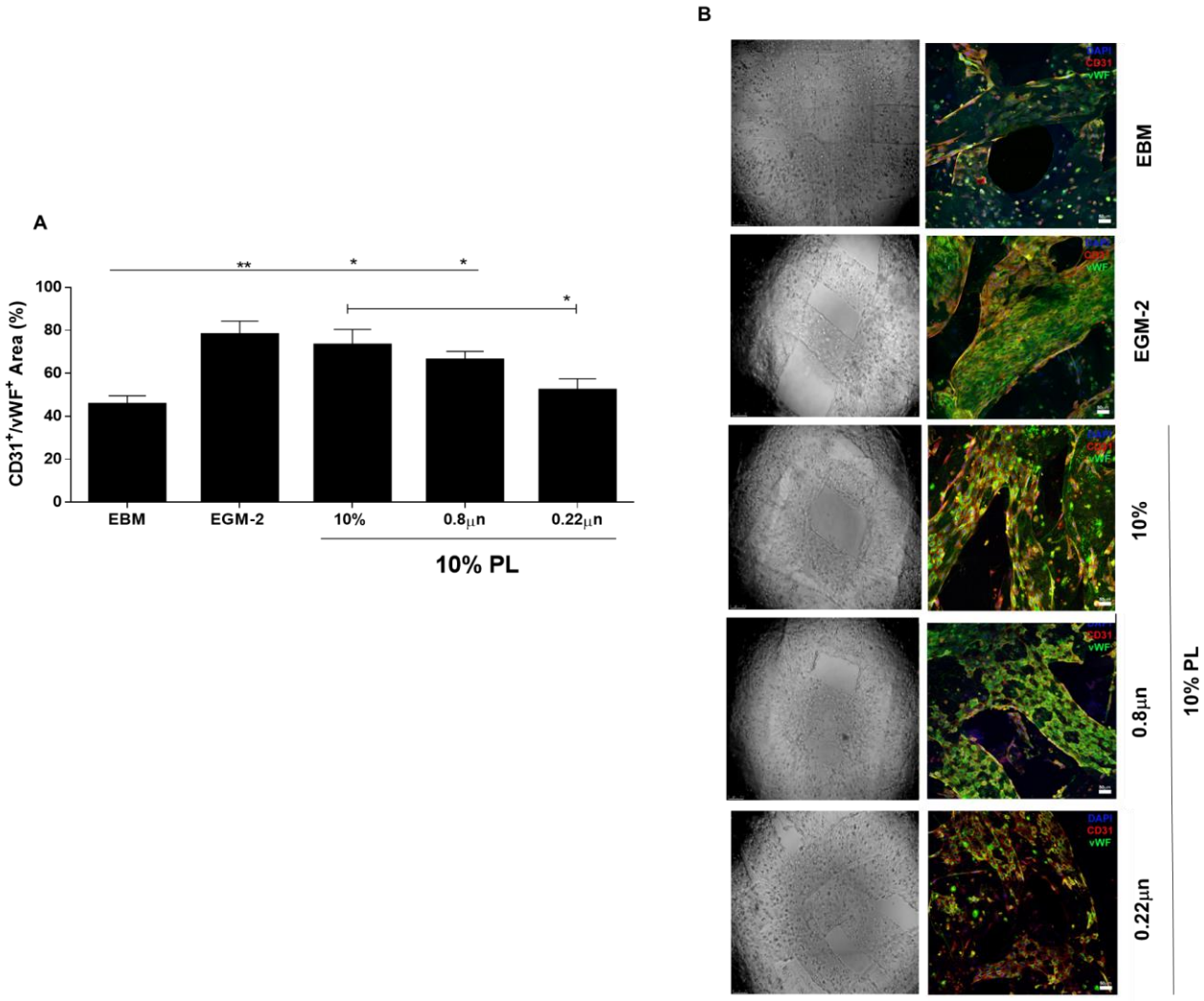


Figure15 A-B 3D bioprinting

A)The graph show the percentage of CD31⁺/vWF⁺ endothelial positive area after treatment with EBM, EGM-2, 10% PL, 0.8µm filtered 10% PL or 0.22µm filtered 10% PL that are significative different in EBM compared to EGM-2, 10% PL, 0.8µm filtered 10% PL and 10% PL compared to 0.22µm filtered 10% PL. B) Representative image to confocal microscopy of HUVEC on the 3D tubular structures. In the left column, the image was taken on the bright field and in the right column, the image was in FITC and TRITC channel. 4X magnification, scale bar 50µm.

Given that endothelial cells were able to uptake EV, which likely release their cargo in the cytoplasm and activate specific biological/molecular pathways as commonly reported in literature [141], we asked which potential angiogenesis-associated signaling EV of PL origin might have contributed.

As a large amount of studies have demonstrated the key contribution of hydrogen peroxide in modulating and preserve vascular endothelial function, homeostasis and integrity of the vascular network and angiogenesis beyond pathological scenarios [142], we verified if the depletion of EV could influence the whole production of hydrogen peroxide by endothelial cells. Results displayed that both the 0.8 and 0.22µn filtration significantly decreased the levels of hydrogen peroxide compared to 10% PL in HUVEC derived conditioned media (Figure 16A, $p=0.0151$ 0.8µn filtration and $p=0.014$ 0.22µn filtration $p=0.014$). The treatment with 10% PL enhanced the physiological production of hydrogen peroxide in HUVEC respect to negative and positive controls (Figure 16A , $p=0.0004$ *vs* EBM and $p=0.0014$ *vs* EGM-2). The hypoxic conditioning leveled out the pro-angiogenic effect in all filtered conditions compared to 10% PL (Figure 16B, $p>0.05$). High levels of hydrogen peroxide were still maintained after treatment with the sole 10% PL respect to EBM ($p=0.0078$) and EGM-2 ($p=0.0058$).

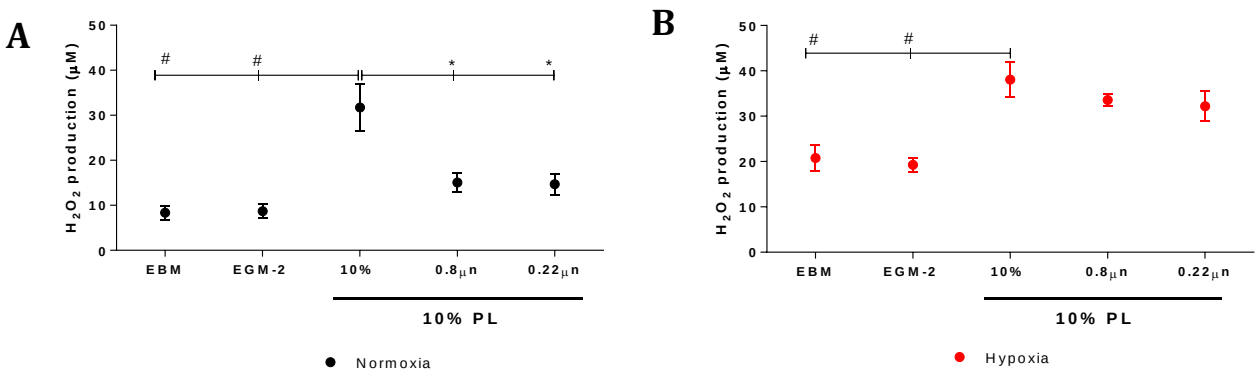
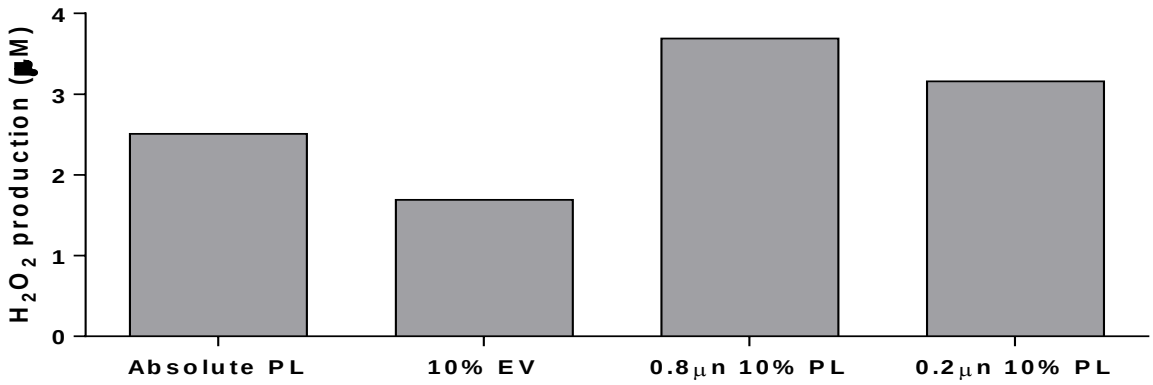


Figure16 A-B

A)In normoxia state, H_2O_2 significantly decreased in 0.8 and 0.22µn conditions compare to 10%LP. B) The 10% PL treatment present high levels of hydrogen peroxide respect to controls.

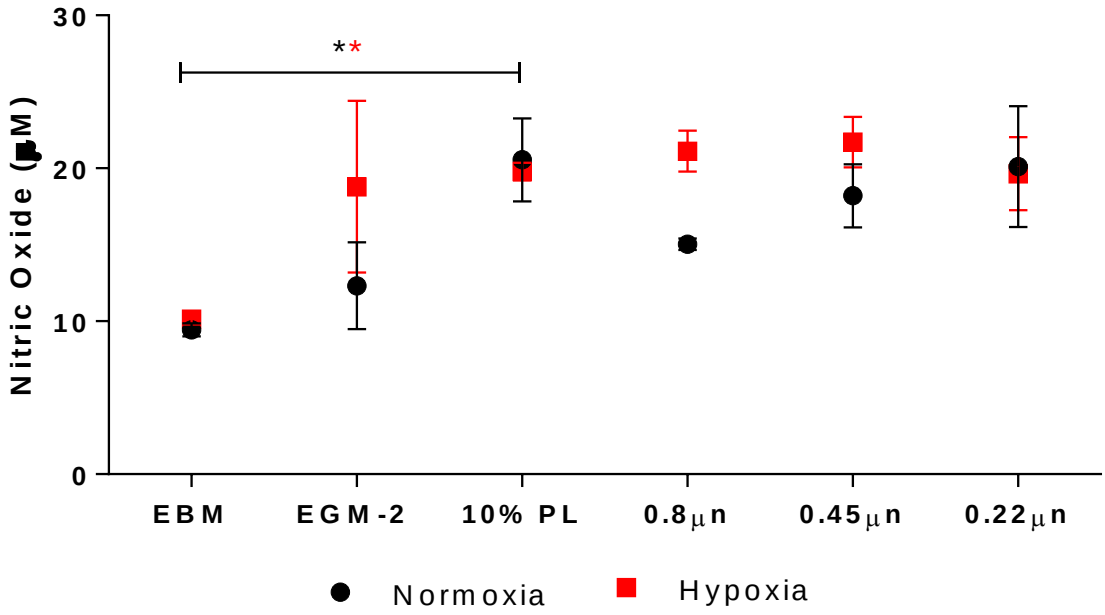
Notably, both 10% PL and the filtered forms contained a very low amount of hydrogen peroxide, ranging from 1.7-3.7 µM (supplementary Figure 17).



Supplementary Figure 17

The medium (PL, 10% EV, and 10% filtered PL) before treatment on HUVEC, present a low level of H_2O_2 production.

Given that the nitric oxide (NO) is a downstream molecule of hydrogen peroxide, we also investigated changes in NO production. Results have shown that neither normoxia nor hypoxia increased the levels of soluble NO after filtration compared to 10% PL (Supplementary Figure 18, $p<0.05$), that differently was able to enhance the soluble NO compared to EBM ($p=0.0154$ normoxia, $p=0.0001$ hypoxia), as we already observed in different cell culture in vitro models [127].



Supplementary Figure18 NO production assay.
In normoxia and hypoxic conditions, the levels of soluble NO do not change after filtration compared to 10% PL but NO level enhances the soluble NO compared to EBM.

As Nox4 is considered the main and specific NADPH isoform responsible of the direct production of hydrogen peroxide in endothelial cells [143,144], we investigated its potential involvement. To this aim, we asked if the observed

alterations in hydrogen peroxide production and angiogenesis concomitant to the depletion of the >200nm size EV in PL, could reflect changes in Nox4 expression, mainly regulated at transcriptional level [145].

Results based on depletion displayed that the 0.22μn filtration was able to significantly enhance Nox4 mRNA levels compared to 10% PL (Figure 19A, p=0.0021). The hypoxic conditioning was able to equalize the levels of Nox4 in all conditions (Figure 19A, p>0.05 all). Moreover, to further investigate the association between alterations in transcriptional levels of Nox4 and medium-large EV (>200nm), we treated the cultures directly with the sole EV. Results showed that the whole pool of EV (10%) decreased Nox4 mRNA levels compared to 10% PL (Figure 19B, p=0.04), but the removal of the >200nm fraction from the whole pool of EV did not alter this scenario, suggesting that the entire pool of EV is likely to contribute to the modulation of Nox4 rather than a specific set. Interestingly, hypoxia overturned this scenario, by flattening these differences among conditions (Figure 19B, p>0.05 all).

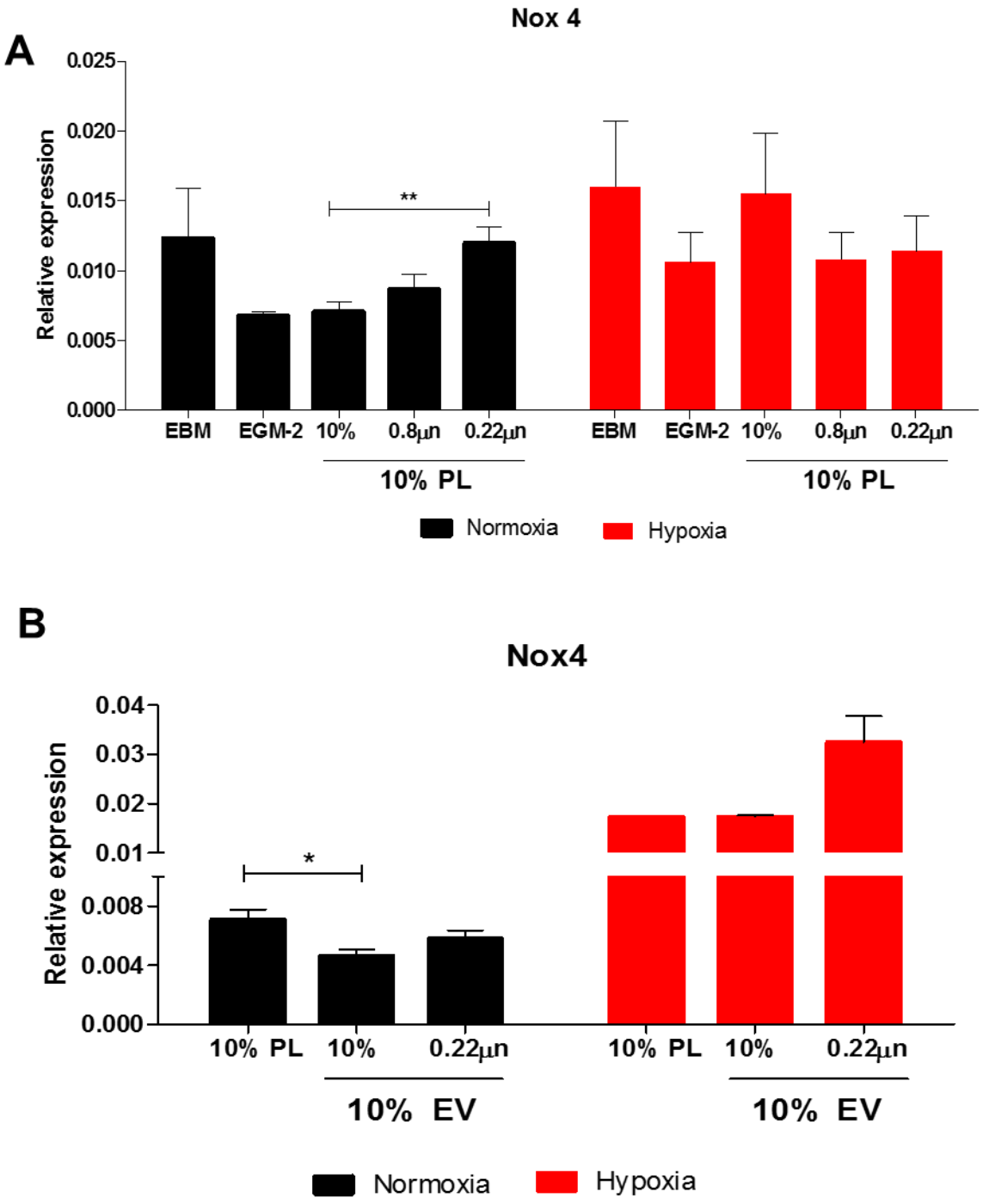


Figure19 A-B Real Time PCR relative NOX4 expression

A)The graph displayed that the 0.22µn filtration was able to significantly enhance Nox4 mRNA levels compared to 10% PL in normoxia status but in the hypoxic

conditioning, the levels of Nox4 in all conditions is equalized. B) Results showed that the whole pool of EV (10%) decreased Nox4 mRNA levels compared to 10% PL, but not significative differences in EV-0.22µn filtration. In hypoxia condition there are flat results in all conditions (10%PL and 10% EV).

Some key functions of platelets such as aggregation and activation are known to be mediated by miRNAs [146,147]. This ability can also include EV, known to transfer information to target cells through miRNAs[148], and therefore determining the diversity of the biological effects in relation to the cargo within the vesicles. Coherently, we hypothesized the presence of miRNAs even in PL based formulations, by analyzing the miRNoma profile of two different batches for a total of 4 replicates. So far, the presence of miRNAs within PL based preparations has never tested.

Results have shown that the majority of the small RNA content is represented by miRNAs (43%), followed by Y RNAs (17%), antisense RNAs (10%) and lincRNAs (8%) (Figure 20A). After clustering, we selected those miRNAs fulfilling the requirement of being highly and consistently expressed among the 4 analyzed replicates (Figure 20B).

We then computationally explored the role of the top 40 miRNAs using the mirPath v.3 online tool. We interrogated the database by employing six Gene Ontology lists containing miRNAs related to specific GO terms, such as:

-*ENDOTHELIAL CELL ACTIVATION:* GO_0042118

-*ENDOTHELIAL MORPHOLOGY:* GO_0061154

-*VASCULATURE DEVELOPMENT:* GO_0001944

-*VASCULOGENESIS:* GO_0001570

-*NADPH OXIDASE COMPLEX:* GO_0043020

-*NADPH OXIDASE:* GO_0016174

The GO terms selection was performed according to the main observed effect of PL treatment on endothelial cells and therefore angiogenesis. The role of NADPH oxidase was also included.

After intersecting each list to the PL top 40 miRNA list, we extrapolated potential eligible candidates for angiogenic roles. We found that the highest overlap was with the vasculature development gene list. Afterward, we compared the results and shortlisted 5 miRNAs playing a role in both NADPH oxidase function and vascular-related pathways. The high-priority list of the abovementioned miRNAs is reported as in Table 1:

miRNAs	MIMAT entry	Average count
hsa-miR-320a	MIMAT0000510	9702
hsa-miR-148b-3p	MIMAT0000759	7451
hsa-miR-25-3p	MIMAT0000081	5237
hsa-miR-26b-5p	MIMAT0000083	2632
hsa-miR-152-3p	MIMAT0000438	1111

Table 1. *List of top 5 miRNAs in PL-formulations involved in vascular and endothelial responses*

Additionally, we found that miR126, also known as angiomiRNA and selectively expressed on endothelial cells[149], was in the top 40 miRNAs expressed in PL. Accordingly, we validated its presence in Mesengen®.

Results have confirmed that PL is enriched of miR126 and that the expression levels increased according to the increasing amount of PL tested (Figure 20C, p=0.043).

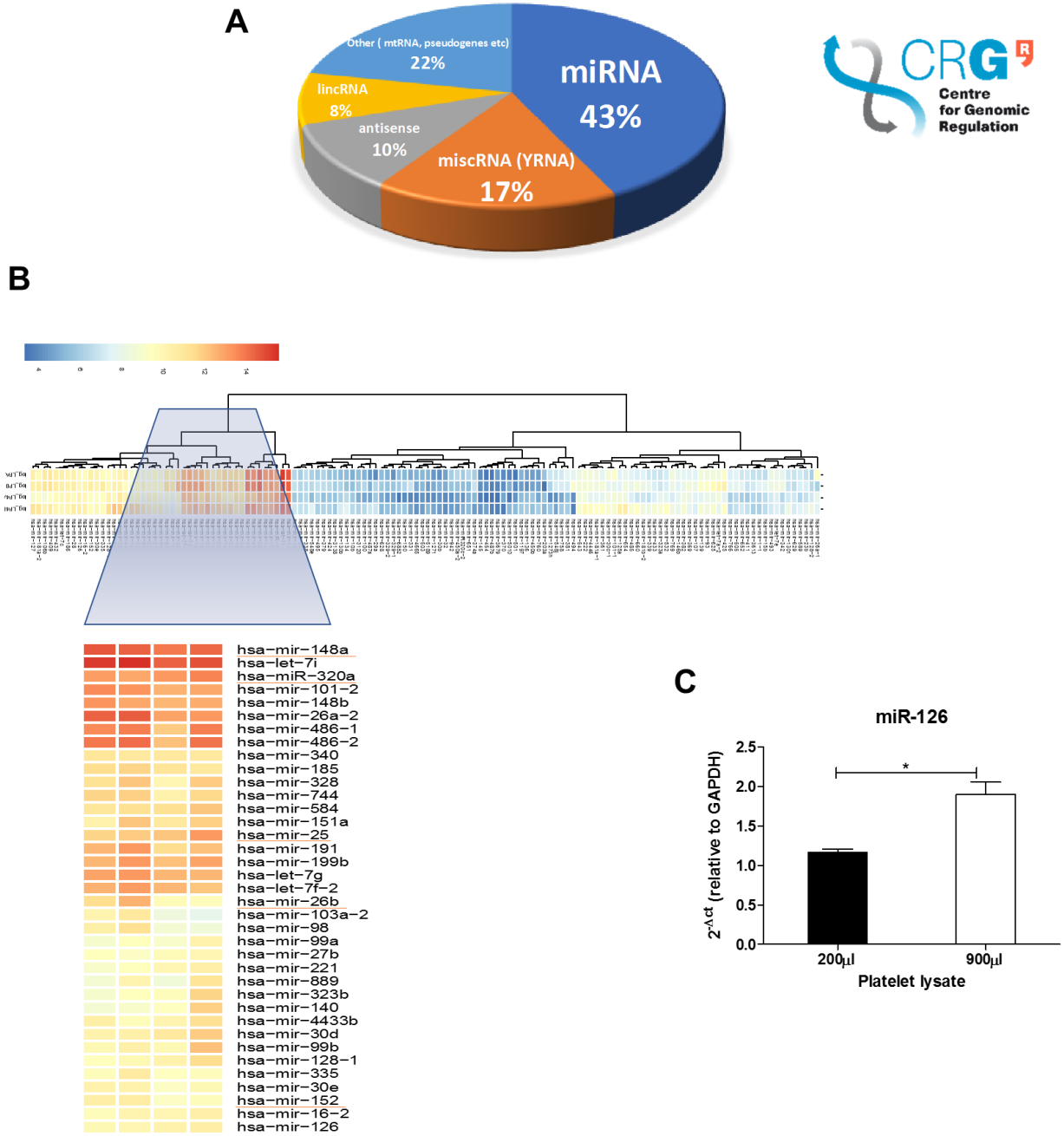


Figure 20 A-C

a)The pie chart represents the percentage distribution of small RNA, obtained by Centre For Genomic Regulation (CRG). B) The mir-PL heatmap represents miRNAs highly and consistently expressed among the 4 analyzed replicates. Specifically, the range of red-yellow color shown the miRNAs with high average copy count, instead the blue one is the lower expressed. C) Relative expression validation of miRNA-126 in different volumes of PL.

5 DISCUSSION

This study demonstrates that PL formulations contain EV which are biologically active and possess angiogenic effects on endothelial cells. We identified that the dimension of EV in PL is extremely heterogeneous but mainly composed by medium-large EV (200->800nm)[150]. Nevertheless, a limit in our technical detection by FACS of smaller EV (<200nm) parallel to a major challenge to analyze exosomes needs to be considered. Although the cytofluorimetry still remains a main acknowledged approach to study EV[128,151], however it still provides some limitations such as the employment of beads displaying different physical properties,(especially the refractive index) not appropriately reflecting those of EV.

In fact, the TEM analysis displayed a smaller EVs. Despite this, even assuming that exosomes are part of PL formulations, their presence seems to play in our system a minor role in determining the biological effects ascribable to EV within PL preparations. This aspect is also strengthening by

the phenotypic analysis of EV. The majority of detected EV expresses CD41, which represents the source marker and therefore their origin from platelets. Only a very low percentage of EV is also positive for CD63 (CD41+/CD63+), mirroring its faint expression in the whole PL, and therefore suggesting the scarcity of exosomes in the preparations. It is important to highlight that unequivocal markers to define the phenotype of EV is currently unavailable but only a panel of multiple, not exclusive and often overlapping markers[150]. The phenotypic expression of EV cannot be assumed as homogenous among laboratories and it strictly depends on the source of EV and pathological conditions. For instance, two Annexin V positive and negative distinct subpopulations can coexist among EV from the same platelet origin[152]. Accordingly, we found that Annexin V is very low expressed in Pl derived EV. In cancer patients, the levels of CD31 increase in patients with lupus erythematosus and carotid artery diseases[153,154]. These apparent discrepancies can be only explained by the fact that we are likely analyzing similar but not identical sources of EV. In other words, although EV could be obtained from blood or from platelets, they cannot be considered equal to EV derived from PL preparations obtained from a pool of donors and subjected to manufacturing processes where platelets are lysed. We themselves found differences in the expression of CD41 among batches of PL (high standard deviation on CD41 expression), highlighting the key influence of the starting material and manufacturing of PL on the phenotypic composition of EV.

Our results also demonstrate that endothelial cells internalize EV contained in PL formulations. Despite this, the intracytoplasmic uptake does not guarantee a direct contribution to all biological responses. In fact, the depletion of medium-large EV alters neither migration nor proliferation of

HUVEC. This observation can be explained by the fact that key soluble mitogenic and chemotactic mediators in PL such as VEGF, PDGF, TGF-b1, NPY, and bFGF [76,103,127,139,140] (main responsible of proliferation and migration), are not depleted in our experimental system. Besides, hypoxia employed to mimic the vascular insult, boots the migratory capacity of these mediators, increasing even more the effect. This is in line with the hypoxic effects normally exerted on endothelial cells as observed in other studies[155,156]. Notably, the system depletion helped us to understand the role of EV within the context of PL based preparations and not only as individual contributors when EV are administered to endothelial cells.

Certainly, the most significant effect is represented by the influence of PL derived EV on angiogenesis, which is not an exclusive property mediated by platelets as other cell sources can promote angiogenesis trough EV [157,158]. Our experiments on depletion and those with the enrichment of vesicles suggested that the whole pool of EV is able per se to enhance the in vitro angiogenesis (to a lesser extent than PL as expected), however this capacity is restricted to the presence of the medium-large EV set in PL. In fact, by depleting the >200nm EV fraction both in the 10% PL preparation and directly on 10% EV, the angiogenic property is nullified. Thus, it is conceivable that PL derived EV play a role in implementing the angiogenic property that PL normally possesses. The 3D bioprinted system strengthens the angiogenic role of PL derived EV, highlighting that their absence from PL formulation negatively impact the ability of endothelial cells to functionally organize in 3D tubule-like structures. Remarkably, we also show for the first time the employment of PL in a 3D model showing the ability of these formulations to foster the formation of endothelial tubule-like network and

therefore providing new in vitro and validated systems in order to facilitate an efficient replacement of animal models. Notably, hypoxia, employed as a vascular insult, boosts rather than decreases the angiogenic properties of HUVEC after enrichment and even in depletion of EV. This is likely due to the intrinsic effect of low concentration of oxygen priming angiogenesis through HIF-1□ and their downstream key targets such as VEGF, which are enhanced in an attempt of the tissue to repair the vascular damage [159]. Other studies have observed the positive influence of hypoxia on microvesicles and exosomes [160,161]. However, in our system, the hypoxic effect might be even more increased by PL already enriched of main bioavailable angiogenic soluble mediators including the same VEGF. Accordingly, the precondition of MSC with hypoxia has been reported to increase the paracrine factors contained in exosomes[162]. The increased angiogenesis after treatment with EV upon hypoxia supports their potential protective effect and suitability during vascular insult.

For the first time in this study, we attempt to describe a molecular target of angiogenesis ascribable to PL derived EV.

Notably, PL and the depleted fractions of EV contain per se very low levels of hydrogen peroxide. When HUVEC are treated with the whole PL, the levels of hydrogen peroxide increase, but in a physiological concentration range reported to regulate the beneficial angiogenic effects in endothelial cells [143,163,164], affecting arteries relaxation in a non-NO mechanism [165] and also in presence of ischemic injury [166]. Differently, only supraphysiological levels of hydrogen peroxide in endothelial cells (>100 μM)[164] result in oxidative stress and impair vascular functions, confirming the double face role of this molecule during angiogenesis [144,167]. Micromolar levels of hydrogen peroxide

have been reported to exert a similar double effect in platelets, by enhancing their activation [168] or aggregation and subsequent disaggregation upon stimulation with agonist as ADP [169] with potential effects on the microenvironment.

Thus, it is plausible that PL enhances angiogenesis by preserving the physiological levels of hydrogen peroxide in an antioxidant fashion. Additionally, our results highlight that by selective depletion of PL derived from EV, the profile of the production of hydrogen peroxide by endothelial cells mirrors that observed during angiogenesis assays either in normoxic and hypoxic conditions suggesting a potential association between the two phenomena. Hypoxia increases per se oxidative stress. Thus, it is not surprisingly if a low percentage of oxygen rescues the effect after depletion, although the levels of hydrogen peroxide remaining in the physiological concentration range.

The Nox4 isoform (the most important specific NADPH in endothelial cells) produces reactive oxygen species but it is also a sensor of the hydrogen peroxide axis, although not all studies confirm this direct relation [143,170,171]. The gene expression profile of Nox4 in experiments with depletion is not compatible with the origin of hydrogen peroxide found in the endothelial cells conditioned media. In fact, when we treat HUVEC with PL, we have observed higher production of hydrogen peroxide, however corresponding to lower transcriptional levels of Nox4. After depletion of a specific set of EV from 10% PL, this scenario is overturned, as the production of hydrogen peroxide decreases parallel but transcriptional levels of Nox4 remain elevated. Nevertheless, the direct treatment of HUVEC with the whole pool of EV implies a lower amount of Nox4 mRNA compared to PL. The depletion

of >200nm EV from the entire pool of EV did not alter the effect. Overall, these results lead us to conclude that the Nox4 isoform does not represent the primary source of hydrogen peroxide in our system. We believe that Nox4 is a downstream target negatively regulated by hydrogen peroxide. This has indicated us that the combination of Nox4 and hydrogen peroxide lead to a mutual negative feedback loop. Thus, the transcriptional effects on Nox4 are subsequent to the production of hydrogen peroxide whose production is not strictly dependent on NADPH oxidase.

In light of this, it is acknowledged that Nox4 does not represent the only and unique source of hydrogen peroxide within the intracellular compartment. Accordingly, it is plausible that spontaneous described mechanisms or additional redox systems including superoxide dismutase, glucose oxidase or xanthine oxidase [172] all responsible to generate hydrogen peroxide) might play a role in our system. We should also consider that the hydrogen peroxide-Nox4 mechanism belongs to a wider array of multiple pathways to induce angiogenesis by EV. Moreover, given that EV of platelet origin have been reported to enhance angiogenesis by the ROS-VEGF axis [173], the involvement of this last growth factor contained in PL preparations cannot be ruled out.

To date, from a molecular standpoint the modality by which clinical preparations obtained from platelets enhance regenerative angiogenesis, remains not fully explored. Platelets contain a wide range or miRNAs whose investigations so far have been restricted to functions related to aggregation and activation [174]. Current evidence reports that platelets derived miRNAs can be transferred to target cells or to the microenvironment [106,175,176]. Thus, it is conceivable that free miRNAs contained in platelets derived preparations

may represent a further mechanism to modulate angiogenesis. So far, proper screening of the miRNA profile has been performed only in intact or hyperreactive platelets, normally obtained from healthy subjects or in presence of defined cardiovascular pathologies. These reports have shown significant and differentially changes in the composition and number of miRNAs, therefore highlighting an active modification of the mRNA translation and validating their potential use as predictive biomarkers in vascular disorders [177]. In this context, PL based formulations have never been tested before, although PRP has been screened and found containing a high amount of miRNAs [178]. Interestingly, when we profiled our PL, data have displayed that the formulation reflects a similar mature repertoire of miRNAs found in human platelets and described in literature [146,147]. For instance, miRlet-7, a well-known marker of platelet differentiation and maturation [179], confirming the origin of the hemoderivative PL, is abundantly present together with additional miRNAs including defined microRNA families (miR25 and 103) [147]. This result likely suggests a common basal source cargo between platelets and platelets derived products. Thus, it is conceivable that miRNAs represent a mechanism to modulate angiogenesis even in PL derived EV, which have been already reported to act as cell-to-cell communicators by nucleic acids [180]. To date, the modality by which the platelet-derived EV cargo is determined as well as its biogenesis and release upon specific stimuli, resulting in defined biological responses, remains unclear. Accordingly, for the first time, our bioinformatic approach has allowed us to associate the angiogenesis effects of PL with the expression of defined miRNAs. This strategy has limited the wide range to only a few miRNAs including the miR320a, 25-3p, 26-b-5p, 152-3p, 148-b-3p. In

literature, very few studies have investigated the role of these top 5 miRNAs in close association to more general angiogenesis not specifically related to that mediated by platelets. Interestingly, the miR25 family has been clearly involved in the homeostasis of the physiological process and in particular to autophagy, cell proliferation, oxidative stress and inflammation [181]. Moreover, as negative modulator of the cardiac muscle growth, miR25 plays a pivotal role during cardiac hypertrophy and vascular disorders[182-184]. The Plasmacytoma variant translocation 1 (PVT1) long noncoding RNA (lncRNA) regulates angiogenesis and metastasis trough miR26[185]. The miR152 modulate endothelial differentiation of human mesenchymal stromal cells trough epigenetic mechanisms mediates by Dnmt1[186]. Additional reports are still not available. So far, we have validated miR126, also described as angiomiRNA [187,188]. Sharing with miR320 the unique expression in endothelial cells, miR126 is able to downregulate adhesion molecules such as VCAM-1 upon influence of specific cytokines, therefore contributing to vascular inflammation[149]. Strictly correlations with miR126 expression levels and patients with acute ischemic stroke have been found[189].

The wide array of miRNAs found both in platelets and PL would strengthen the concept of their novel extracellular role in modulating biological functions. We have confirmed not only the presence of miRNAs but more importantly their stability in PL preparations as described in different body fluids (i.e. serum, plasma or urine). As PL represents a cell-free system, it is plausible that miRNAs also might preserve their own biological function thanks to their high stability. Despite this, it would be crucial to understand if the 5 specific angiogenic miRNAs highlighted by the miRNoma profile are also contained in EV and in the medium-large fraction. This issue will require

the sorting of selected classes of EV by additional methodology. Besides, we will also address whether these miRNAs are modified according to adenylation or uridylation activity of their sequence.

Platelet concentrates destined for transfusion accumulate EV during prolonged storage [127], emphasizing the employment of PL-base preparations as a potential major reservoir of vesicles with angiogenic ability. Notably, our study has been focused on PL as Platelet-rich plasma (PRP) is not equally effective on angiogenesis and tissue regeneration[190], likely due to the excessive variability among batches but also due to the fact that PL is a concentrated hemoderivate and therefore free form soluble molecules are already available in the preparation compared to PRP.

To date, the individual contribution of growth factors, cytokines, EV and additional soluble mediators within PL, has not fully elucidated in terms of regenerative angiogenesis. Certainly, the methodology to manufacture PL severely impacts the quantity and in particular the quality of EV within the formulations. This criticality is in line with main issues regarding more standardized and desirable preparations of PL, which still strictly depend from the choice of donors (age, sex lifestyle habits) and more importantly from the method use to concentrate, to lyse or activate platelets[191], all main factors to determine the final composition of EV in the formulation. Accordingly, PL formulations with excessive heterogeneity of EV might result in parallel different downstream signaling and pathways activated with a wide range of unpredictable biological effects, also depending from cells potentially targeted by clinical PL preparations.

In conclusion, PL based formulations are a source of biologically available EV and with angiogenic functions on endothelium. One of the multiple potential

mechanisms by which EV may enhance angiogenesis is mediated by hydrogen peroxide, which acts by a negative loop on the transcription activity of Nox4. The PL is enriched of several miRNAs that define the hallmark of their platelet origin. So far, we could list a small number of miRNAs (25-3p, 26-b-5p, 152-3p, 148-b-3p) with angiogenic and oxidative functions and whose role in association to platelets derived EV remain unclear. Future investigations will be required to unveil these issues.

This study supports the angiogenic proficiency of PL in fostering an angiogenic network of endothelial cells after vascular damage, confirming the cell-free therapy approach as a more secure strategic tool in clinic applications.

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