Universidad de Navarra

School of Sciences

ROLE OF MATRIX METALLOPROTEINASE-10 AFTER HINDLIMB ISCHEMIA

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Doctoral dissertation presented by Violeta Gómez Rodríguez to fulfill the requirements for the degree of Doctor by the University of Navarra.

The present work has been developed under our supervision at the Division of Cardiovascular Sciences in the Center for Applied Medical Research (CIMA) and we hereby authorize its presentation for the defense.

Pamplona, February 2015

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To my parents,

To Manu,

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ABBREVIATIONS

Abbreviations

ABI: ankle - brachial index
ActD: actinomycin D
AMI: acute myocardial infarction
APC: allophycocyanin
ApoE: apolipoprotein E
bFGF: basic fibroblast growth factor
CLI: chronic limb ischemia
CRP: C-reactive protein
CVDs: Cardiovascular diseases
DAPI: 4',6-diamidino-2-phenylindole
DNA: deoxyribonucleic acid
ECM: extracellular matrix
ECs: endothelial cells
EVs: extracellular vesicles
FITC: fluorescein isothiocyanate
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
H&E: Hematoxylin and eosin
HDAC7: histone deacetylase 7
HDL: high density lipoproteins
IC: intermittent claudication
ICAM-1: intercellular adhesion molecule 1
IL: interleukin
IMT: intima-media thickness
KC: keratinocyte chemoattractant
KO: knock-out
LDL: low density lipoproteins
MCP-1: monocyte chemoattractant protein 1
MMPs: matrix metalloproteinases
MP: microparticle
mRNA: messenger ribonucleic acid
PAD: peripheral arterial disease
PAI-1: plasminogen activator inhibitor 1
PAR-1: protease activated receptor 1
PCR:polymerase chain reaction
PDGF: platelet-derived growth factor
PE: phycoerythrin
PS: phosphatidylserine
RBC: red blood cell
rhMMP-10: recombinant human matrix metalloproteinase-10
ROS: reactive oxygen species
SEM: Standard error of the mean
SMCs: smooth muscle cells
TA: tibialis anterior
TBST: tris buffered saline with Tween
TF: tissue factor

TGF-β: transforming growth factor β
TIMPs: tissue inhibitors of metalloproteinases
TNFα: tumor necrosis factor α
tPA: Tissue plasminogen activator
VEGF: vascular endothelial growth factor
VLDL: very low density lipoproteins
WT: wild type

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1-INTRODUCTION

1.1 Cardiovascular diseases of ischemic nature

Cardiovascular diseases (CVDs) derived from ischemic events are the world leading cause of death, mainly owed to heart disease, stroke and peripheral arterial disease (PAD). An estimated 17.3 million people died from CVDs in 2011, representing 30% of global mortality and are projected to remain the single leading causes of death worldwide. It is predicted that by 2030, almost 23.3 million people will die from CVDs [1,2].

CVDs also represent a major economic burden on health care systems in terms of direct (e.g. hospitalizations, rehabilitation services, physician visits, drugs) and indirect costs associated with mortality and morbidity (e.g. losses of productivity due to premature mortality and short- or long-term disabilities). Europe spent 12% of the total European health care expenditures in CVDs in 2006. Hospitalizations and drugs represented 57% and 27% of the overall direct costs, respectively. Indirect costs associated with productivity loses (21%) and informal care (17%) corresponded to 38% of the total cost [3].

The aetiology of the ischemic cardiovascular pathologies is diverse, although it is mainly related to the development of atherosclerosis. Atherosclerosis is a general pathological process with a years-long development which presents eccentric and focal thickening of the intima and media artery layers, establishing the so called atherosclerotic plaque [4]. The gradual growth of the plaque deals with a progressive obstruction of the arterial lumen and with the constant threat of plaque rupture, with the ulterior thrombus formation, release and artery blockage; thus, in case of critic stenosis rate or thrombosis, the arterial flow shortage will lead to ischemia of the downstream tissue.

Epidemiological studies over the past 50 years have revealed numerous risk factors for atherosclerosis development. Some of them present an important genetic component, and others are largely environmental. As concluded in the INTERHEART study, increased lipids, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, alcohol, low consumption of fruits, vegetables and a sedentary lifestyle account for most of the risk of myocardial infarction worldwide in both sexes and at all ages in all regions [5].

The slow course of atherosclerosis is related with the absence of symptoms until a major obstruction -via either extreme plaque growth or thrombus formation, turns up.

Clinical symptoms are directly related to the location of the obstruction, mainly the coronary, the cerebrovascular and the peripheral arterial bed [6].

1.1.1 Atherosclerotic plaque development

Previously considered as a mere accumulation of cholesterol in the vessel wall, atherosclerosis is nowadays understood as a chronic inflammatory disease of the medium and large arteries. For its onset a variety of cells including circulating and resident cells of the artery wall are needed [7].

The loss of the correct endothelial function is the cornerstone for the atherosclerotic plaque development. High levels of circulating low density lipoproteins (LDLs) trigger the atherosclerotic process since LDLs passively diffuse through the endothelial cells (ECs). The infiltration and retention of LDLs in the arterial intima initiates an inflammatory response in the artery wall. LDLs undergo modification, including lipid oxidation as a result of exposure to the oxidative waste of vascular cells [8,9].

The accumulation of oxidized LDLs induces endothelial dysfunction including changes in permeability, cell adhesion and secretion of vasoactive molecules [7]. Blood leukocytes adhere poorly to the normal endothelium but if the endothelial monolayer becomes inflamed, it expresses adhesion molecules which bind ligands on leukocytes surface. Selectins, integrins and proinflammatory cytokines promote the recruitment of leukocytes into the intima. Once adhered to the activated endothelial layer, monocytes diapedese between ECs to penetrate into the tunica intima [10]. In this layer, the monocytes acquire attributes of tissue macrophages. Inflammatory mediators can augment the expression of macrophage scavenger receptors leading to the uptake of modified lipoprotein particles and the formation of foam cells [10] (Figure 1).

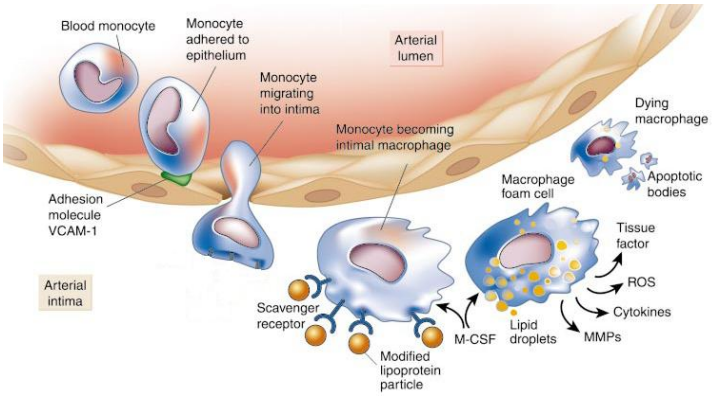


Figure 1. Formation of foamy cells in atherosclerosis. Once in the intima, monocytes differentiate to macrophages. The presence of inflammatory mediators induces the phagocytosis of the modified lipoproteins (Libby, *Nature* 2002).

The formation of foam cells and the lipid accumulation lead to the formation of a lipid-rich core as the artery enlarges in an outward, abluminal direction to accommodate the expansion of the intima. If inflammatory conditions prevail and risk factors such as dyslipidemia persist, the lipid core expands [10]. Cytokines and growth factors secreted by macrophages and T cells induce smooth muscle cells (SMC) proliferation, migration from the media to the intima, and extracellular matrix (ECM) production which aids in the formation of a fibrous cap which stabilizes the plaque [11]. As atheroma progresses, the fibrous cap tends to cover a mixture of necrotic cellular debris, macrophages, foam cells and extracellular lipid pools called necrotic core [12].

Atherosclerotic plaques can continue to grow and develop over significant periods of time (Figure 2). Providing that the plaque structure remains stable, rupture is unlikely and therefore without significant clinical consequences [13].

Activated macrophages within the plaque express several extracellular proteases such as serine proteases, cathepsins, and matrix metalloproteinases (MMPs) that disrupt macrophage and SMC function/survival and promote enzymatic degradation of the fibrous cap [14,15]. Sudden thrombosis occurs as a consequence of rupture of the plaque's fibrous cap or superficial erosion of the intimal surface [10,16]. When blood gets in contact with the tissue factor (TF) present in the plaque it instigates thrombus formation (Figure 2). Key features of rupture-prone unstable plaques are a thinned fibrous cap overlying a large necrotic core in the setting of an active inflammatory infiltrate [16,17]

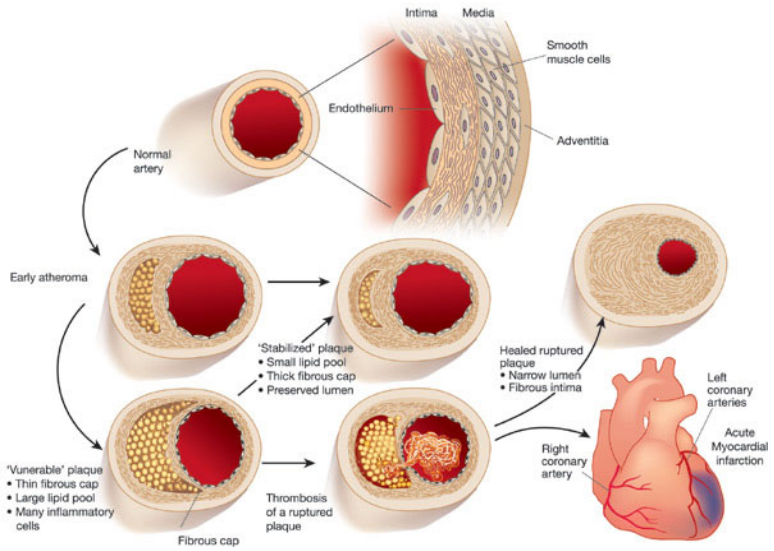


Figure 2. Schematic of the life history of an atheroma. Vulnerable plaques present thin fibrous caps, large lipid cores and great inflammatory infiltration. These plaques are at a high risk of developing thrombosis which causes ischemia at the downstream tissues (Libby, *Nature* 2002).

1.1.2 Clinical manifestations

The process of atherosclerosis begins early in life and continues at various rates, with simultaneous but different manifestations in several arterial beds [9] resulting in coronary artery disease, cerebrovascular disease and PAD [18]. Patients with established atherosclerotic disease normally present more than one vascular bed affected, defined as polyvascular disease. Its prevalence among the ischemic diseases increases from nearly 25% in coronary artery disease to 40% in stroke and to 61% in PAD patients [19].

Coronary artery disease

Coronary artery or ischemic heart disease is the clinical manifestation and the anatomopathologic consequence of ischemia at the level of the coronary arteries of the heart. It is one of the most important health problems worldwide and the leading cause of death in the CVD field [20]. In the United States specifically, 1 of every 6 deaths is caused by this pathology [2]. In the majority of cases, the reduction of the coronary blood flow is due to atherosclerotic lesions [9]. Acute and maintained ischemia causes the worst case of coronary artery disease named as acute myocardial infarction (AMI). Its most frequent cause is the intracoronary thrombosis which follows the rupture of an atherosclerotic plaque.

The pathophysiology of coronary artery disease involves the loss of cardiomyocytes function. Right after an acute ischemic insult, the affected myocardial segment ceases contracting, becomes electrocardiographically silent and begins slowly to develop a passive stretching which is imposed by the non-ischemic surrounding tissue. The immediate consequence of this contractility loss is the reduction of the ejection fraction and the concomitant cardiogenic shock [21].

Cerebrovascular disease

Around 6 million people die globally each year from stroke [22]. Stroke or cerebrovascular accident is the consequence of a sudden interruption of blood flow in the brain. This disruption can be due to the presence of a blood clot (ischemic stroke) or the rupture of a blood vessel inside the brain in which blood is spread throughout the cerebral tissue (hemorrhagic stroke). The vast majority of the stroke cases belong to the ischemic-type (87%) and most are caused by atherosclerosis. The entailing ischemia produced after a stroke can lead to irreversible neuronal damage and death in the worst cases [23]. Time is the most important variable to take into account since the longer it takes for the clot to be removed the worse clinical outcome the patient will present [21].

When the cerebral blood supply is restricted, only the tissue that was strictly supplied by the affected arterial bed gets necrosed. Surrounding the infarct area, there is a tissue area which maintains sufficient residual blood flow to preserve cell viability but not its energetic metabolism or its normal function [23]. This area is called the *penumbrae* zone and is characterized for being temporarily recoverable [24]. After stroke, neuronal damage progresses over time as consequence of harmful ischemia-triggered reactions which are maintained active even after reperfusion and worsen by the latter [25].

Peripheral Arterial Disease

Peripheral arterial disease (PAD) refers to a cluster of conditions in which atherosclerosis occurs in the peripheral circulation, particularly in the lower extremities at the levels of the femoral, popliteal and saphenous arteries [26] (Figure 3). . Its prevalence is in the range of 3% to 10% in people over 55 years, although it increases to 15-20% in persons over 70 years of age [27]. However, the prevalence of asymptomatic PAD is estimated as high as 20% of the adult population [28]. Unlike AMI or stroke, non-critical PAD patients have a relatively low risk of death although it causes substantial disability since the affected limbs are at higher risk of infection and

amputation. However, critical limb ischemia patients, present a dramatically high risk of death [29].

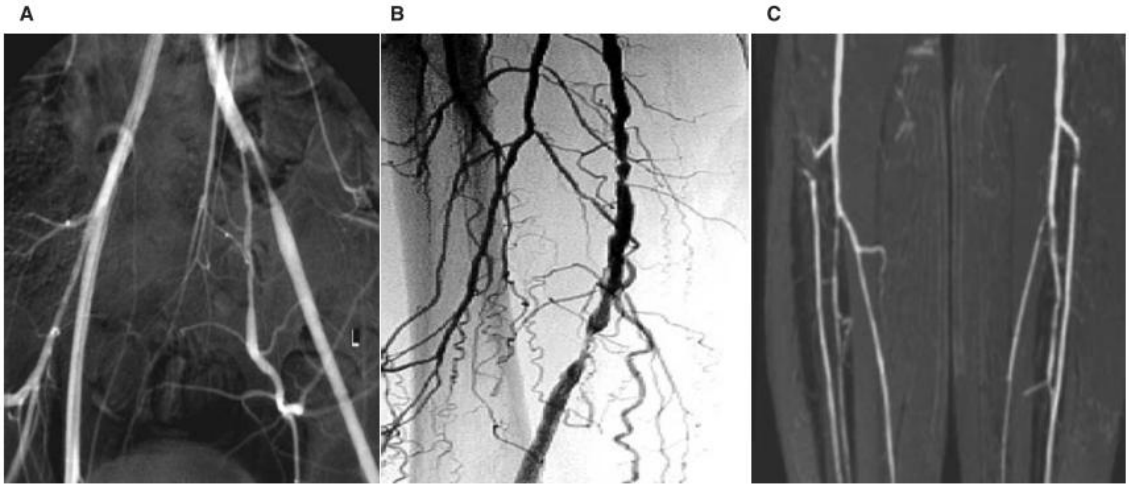


Figure 3. Typical distribution of arterial lesions in peripheral arterial disease. A) Stenosis of the left external iliac artery. B) Several stenoses in the superficial femoral artery. C) Extensive disease of the tibial vessels (Hiatt, *Cardiology Rounds,* 2006).

The reduction of the blood flow in these patients depends not only on the occluded or narrowed segment but also on the ability of the organism to counterbalance the increase in cardiac output, development of collateral vessels and dilation of the peripheral arteries [21]. The loss of blood supply to the limbs can seriously affect the patient's everyday life by impairing their ability to walk, by causing permanent pain or by undergoing amputation in the worst cases.

There are two classification models for PAD patients based on the symptomatology they present, the Fontaine classification (the most used by physicians) and a more detailed and renewed categorization, the Rutherford classification (Table 1). The limit between symptomatic and asymptomatic patients in both scales is given by the presence of intermittent claudication (IC), the hallmark of PAD. IC refers to impairment in walking which is defined as reproducible fatigue, discomfort, or pain that occurs in specific limb muscle groups during effort resulting from exercise-induced ischemia, which goes away with rest [30].

Table 1. Fontaine and Rutherford's classification models for PAD patients.

Fontaine		Rutherford		
Stage	**Clinical description**	**Grade**	**Category**	**Objective description**
Stage I	Asymptomatic	Stage 0		Asymptomatic, normal treadmill test
Stage IIa	Intermittent claudication, pain-free walking distance > 200 m	Stage I	Grade 1	Mild intermittent claudication, treadmill exercise limited to 5 min; ankle pressure after exercise < 50 mmHg, but at least 20 mmHg lower than at rest
Stage IIb	Intermittent claudication, pain-free walking distance > 200 m		Grade 2	Moderate intermittent claudication, between Rutherford 2 and 3 disease
Stage II (complicated)	Trophic lesions with intermittent claudication but without critical leg ischaemia		Grade 3	Severe intermittent claudication, treadmill exercise limited to > 5 min; ankle pressure after exercise > 50 mmHg
Stage II	Rest pain	Stage II	Grade 4	Rest pain, ankle pressure > 40 mmHg and /or great toe pressure > 30 mmHg; pulse volume recording barely pulsatile or flat (chronic limb ischemia)
Stage IV	Ischaemic lesion (ulcer, gangrene, necrosis)	Stage III	Grade 5	Limited ischaemic lesion, ankle pressure > 60 mmHg and/or great toe pressure > 30 mmHg; pulse volume recording barely pulsatile or flat (chronic limb ischemia)
			Grade 6	Extended ischaemic lesion (above metatarsal level). Chronic limb ischaemia

PAD patients present distinct symptomatology depending on the severity of the stenosis. At the beginning, the pathology is mostly asymptomatic but as it worsens, complications arise. The perfusion to the limbs decreases as the disease progresses, a fact that is clearly apparent during exercise or walking when the oxygen demand from the muscles increases. At this point patients present pain at the level of their lower extremities pushing them to rest in order to be able to continue with the activity (IC) [30]. When the ischemic conditions are severe (critical limb ischemia or CLI), patients present pain at rest and in the worst cases amputation of the limb must be performed since gangrene could cause death [31].

Doppler ultrasound measurement of the ankle artery pressure and its relation to the branchial pressure (ABI test) has proved to be the most effective, accurate and practical test for detecting PAD. Ratios <0.9 indicate a lower pressure in the limbs that is normally the result of the presence of atherosclerotic plaques [32]. The treadmill walk is also a very useful tool for the diagnosis of those patients with apparently normal ABI. If the patient presents plaques at the lower limbs, his ankle pressure levels will exhibit a

drop greater than 20-mmHg after 1 minute of this exercise. Once PAD is diagnosed, colour-flow duplex imaging and magnetic resonance angiography are excellent methods for evaluating the anatomy of the lower extremities and will help in determining the most convenient treatment for the patient [26].

Depending on the symptoms, the treatment will differ but in most cases the main action points are control of the risk factors, antiaggregation treatment, improvement of the limb vascularisation through controlled exercise and in the worst cases surgical bypass or endarterectomy [33].

1.2 Pathophysiologic mechanisms in peripheral artery disease

The skeletal muscle is the tissue in the limb that is most vulnerable to ischemia. Most human skeletal muscles contain a mixture of three different types of myofibers. Type 1 are slow-twitch and fatigue-resistant oxidative fibers, type 2A are fast-twitch and moderately fatigue resistant oxidative fibers and type 2B myofibers are fast-twitch and not fatigue-resistant with low oxidative activity and low mitochondrial presence. The proportion of the different types of fibers in each muscle determines its overall metabolism and its reaction to damage [34].

When ischemia occurs, there is extensive energy depletion in the muscle that correlates with enhanced muscle necrosis. The necrosis of the fibers triggers the beginning of different overlapping processes, including inflammation and tissue necrosis that will lead to the regeneration phase where the skeletal muscle will recover its morphology and function. The first reaction of the injured muscle is the release of chemokines and pro-inflammatory cytokines for the attraction and activation of inflammatory cells [35]. The damaged blood vessels of the necrotic area allow the invasion of these cells, which mainly belong to the innate immune response [34,36]. Polymorphonuclear cells have been shown to be the first to arrive at the damaged area [37] and once in the muscle, they start phagocyting dying myocytes and releasing different cytokines and chemokines for the recruitment of monocytes and other inflammatory cells that will replace neutrophils in the inflammatory response [36,38]. The correct balance between pro- and anti-inflammatory cytokines is necessary to promote the correct activation of the regenerative process. The maintenance of this balance determines if the final outcome of the inflammatory response would be detrimental or beneficial [35].

At the same time, the formation of new myofibers starts by the activation of the skeletal muscle stem cells (satellite cells) by the release of different growth factors (e.g., FGF, TGF-β, HGF, IL-6). Satellite cells are normally quiescent inactive cells which are located underneath the basement membrane of adult myocytes surrounding each myofiber. When activated, they enter the cell cycle, migrate, proliferate and differentiate into myoblast, which enables their access to damaged areas and their further differentiation into myocytes. The latter either fuse together or to damaged fibers to form new adult multinucleated myofibers leading to tissue repair (Figure 4) [35].

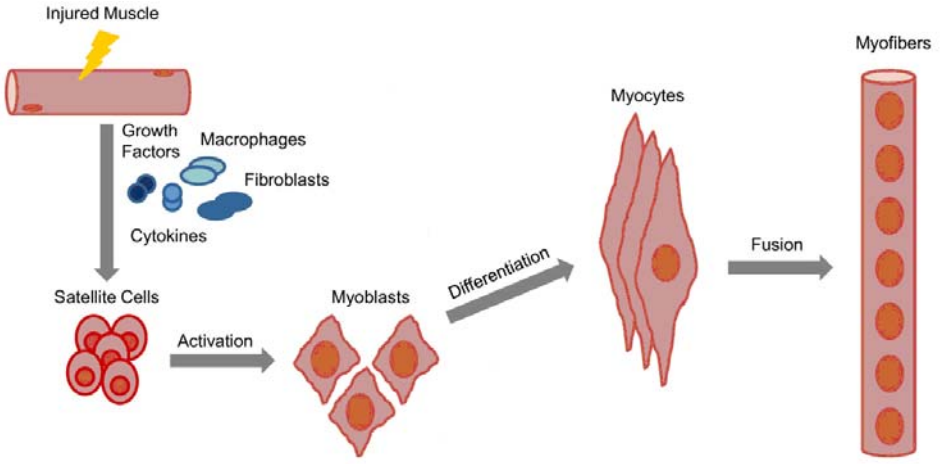


Figure 4. Skeletal muscle regeneration process. After injury, different growth factors and cytokines are released from the damaged muscle fibers and leukocytes in order to activate satellite cells. These cells become then myoblasts which are muscle cells capable to differentiate into myocytes, the myofibers basic structure. The fusion of the myocytes leads to the formation of new myofibers which will then grow becoming adult and completely functional skeletal muscle cells (modified from Burks et al., *Skelet Muscle* 2011).

Moreover, myofibroblasts will start producing ECM components (fibronectin, type I and II collagen) as an initial scaffold for the muscle after phagocytosis of the dying tissue. This deposition of ECM results in the formation of an acellular scar that in light to moderate injuries will be removed; but that in contrast, in large muscle injuries like the one resulted from acute ischemia, will prevail limiting full muscle regeneration [34].

A crucial step after a sudden drop in perfusion is the recovery of blood supply to the downstream tissue in order to provide cells with oxygen and nutrients. For this purpose, two process are required: the enlargement of pre-existing collateral vessels (arteriogenesis, Figure 5A) and the development of new capillaries (angiogenesis, Figure 5B) [39].

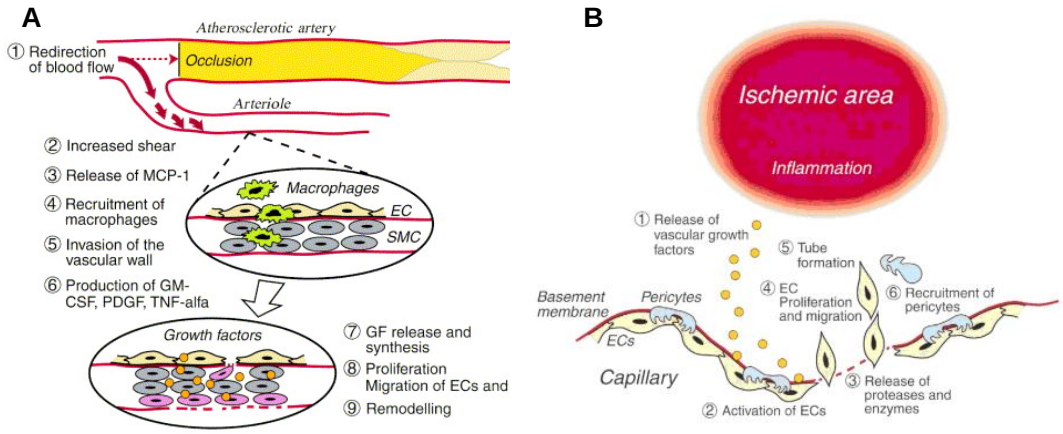


Figure 5. Arteriogenesis (A) and angiogenesis (B) after limb ischemia. In order to restore the blood supply to the ischemic tissue different angiogenic and arteriogenic factors are released by ECs and macrophages for the formation of new blood vessels and the enlargement of preexisting ones (Wahlberg et al, *J Vasc Surg* 2003).

When stenosis in a large main artery becomes hemodinamically significant, blood flow is redirected toward surrounding arterioles leading to shear stress and starting the arteriogenesis process (Figure 5A) [39]. As a result, ECs express cytokines for the recruitment of monocytes (predominantly MCP-1) and monocyte adhesion molecules (such as ICAM-1) in order to allow their invasion into the vascular wall. The recruited monocytes transform to macrophages and produce ECM components and proteases to promote tissue remodelling. Macrophages also produce large amounts of growth factors, essentially from the FGF family, which will stimulate ECs and SMC proliferation. The proliferation of these cells is followed by the disruption of the elastic internal lamina and the migration of SMC in order to form a neointima layer. The adventitia is then broken down by MMPs and plasmin providing space for the growing vessel. In addition, fibroblasts are stimulated for the production of new ECM layers that will be added to the vessel wall for the enlargement of the vessel [40,41].

As for angiogenesis, the initial step is the stimulation of ECs in the vicinity of the ischemic insult by vascular growth factors, mainly the vascular endothelial growth factor (VEGF) family of proteins (Figure 5B). VEGF increases vascular permeability enabling extravasation of plasma proteins and degradation of ECM, creating a new environment to support ECs proliferation and migration. Once in the ECM, ECs form cords with lumen by thinning the cells and by fusion with existing vessels. Different growth factors like TGF-β and PDGF allow the stabilization of the new vessels by inducing ECM production and pericyte recruitment [41,42].

The overall restoration of blood flow accompanied by a balanced inflammatory response and the formation of new myofibers leads to the recovery of the muscle morphology and function, making it undistinguishable from a non-injured one [43].

1.3 Matrix metalloproteinases

The ECM is a highly insoluble suprastructure which classically provides structural support to the tissues and is composed of a basement membrane and a stromal matrix. The basement membrane is a sheet-like structure that is in contact with the cells and is mainly made of collagen type IV, fibronectin, heparansulfate proteoglycans and laminin [44,45]. The stromal matrix constitutes the bulk of the ECM, and is comprised of larger fibrous structures (predominantly collagen type I, hyaluronan and glycosaminoglycans), that typically provides structural and biochemical support to the surrounding cells [46] . The degradation of the ECM is a common feature of normal growth and development (e.g., tissue repair, aging and cell migration [47]) although its breakdown is also modulated in many pathological conditions. The degradation process is tightly regulated by a complex interplay of cell-cell and cell-matrix interactions involving the production of activating molecules, inhibitors and regulatory molecules such as cytokines and growth factors. MMPs have been traditionally described as leading enzymes in the proteolysis of the ECM since they posses the ability to degrade and process all its components [44,48,49]. Nevertheless, the activities of these enzymes are not restricted to ECM degradation. MMPs have been found to be key regulators in cell behaviour and signalling pathways by binding and processing chemokines, cytokines, hormones, adhesion molecules and other membrane receptors [44,49–54] .The importance and the widespread biological activities of MMPs strongly suggest that their regulation could be an interesting target for drug development.

MMPs are produced in the majority of cell types, including ECs, SMCs, leukocytes and different tumour cells. Their activity is tightly regulated at three levels: transcriptional, post-translational and by the presence of inhibitors. Growth factors, cytokines and hormones induce MMP expression at the transcriptional level, while heparin and corticosteroids have an inhibitory effect [55]. MMPs are produced as zymogens requiring their activation in the tissue by cleavage of the amino-terminal pro-domain, mainly by plasmin and also other MMPs. Most MMPs are secreted enzymes although some of them (named as membrane-type MMPs or MT-MMPs) are found bound to the cell membrane. MMP activity in the tissue is regulated by the presence of two major types

of endogenous inhibitors, α2-macroglobulin, a plasma protein that acts as a general proteinase inhibitor, and tissue inhibitors of metalloproteinases (TIMPs), specific MMP-inhibitors which irreversibly bind to the catalytic domain of MMPs causing its blockage [55]. The four TIMPs characterized so far (TIMP-1, -2, -3 and -4) share similar structural features and, usually, their transcriptional regulation parallels to that of MMPs.

Therefore, overall proteolytic activity depends on the relative concentration of the active enzymes and their inhibitors. MMP-TIMP interaction is not selective, with the exception of the strong affinity of TIMP-1 for MT-MMPs. TIMP-1,-2 and -4 are secreted in soluble form while TIMP-3 is associated with the ECM. TIMPs are secreted by different cells, including SMCs, macrophages, and platelets. Their activity is stimulated by PDGF and TGF-β and regulated by several cytokines [55]. The equilibrium between MMPs and TIMPs is critical in the maintenance of tissue integrity including the cardiovascular structure [55,56]

1.3.1 MMPs: classification and structure

At present, 25 different MMP members in mice and 24 in humans have been identified. MMPs are a family of endopeptidases characterized by a conserved Zn^{2+} binding motif in the catalytic domain and several conserved protein domains. MMPs are classified in at least seven subgroups depending on their ECM substrate specificity (Table 2) [14]. MMP-18 is not present in humans and MMP-4, -5, -6, and -22 are missing in the list since they were shown to be identical to other members.

Table 2. Classification of human MMPs and their substrate specificity.

MMP (Type)	Name	ECM Substrate
Collagenases		
MMP-1	Collagenase-1	Collagen I, II, III, VII, VIII, and X, gelatin, proteoglycans, tenascin, entactin
MMP-8	Collagenase-2	Collagen I, II, III, V, VIII, and X, gelatin, aggrecan
MMP-13	Collagenase 3	Collagen I, II, III, IV, IX, X, and XIV, gelatin, tenascin, fibronectin, aggrecan, osteonectin
Gelatinases		
MMP-2	Gelatinase A	Collagen I, IV, V, VII, X, XI, and XIV, gelatin, elastin, fibronectin, laminin, aggrecan, versican, osteonectin, proteoglycans
MMP-9	Gelatinase B	Collagen IV, V, VII, X, XIV, gelatin, elastin, aggrecan, versican, proteoglycans, osteonectin
Stromelysins		
MMP-3	Stromelysin-1	Collagen III, IV, V, and IX, gelatin, aggrecan, versican, proteoglycan, tenascin, fibronectin, laminin, osteonectin
MMP-10	Stromelysin-2	Collagen III, IV, V, gelatin, casein, aggrecan, elastin, proteoglycans
MMP-11	Stromelysin-3	Casein, laminin, fibronectin, gelatin, collagen IV, transferrin
Membrane type		
MMP-14	MT1-MMP	Collagen I, II, and III, casein, elastin, fibronectin, vitronectin, tenascin, proteoglycans, laminin, entactin
MMP-15	MT2-MMP	Tenascin, fibronectin, laminin
MMP-16	MT3-MMP	Collagen III, gelatin, casein, fibronectin
MMP-17	MT4-MMP	ND
MMP-24	MT5-MMP	ND
MMP-25	MT6-MMP	ND
Others		
MMP-7	Matrilysin	Collagen IV and X, gelatin, aggrecan, proteoglycans, fibronectin, laminin, entactin, tenascin, casein, transferrin, integrin b_4, osteonectin, elastin
MMP-12	Metalloelastase	Collagen IV, gelatin, elastin, casein, laminin, proteoglycans, fibronectin, vitronectin, entactin
MMP-20	Enamelysin	amelogenin
MMP-23A	MMP-21	ND
MMP-23B	MMP-22	ND
MMP-26	Matrilysin 2	Collagen IV, fibrinogen, fibronectin, casein
MMP-27	ND	ND
MMP-28	Epilysin	Casein

ND: not determined (Rodríguez JA et al., *Rev Esp Cardiol*, 2007)

Their protein structure is composed of five domains: the signaling peptide, the hydrophobic propeptide domain, the catalytic domain, the hinge region, and the hemopexin-like domain (Figure 6).

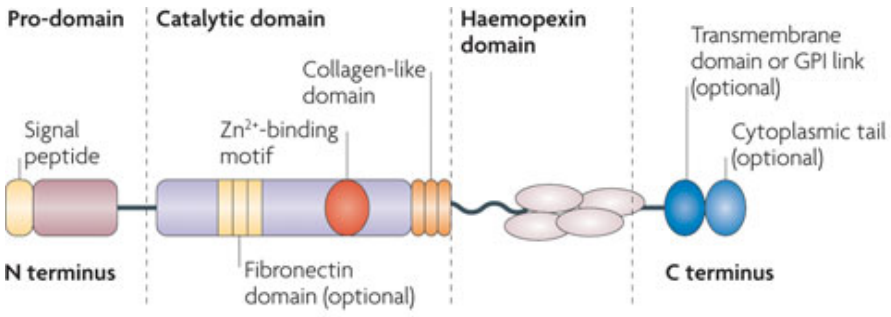


Figure 6. Structure of the MMPs family (Sorokin, L., *Nature Rev Immunol,* 2010).

The signaling peptide, at the N-terminal region, allows the association of the enzyme with the endoplasmic reticulum and its transport out of the cell. Close to the signal peptide there is a hydrophobic propeptide domain, containing a cysteine residue that shields close to it the catalytic domain, which contains a Zn^{2+} ion. The catalytic domain of gelatinases differs from that of the other MMPs because it contains three fibronectin-type II-like domains that form a collagen-binding region that allows the binding and subsequent cleavage of type IV collagen or denatured collagen (gelatin) [57]. The hemopexin-like C-terminal region is connected to the catalytic domain by a flexible hinge region, and allows the binding to other proteins that may regulate the activity of MMPs such as TIMPs [58]. In addition, some MMPs present a transmembrane region and a short cytoplasmic tail (MMP-14, -15, -16 and -24) or a glycosylphosphatidyl anchor (MMP-17 and 25).

MMPs are synthesized as inactive proenzymes or zymogens (pro-MMPs). Activation of proMMPs requires the dissociation of the cystein group in the propeptide domain from the catalytic Zn^{2+} ion. The cysteinyl residue and the Zn^{2+} catalytic domain, common to all MMPs, are coordinated and the interruption of this interaction is believed to be a mechanism of activation common to all MMPs [57]. Once the active site is freed of the propeptide it binds the substrate and the Zn^{2+} ion becomes available for the binding of a hydrolytic water molecule that is essential for catalysis [51].

1.3.2 Physiological roles of MMPs

Many physiological processes require the activity of MMPs. This is the case of embryogenesis, where these endopeptidases play a crucial role. In early skeletal muscle development, processing of specialized cartilage and bone matrices is precisely coordinated. Several MMP knockout models confirm that MMPs play a major role in bone metabolism, e.g., MMP-14 (MT1-MMP) deficient mice (*Mmp14*$^{-/-}$) present gross connective tissue and skeletal abnormalities and do not survive more than 13 weeks *postpartum* [49,59,60] and MMP-9$^{-/-}$ mice have abnormal expansion of the zone of hypertrophic chondrocytes [49,61]. As for the development of the vascular system, some studies point out the complex repertoire of signalling functions MMPs have, as reflected in their wide range of pro- and anti-angiogenic roles in blood vessel development (branching morphogenesis) and homeostasis, e.g.,MMPs-1, -3, -7, -9, -16 and -19 improve VEGF bioavailability, and MMP-1 and MMP-3 release bFGF in ECs, both potent pro-angiogenic factors [49,62–65].

In adulthood, bone tissue still undergoes dynamic remodelling for repair. Some studies have also demonstrated the importance of MMPs in adult bone remodelling as it is the case of the delayed bone fracture repair in the absence of MMP-9 activity [62]. Furthermore, the constant renewal of the ovary and uterus matrices throughout each reproductive cycle in the mammalian species claims for extensive tissue remodelling. The dynamic changes in their architecture are regulated, in part, by MMPs [66,67]. In addition, MMPs have been widely studied in the field of wound healing; and although MMPs' functions were classically restricted to scar resorption [68,69] recent data support the evidence that they are also involved in inflammation and re-epithelization [69–71].

1.3.3 Pathological roles of MMPs

Although the relevance of MMPs in the physiological processes is notorious, it is in pathological conditions when they seem to play a key role. The study of their regulation has emerged as a new attractive field for the study of different pathological conditions since their activity is decisive for various diseases outcomes. For instance, MMPs have been described to be involved in the pathologic tissue destruction and in the cell-cell signalling pathways associated to certain cancers and vascular diseases.

Cancer

Cancer is one of the leading causes of mortality worldwide [1]. As already stated, MMPs activity has broaden over the simplistic idea of just ECM-degrading enzymes. Over the years, their involvement in the regulation of tumor microenvironment and the observation of their increased expression and activation in almost all human cancers has been shown [72]. It is currently demonstrated that MMPs bind and process many different molecules, including growth factors, apoptosis-related ligands/receptors and angiogenic/antiangiogenic molecules, among others. This feature makes them crucial factors in the modulation of the tumor microenvironment, including processes like cell growth, invasion, cell survival, adipogenesis and angiogenesis [72] (Figure 7).

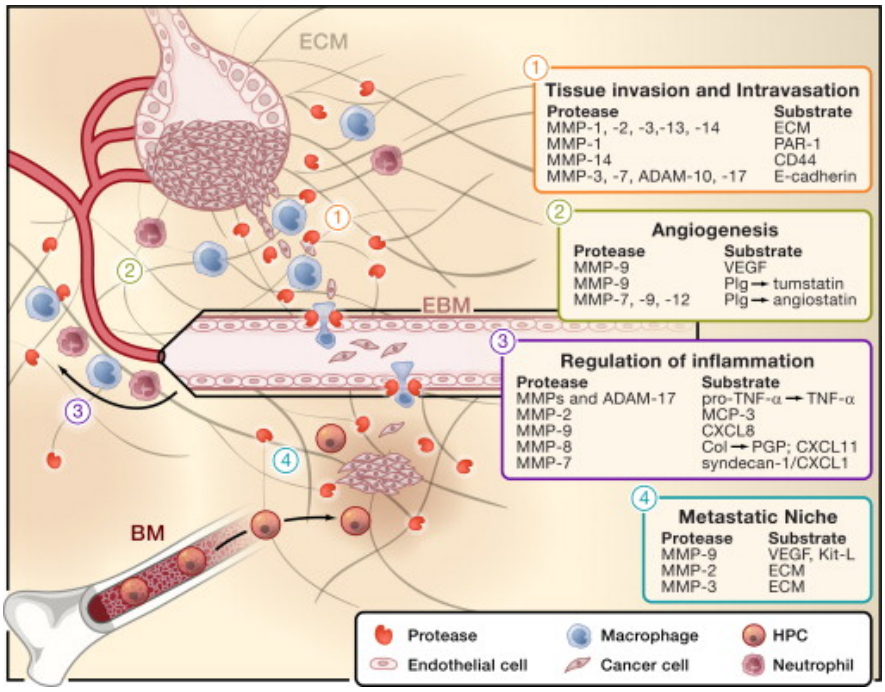


Figure 7. Multiple functions of MMPs in the tumor microenvironment. MMPs are normally provided by non-malignant stromal cells such as neutrophils, macrophages and ECs. These MMPs are able to modulate tumor progression and metastasis at their different stages (Kessenbrock et al., *Cell* 2010).

In the clinic MMPs have been proposed as diagnostic and/or prognostic markers in different cancer types. Gelatinases are the most studied ones in this field. Increased circulating levels of these peptidases have been proposed as a prognostic marker of ovarian cancer [73] and correlated to cell invasion and metastasis in cervical uterine cancer [74]. MMP-9 by itself has also been proposed as diagnostic marker in esophageal cancer [75] and as prognostic marker in pancreatic cancer [48] . Many other MMPs and some TIMPs have been related to different cancer malignancies. For instance, MMP-14 is related to overall survival in breast cancer, MMP-13 has been described to promote survival of squamuos carcinoma and TIMP-1 could be used as a diagnositic marker in pancreatic cancer [48,76–78].

Atherosclerosis

Different MMPs take essential part at various steps in atherosclerotic plaque formation and rupture [56,79] (Table 3).

By disrupting and remodelling ECM, MMPs allow the diapedesis of inflammatory cells and the influx of plasma proteins, including lipoproteins, through the vessel wall. Monocytes penetrate the intima and turn into macrophages, scavenge oxidized LDLs

and become foam cells, which are a rich source of MMPs. Their proteolytic activity allows SMCs migration through the internal elastic lamina into the intima, thus contributing to the growth of the atheroma [80]. The evidence for an upregulation of, for instance, MMP-2 and -9 during neointima formation after vascular injury is overwhelming (Table 3), however, TIMPs are concomitantly upregulated in order to prevent excessive ECM degradation [81].

Table 3. Key matrix metalloproteinases contributors to atherosclerosis (modified from Roycik et al., *Curr Mol Med*, 2013).

MMP	**Contribution(s) in atherothrombosis**
Collagenases	
MMP-1	Plaque destabilization; potential roles in plaque initiation and progression
MMP-8	Plaque destabilization; angiotensin I cleavage leading to VCAM-1 up-regulation and macrophage accumulation
MMP-13	Protective: cleavage of ICAM-1 Destructive: Potential roles in plaque infiltration, progression, and destabilization
Gelatinases	
MMP-2	Facilitation of SMC migration
MMP-9	Protective: Limits plaque growth; plaque stabilization Destructive: Lesion growth and collagen accumulation via proteolysis of basement membrane
Stromelysins	
MMP-3	Plaque stabilization, possibly through MMP-9 activation
MMP-11	Increased neointima formation, possibly through facilitation of SMC migration
Others	
MMP-7	N-cadherin shedding; ApoE proteolytic processing

MMP-12	ECM degradation; macrophage infiltration; plaque destabilization; MMP-2 & -3 activation; N-cadherin shedding
MT1-MMP	MMP-2 activation; ApoE proteolytic processing

SMC = Smooth muscle cells; VCAM-1 = Vascular cell adhesion molecule-1; ICAM-1 = Intercellular adhesion molecule-1; ApoE = apolipoprotein-E; ECM = Extracellular matrix.

ECM degradation by MMPs could reduce fibrous cap thickness and collagen content, which are typical features of plaque vulnerability. Several MMPs, including MMP-1, -3, -9 and -10, have been found expressed at the rupture prone zones of the human atheroma [82–86]. Furthermore, the inhibition of MMPs in mice using TIMPs leads to increased plaque stability [87,88]. The contribution of individual MMPs to atherosclerosis is usually studied in double deficient ApoE$^{-/-}$:MMP mice to enable plaque formation. The genetic deletion of MMP-8, -12 and -13 in ApoE$^{-/-}$ mice leads to more stable plaques [89–91], while the absence of MMP-3 produces less stable lesions [56]. In some cases the same MMP can lead to different observations depending on the artery of study. MMP-3 deletion, for instance, presents more stable plaques in the aorta [92] but less stable ones in the brachiocephalic artery.[90] Likewise, MMP-9 deficiency promotes plaque stability in the aortic root [93] and increased plaque size at the level of the brachiocephalic artery [90]. These results highlight the complexity of MMP family and endorses the study of each family member independently.

Taking in consideration that an exacerbated MMP activity has been mainly related to plaque development and vulnerability it appears rational to try to inhibit their activity in patients with atherosclerosis. However, the use of broad-spectrum inhibitors of MMPs did not have beneficial effects in the disease [87]. More potent and selective agents should be developed and the most appropriate timepoint for the treatment should be calculated [79].

The role of MMPs in thrombosis is not restricted to thrombus formation after the rupture of the fibrous cap of the plaques. Recent studies reveal their involvement in the thrombosis/fibrinolysis system. MMP-1 and MMP-13 have been shown to activate the thrombin receptor PAR-1 by its cleavage at non-canonical sites producing procoagulant effects [54], and the fibrinolysis inhibitor TAFI has been shown to be a target for MMP-10 cleavage favouring the fibrinolytic activity of tPA [94].

Clinical studies have shown increased circulating levels of MMPs in different atherosclerotic-related diseases, and their possible use as predictors of severity and

future cardiovascular events [95–100]. Likewise, increased circulating levels of gelatinases [101–104] and other MMPs like MMP-1, -7, -8 and -12 [68,96,97,105–107] have been reported in coronary artery disease patients. After acute ischemic stroke MMP-1, -3, -7, -9 and -10 augment in the blood stream and their levels are associated with neurological symptoms [99,108–111]. There are not many studies in respect of MMPs and PAD but the few of them also show increased circulating MMP-2, -8 and -9 levels in PAD patients compared to healthy volunteers [100,104].

Limb ischemia

The skeletal muscle undergoes enormous remodelling after an acute ischemic insult. Hypoxia is accompanied by a vast immune response directed to the ischemic tissue [34–38,41] and triggers the angiogenic and arteriogenic response to restore blood supply. Satellite cells are then activated and released from the basal membrane becoming myoblasts [34]. MMPs are involved throughout all these processes.

After the shortage of blood supply, the hypoxic tissue becomes a battle field where many different cell types are involved. Neutrophils and macrophages phagocyte the dying myocytes, secrete chemokines for the recruitment of more leukocytes and, in cooperation with myofibroblasts, produce ECM components and MMPs [34,41]. While the skeletal muscle regenerates, the ECM that is used as a provisional scaffold is slowly reorganized by MMPs around the newly formed myotubes and vessels [112].

MMPs are also involved in the activation of satellites cells [113,114]. Moreover, MMPs participate in fiber formation as the expression of several MMPs, e.g. MMP-1, -2, -9 and -10, has been reported during the differentiation of myoblasts to myocytes [114–117]

MMPs are key molecules for the migration of ECs and SMCs and are also needed for the remodelling of the artery wall [41,118]. *In vitro*, some MMPs enhance proliferation, migration and tube formation ability of ECs, e.g. MMP-7, -9 [118,119], while some others induce tubular network collapse and regression, e.g. MMP-1 and -10 [120]. Moreover, angiogenic factors can induce the expression of certain MMPs in ECs [118] as it is the case of MMP-9 after bFGF stimulation [121]. *In vivo* studies have demonstrated the beneficial and detrimental effects of MMP activity in the ischemic muscle. There is evidence of increased MT1-MMP, MMP-2 and -9 activity in the ischemic muscle after femoral artery ligation, moreover deficiency in the two gelatinases impairs neovascularization of the ischemic lower limbs and presents reduced leukocyte infiltration [122,123].

1.4 MMP-10

MMP-10, also known as stromelysin-2 (Figure 8), is a metalloproteinase that degrades multiple components of the ECM, such as proteoglycan, gelatin types I, II, IV and V, laminin, fibronectin, and collagen III, IV and V [58]. This metalloprotease contains 4 hemopexin-like domains and presents a molecular weight of 54 KDa as proenzyme and 46 KDa when activated in humans. The conserved cysteine present in the cysteine-switch motif binds the catalytic zinc ion, thus inhibiting the enzyme. MMP-10 is thought to be activated by a number of serine proteases, namely plasmin, kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase, and chymase [114,120]. The dissociation of the cysteine from the zinc ion upon cleavage of the pro-peptide activates the enzyme [124]. MMP-10 is able to activate other MMPs like MMP-1, -7, -8, -9 and -13 and is inhibited mainly by TIMP-1 but also TIMP-2 at some extent [124].

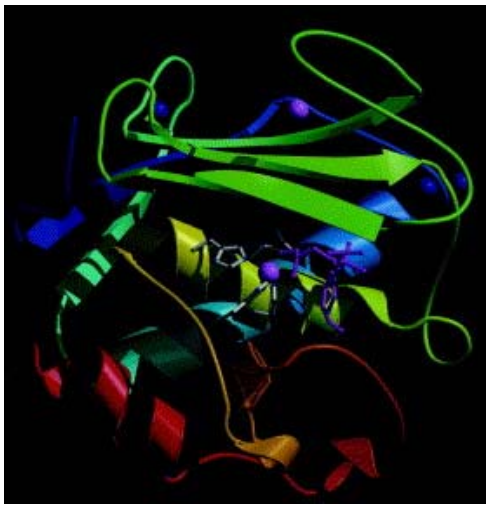


Figure 8. Stereo view of the catalytic domain of the human MMP-10 protein represented as ribbons. The catalytic and the structural zinc ions and the three calcium ions are shown as pink and blue spheres of arbitrary radius, respectively (modified from Bertini et al. *J Mol Biol*, 2004).

Although its similarity degree is as high as 82% with one of the other stromelysin (MMP-3) and both share ECM substrate specificity, these stromelysins present differential patterns of transcriptional regulation and tissue distribution that hint at distinct physiological functions. MMP-10 gene expression is mainly regulated at the transcriptional level, and a number of cytokines and growth factors have been shown to induce or stimulate its synthesis, including IL-1, TNFα, TGF-β, PDGF and thrombin,

whereas others, such as heparin and corticosteroids, have an inhibitory effect [55]. MMP-10 is produced in a variety of cell types, including keratinocytes, osteoclasts, T-lymphocytes, monocytes, neurons, ECs and myoblasts, and it is normally secreted into the extracellular space.

MMP-10 participates in different physiological processes like wound healing. Transgenic mice expressing a constitutively active MMP-10 mutant in keratinocytes have shown reduced deposition of new matrix and increased cell apoptosis in the healing epithelium [125], suggesting that a tightly regulated expression of MMP-10 for proper wound healing is needed [58]. Moreover, active MMP-10 is expressed at high levels in certain human carcinomas compared with normal tissues and involved in the ECM remodeling associated with tumor growth [126]. In addition, recent reports show the active role of MMP-10 in mouse models of skeletal muscle repair after injury and hepatic wound healing [117,127,128].

MMP-10 and the cardiovascular system

Regarding the vascular pathophysiology, there are scarce reports of the involvement of MMP-10. It has been shown that histone deacetylase 7 (HDAC7) is specifically expressed in the vascular endothelium during early embryogenesis, where it maintains vascular integrity by repressing MMP-10 expression [129]. Moreover, a possible role in angiogenesis has been described, as MMP-10 overexpression and activation by several serine proteases has been associated with capillary tubular network collapse and regression in 3D collagen matrices [120].

Vascular inflammation plays a key role in the onset, progression and thrombotic complication of atherosclerotic lesions [10]. Inflammation heightens production of biomarkers, such as C-reactive protein (CRP), which has been proposed to be a powerful and independent predictor of AMI, stroke and vascular death in a variety of clinical settings [130]. It has been suggested that CRP has direct proatherosclerotic effects on cellular functions implicated in the atherosclerotic lesion formation [84]. Previously, our group reported that CRP induces endothelial MMP-1 and -10 expression, both at the mRNA and the protein level, and that its expression colocalizes with that of CRP in rupture-prone zones [131] (Figure 9). Recently, our group has also demonstrated that thrombin, a prothrombotic/proinflammatory protein, also induces endothelial MMP-10 expression and secretion, through PAR-1-dependent mechanism [132].

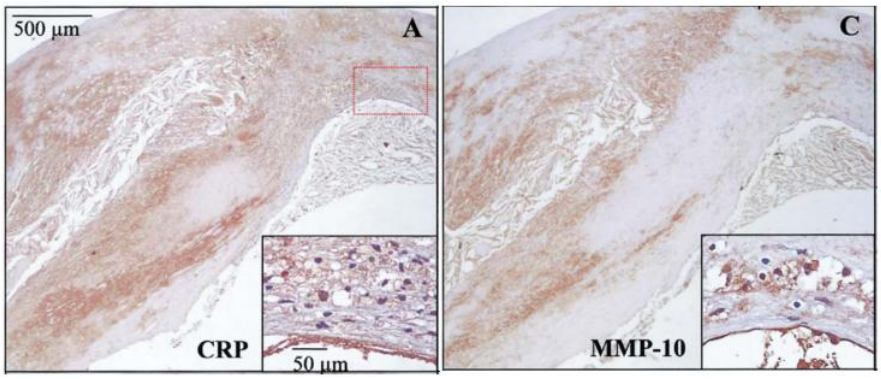


Figure 9. Representative immunohistochemical analysis of C-reactive protein (CRP) and MMP-10 in adjacent sections of human atherosclerotic plaques. Area squared in red corresponds to magnified details (modified from Montero et al., *J Am Coll Cardiol*, 2006).

Genetic studies carried out in patients with abdominal aortic aneurysm, a vascular pathology characterized by histological signs of chronic inflammation, vascular destructive remodeling of ECM, and depletion of SMCs. The studies reported that the nt+180 polymorphism of the MMP-10 gene, in which an adenosine is substituted by a guanine in the nucleotide 180, is associated with the presence of the disease [133].

Several studies of our group have demonstrated the close relationship between atherosclerosis and MMP-10. Among asymptomatic subjects free from CV disease, MMP-10 circulating levels have been associated with increased carotid intima-media thickness (IMT) suggesting MMP-10 as a surrogate marker of atherosclerosis [95]. Moreover, MMP-10 levels increase along with the number of cardiovascular risk factors and there is an independent association between smoking and MMP-10 concentration in asymptomatic individuals [95,134]. Raised serum MMP-10 levels are also associated with systemic inflammatory markers, such as fibrinogen and hs-CRP [95,131], suggesting a close relationship between ongoing inflammatory markers and systemic proteolytic activation with subclinical atherosclerosis. In addition, MMP-10 circulating levels are described to be augmented in other vascular pathologies associated with impaired vascular remodeling and inflammation, as it is the case of patients with increased thrombin generation, chronic kidney disease and diabetes [98,132,135].

There is also evidence that MMP-10 plays a role in acute ischemic stroke. In 2008, the first data of the involvement of MMP-10 in this pathology were published. Montaner et al. showed that MMP-10, among other MMPs, was upregulated in the infarcted tissue compared to healthy areas in human samples [110]. Moreover, increased serum concentrations of inactive MMP-10, which do not necessarily imply more active MMP-10 levels, are found in ischemic stroke patients and correlated with greater infarct

volume, severe brain edema, neurological deterioration and poor functional outcome at 3 months [111].

Recently, a novel role of MMP-10 has also been proposed by our group in acute ischemic stroke. Tissue plasminogen activator (tPA) is the only drug licensed for use in highly selected patients although it can only be used between 3-4.5 hours after stroke. This drug may unfortunately cause serious bleeding in the brain, thus new therapies for acute ischemic stroke must be developed. Our data indicate that administration of active MMP-10 enhances fibrinolysis and reduces blood reperfusion time and infarct size to the same extent as tPA in a mouse model of thrombin-induced ischemic stroke, however the administration of this protease promotes a shortage in the bleeding time and absence of intracranial hemorrhage [94].

1.5 Animal models of ischemia

There are different models for the study of the events triggered by ischemia. In this doctoral dissertation we studied the underlying mechanisms after the ischemia of the limbs. Therefore, we used models of femoral artery ischemia to study the role of MMP-10 in skeletal muscle repair after hypoxia [136,137].

The hindlimb ischemia model is based on the ligation of the femoral artery resulting in distal ischemia. The location of the artery occlusion dictates the severity of the ischemia in the downstream muscle, thus the closer to the iliac artery, the greater downstream ischemic area. We wanted to analyze the role of MMP-10 in mild and in severe ischemia, therefore we utilize two mouse models. The severe ischemia model, named as the total excision model, was obtained by the excision of the femoral artery proximal to the inguinal ligament, with a perfusion drop of >95 % to the downstream muscles right after surgery. For the study of the less aggressive ischemia, we based our experiments in the mild excision model in which the superficial femoral artery was ligated with a subsequent drop in perfusion of about 50 %.

The ischemic area does not only depend on the occlusion of the artery, in addition, the type of muscle on which the study is focused is also determinant, i.e., high oxidative muscles will present greater ischemia-induced damage than low oxidative ones. As explained in the materials and methods section (see below), we focus our study on the most oxidative muscle of the calf, the soleus muscle. Its high aerobic metabolism makes it the most vulnerable to ischemic conditions, thus in need of greater tissue remodeling and regeneration after damage.

The repair of the skeletal muscle after acute ischemia is well characterized and comprises two phases, a degenerative and a regenerative one. The former occurs right after injury and is characterized by necrosis and inflammation of the tissue. The proper activation of this phase will initiate the regenerative phase, which takes longer but results in the complete regeneration of the muscle tissue (Figure 10).

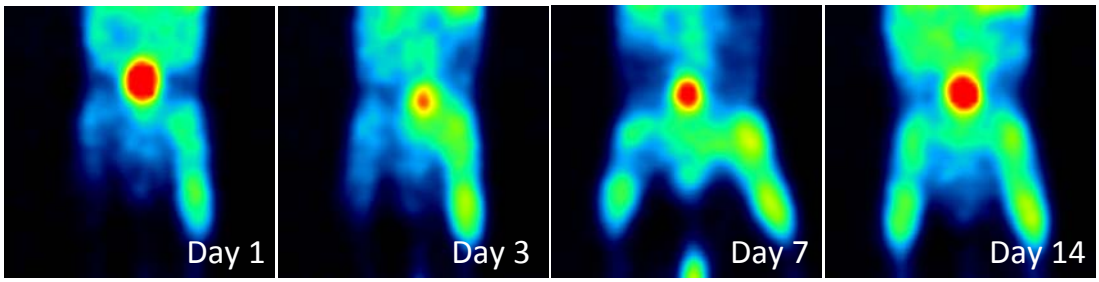


Figure 10. Representative microPET images after mouse hindlimb ischemia of the right limb over time in our model of severe ischemia. At non-excesive ischemic rates, the limb is able to recover its perfusion by angio- and arteriogenesis.

1.6 Extracellular Vesicles

All body fluids, e.g. urine, lacrima, mother's milk and blood, present numerous plasma membrane-derived vesicles released under physiological and pathological conditions by membrane shedding from a variety of cell types, including platelets, ECs, leukocytes and erythrocytes [138,139]. These particles are comprised in a large and heterogeneous family named as extracellular vesicles (EVs) including exosomes, microparticles (MPs) and apoptotic bodies.

Nowadays, the exact limits between the different EVs are becoming blurred leading to controversy in the scientific community. MPs have been described to be different from the other extracellular vesicles like apoptotic bodies, which are described to be larger and to contain DNA, and from exosomes, which have classically been characterized by smaller sizes and different protein composition [138]. MPs are the most widely studied group. In 2005, the Vascular Biology Subcommittee of the International Society on Thrombosis and Haemostasis stated a standard definition, "*Microparticles are 0.1 – 1 micrometer cell-derived vesicles that lack a nucleus or synthetic capacity, may contain cytoskeletal proteins, and expose some quantity of phosphatidylserine on their surface*" [140]. Phosphatidylserine (PS) is a negatively charged lipid localized on the cytosolic side

of the plasma membranes that in response to stimulation or during apoptosis is exposed on the extracellular surface of the membrane.

The presence of PS on the MP surface used to be considered as a universal marker of MPs, since the only mechanism known for the release of these vesicles ended up with PS exposure on the membrane. In this process, variations in the intracellular Ca^{2+} concentration trigger MP production and release. The intracellular Ca^{2+} regulates the activity of the enzymes flippase, floppase and scramblase, which are responsible for maintaining the membrane asymmetry between the two lipid layers. Cell activation leads to an increase in intracellular Ca^{2+} triggering the exposure of PS on the cell membrane, and the activation of intracellular signaling molecules like calpain and Rho kinase, which are responsible of the cleavage of the cytoskeleton resulting in cellular contraction and membrane blebbing [141]. However, as above mentioned, not all MPs express PS and not all of them are formed and released by this mechanism. Likewise, inactivated platelets and ECs release MPs in an αIIβ3 integrin-dependent manner in which nor Ca^{2+} neither calpain are involved [142]. Different hypothesis have been proposed for the formation of these other MPs, like being the result of multiple fusion events between cell debris or small endosomal-secreted vesicles with the medium or plasma components, or by being released by a particular an unknown cytoskeleton cleavage [141].

Necrosis, apoptosis and the response to certain stimuli are well known MP-release triggers [141,143]. However, the number of MPs and their cellular origin may vary depending on the stimulus. For instance, growth factor depletion or stimulation with TNF-α of either macrovascular or microvascular cells resulted in the release of phenotypically and quantitatively distinct MPs [144].

MPs present a wide variety of ligands, receptors, cyto- and chemokines, enzymes, mRNA and microRNA enabling them to interfere not just in blood clotting but also in many different processes like intercellular signaling, inflammation, cell adhesion, waste management, stress protection, and vascular function and integrity (Figure 11) [138]. In addition, increased numbers of total circulating MPs are associated with different pathologies, e.g. diabetes, cancer, sepsis, and pulmonary hypertension [145].

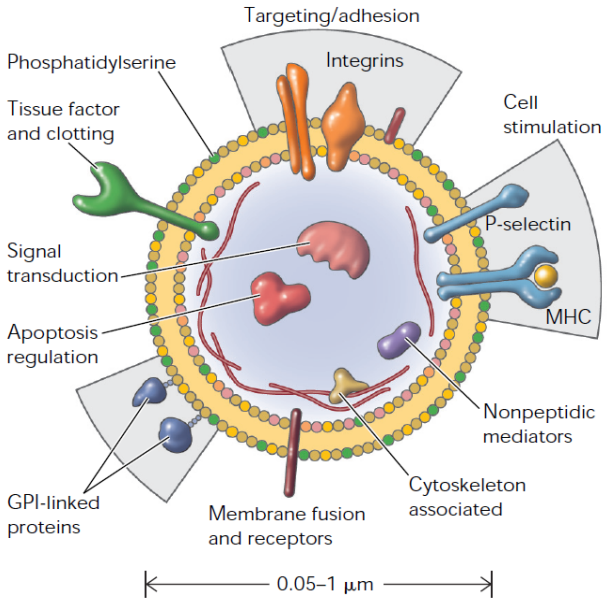


Figure 11. Representation of several microparticle components. MPs are involved in intercellular information exchange and are key mediators in blood hemostasis (Hugel et al., *Physiology, (Bethesda*), 2005).

Although the study of EVs, including MPs and exosomes, has yielded strong interest, the different methodologies for their study remains a challenge. Their small size makes very difficult to detect or distinguish them from the lipoproteins present in blood. Lipoproteins (HDL, LDL, VLDL and chylomicrones) present similar size and density to EVs and are normally co-isolated with the vesicles; therefore interpretation of the data needs to be carefully done. Figure 12 shows the presence of EVs in fractionated plasma [146], a technique that allows the separation of small lipoproteins and protein aggregates from EVs larger than 70 nm.

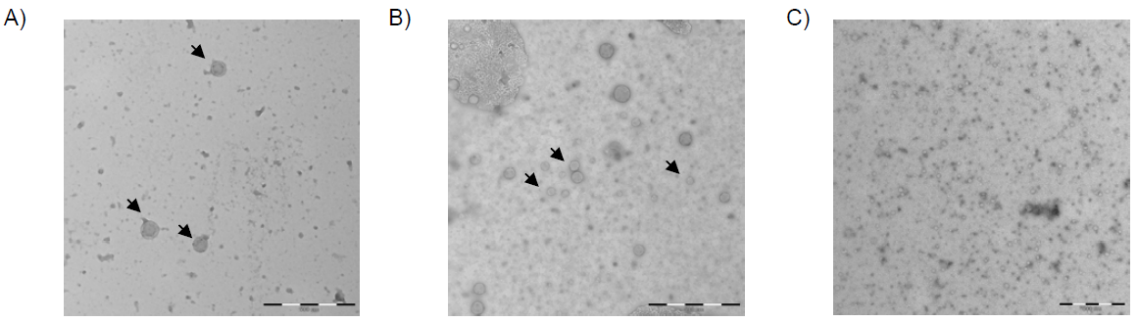


Figure 12. Transmission electron microscopy images of mouse plasma fractionated by size exclusion chromatography. Extracellular vesicles come in the first fractions (A), followed by lipoproteins (B) and protein aggregates (C, black staining). Scale bar is 500 μm.

Microparticles in cardiovascular disease

Circulating MPs may present a marked prothrombotic character when harboring procoagulant molecules. Activated platelets release PS-harboring MPs; in addition, monocytes have been described as the major circulating source of tissue factor-bearing MPs in health and disease, although MPs derived from neutrophils, ECs and platelets may also present tissue factor (TF) on their surface when adequately stimulated [147]. Since PS as well as TF are potent procoagulant factors [147], MPs harboring one, the other or even both, have risen as key modulators of blood haemostasis [139,148–151].

Constitutive levels of circulating MPs have been reported to contribute to overall vascular health and homeostasis in coagulation [145]. However, increased levels of MPs have also been described in the blood stream in different disease states such as diabetes, stable angina and AMI [152–157]. For instance, in acute ischemic stroke patients, circulating EC-derived MP are linked to severity, lesion volume and poor outcome [158]. In high-grade carotid stenosis patients increased levels of leukocyte-derived MPs are associated with unstable plaques [159]; and regarding myocardial infarction, circulating MPs from patients have been related to endothelial dysfunction [160].

In addition, there is increasing evidence of the role that MPs play in atherosclerosis development, progression and stabilization [161]. A recent review has summarized the different and most relevant atherosclerosis stimuli which lead to MP release from circulating or vascular cells [162] (Table 4). Rautou et al. outline that circulating levels of MPs are increased in subjects at high atherothrombotic risk and how these vesicles induce endothelial dysfunction promoting atherosclerosis development. Moreover, locally, MPs within atherosclerotic plaques have been implicated in monocyte recruitment by transferring intercellular adhesion molecules to ECs.[148]. Inside the plaque, a population of CD40 ligand-expressing MPs trigger angiogenesis increasing the number of *vasa vasorum* and promoting intraplaque haemorrhage [162,163].

Table 4. Relevant atherosclerosis-related stimuli for MP release (modified from Rautou et al., *Circ Res*, 2011).

Stimuli	**Cell Type**			
	Endothelial Cell	Platelet	Smooth Muscle Cell	Monocyte/ Macrophage
Cigarette extract	…	…	…	Li et al, 2010
Modified LDL	Nomura et al, 2004	…	Llorente-Cortes et al, 2004	…
HDL cholesterol	Liu et al, 2007	…	…	Liu et al, 2007
Uremic toxin	Faure et al, 2006	…	…	…
Flow conditions	Ramkhelawon et al, 2006	Nomura et al, 2000	Stampfuss et al, 2006	…
Thrombin	Sapet et al, 2006. Simoncini et al, 2009	Barry et al, 1997. Dale et al, 2009. Chang et al, 1993	…	…
Collagen	…	Barry et al, 1997. Boilard et al, 2010. Chang et al, 1993	…	…
Homocysteine	Sekula et al, 2011	Olas et al, 2010	…	…
Activated Protein C	Pérez-Casal et al, 2005	…	…	Pérez-Casal et al, 2005
PAI-1	Brodsky et al, 2002			
Proinflammatory cytokines (TNFα, IL1β) and CRP	Combes et al, 1999. Curtis et al, 2009. Abid Hussein et al, 2005. Wang et al, 2007	Nomura et al, 2000. Piguet et al, 2002	Schecter et al, 2000	Jungel et al, 2007
Oxidative stress	Vince et al, 2009. Szotowski et al, 2007	…	…	…
Fas ligand	…	…	Essayagh et al, 2005	…
PDGF	…	…	Schecter et al, 1997	…

2-HYPOTHESIS AND OBJECTIVES

Hypothesis

After an ischemic insult, an acute inflammatory response is driven to the hypoxic tissue. The development of new blood vessels and new muscle fibers takes place in order to restore muscle functionality. There is substantial evidence that MMPs greatly contribute to tissue remodeling after ischemia throughout the process. Among MMPs, MMP-10 has been suggested to have a predominant role in inflammatory conditions.

On this basis, we hypothesized that MMP-10 could have a relevant role in the degeneration, and the subsequent regeneration of the skeletal muscle after the ischemic injury by direct action on the ECM components, and/or through proteolytic modification of currently unknown targets.

As conveyors of intercellular signalling, EVs play important roles in different biological processes and have been reported to increase during pathological conditions. We hypothesized that MMP-10 could influence EVs release after ischemia.

Objectives

In order to assess the role of MMP-10 in tissue repair following ischemia, we addressed the following objectives:

- To determine MMP-10 function in mild hind limb ischemia throughout the degenerative/regenerative process in WT and *Mmp10*$^{-/-}$ mice.

- To analyze MMP-10 function in severe ischemia by using a model of total excision of the femoral artery in WT and *Mmp10*$^{-/-}$ mice.

- To elucidate the mechanisms by which MMP-10 modulates the ischemic response in skeletal muscle.

- To characterize the EV population present in plasma of WT and *Mmp10*$^{-/-}$ mice during the degenerative and regenerative phases after injury.

3-MATERIALS AND METHODS

3.1 Animal Study- Hind limb ischemia model

Male wild type (WT, C57Bl/6J) and MMP-10 deficient mice (*Mmp10*$^{-/-}$, C57Bl/6J background N8) kindly provided by Dr. W.C Parks from the University of Washington, USA (12 weeks old, n=6-28/genotype/group) were anesthetized with isoflurane (2.5–4%, inhaled) and underwent surgery under sterile conditions. A longitudinal incision was made in the skin overlying the middle portion of the right hind limb. The non-excised left limb was used as control. To induce mild ischemia, unilateral ligation of the superficial femoral artery without damaging the femoral nerve was performed, and the cutaneous vessels branching from the caudal femoral artery side branch were also ligated [164]. To induce severe ischemia, the femoral artery and vein were ligated and excised both proximal to the inguinal ligament and right before the bifurcation to the saphenous and popliteal arteries. The artery and vein and all side branches were dissected and excised. All animals received an anti-inflammatory agent (Ketoprofen, 5 mg/kg subcutaneously) daily for 3 days and an antibiotic (Enrofloxacin, 25 mg/kg, in drinking water) for 3 days after surgery.

For the rescue assay, recombinant human MMP-10 (rhMMP-10) was produced in a mammalian cell system, purified and activated following the previously described procedure [94]. Mice were injected through the femoral vein with active rhMMP-10 (2 nmol/L, ≈6.5 µg/kg) or vehicle (50 mM Tris, 10 mM CaCl, 15 mM NaCl, 0.05 % Brij 35, pH 7.5) as control right after excision (group 1), or 24 hours post-ischemia (group 2).

The research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals (Directive 2010/63/EU), and approved by the Institutional Animal Care Committee of the University of Navarra.

3.1.1 Tissue perfusion measures

Laser Doppler

For the functional perfusion measurements of the collateral region of the mild ischemia model, we used a laser Doppler perfusion imager, Lisca PIM II camera (Gambro) right after ischemia, 2 and 4 days post-excision. The system incorporates a 2-mW helium-neon laser to generate a beam of light that sequentially scans a 12 x 12 cm tissue surface to a depth of 600 p.m. A photodiode collects the back-scattered light, and the original light intensity variations are transformed into voltage variations in the range of 0 to 10 V. A perfusion output value of 0 V was calibrated to 0% perfusion, whereas 10 V

was calibrated to 100% perfusion. The perfusion signal is split into six different intervals, and each is displayed as a separate color.

Micro-positron emission tomography (microPET)

Pefusion data in severe ischemia model were obtained by MicroPET analysis as a more accurate assessement of tissue oxygenation after hindlimb ischemia [165]. 20-min-duration small-animal MicroPET studies were performed 10 min after ^{13}N-ammonia injection as described previously [165]. Briefly, mice were anesthetized with 2% isoflurane for ^{13}N-ammonia injection (75 MBq) in the tail vein and were kept under such conditions during the entire study. PET scans were performed at day 1, 3, 7, 15 and 24 after surgery.

For quantitative analysis and further comparisons among subjects, the evaluation of the perfusion was performed as previously described [165]. In brief, ROIs were drawn on coronal 1-mm-thick small-animal PET images over the hind limbs, and activity concentration per area unit was calculated as a measurement of perfusion. The ratio between the right (ischemic) and left (non-ischemic) hind limbs was used for comparisons.

3.1.2 Histological analysis

Mice were sacrificed by CO_2 inhalation and perfused with saline at different time points. Mice subjected to mild ischemia were sacrificed and their soleus muscles dissected at day 3, 7 and 14 post-excision, while tissues were collected at day 3, 15 and 28 post-excision in the severe ischemia model. Tissues were fixed overnight in 2% phosphate-buffered paraformaldehyde, dehydrated, and embedded in paraffin. Antigens were detected either by detection of a peroxidase-labeled secondary IgG or by immunofluorescence as described in Table 5. Hematoxylin and eosin staining (H&E) was used to evaluate necrosis and the regeneration rate. Sirius red staining was performed in soleus sections to evaluate total collagen deposition.

Table 5. Immunohistochemistry studies.

	Macrophages	Neutrophils	Vessels	Basement membrane	Cxcl1	Collateral vessels	MMP-10
Immunofluorescence	No	No	No	Yes	Yes	Yes	No
Antigen retrieval	Citrate antigen retrieval buffer Dako (S1699)	Proteinase K 20 µg/ml;	10mM Tris, 1mM EDTA pH=9	Proteinase K 40 µg/ml	Proteinase K 40 µg/ml	Citrate antigen retrieval buffer Dako (S1699)	0.01M Citric acid pH=6
Antigen retrieval conditions	95ºC, 30 min	37°, 30 min	95ºC, 30 min	24°, 10 min	24°, 10 min	95ºC, 30 min	95ºC, 30 min
Primary antibody	Rat anti-mouse F4/80	Rat anti-Mouse NIMP-R14	Rat anti-mouse CD31	Rabbit anti-mouse laminin	Rabbit anti-rat KC	Mouse anti-human αSMA	Rabbit anti-mouse MMP-10
Commercial reference	AbD Serotec UK MCA497GA	Abcam ab2557	DIA 310 0.2mg/mL	Sigma-Aldrich L9393	Acris AP08852PU-N	Dako M0851	Acris AP07210PU-N
Working concentration	1:200	1:10000	1:100	1:50	1:20	1:300	1:20
Secondary antibody	Goat anti-rat biotinylated IgG 1:300	Rabbit anti-rat biotinylated 1:200	Rabbit anti-rat biotinylated 1:200	Donkey anti-rabbit-Alexa 555 1:200	Swine anti-rabbit biotinylated 1:200	Goat anti-mouse-Alexa 568 1:200	
Signal amplification	TSA Biotin System (NEL700A001KT)	EnVision anti-rabbit HRP-conjugated (Dako, K4011)	EnVision anti-rabbit HRP-conjugated (Dako, K4002)	No	TSA Biotin System (NEL704A001KT)	No	EnVision anti-rabbit HRP-conjugated (Dako, K4002)
Amplification working concentrations	Streptavidin-HRP 1:150 Biolinyl-tyramide reagent 1:75				Streptavidin-HRP 1:300 Cyanin-3 tyramide reagent 1:150		

Morphometric analysis was performed using a Nikon Eclipse 80i microscope with Cell^D software for collagen and F4/80 analysis and Fiji image analysis software for any other case. For the quantification of the different histological analysis we followed different strategies depending on the antigen we were analysing as described in Table 6.

Table 6: Histological quantification strategies

Analysis	**Quantification strategy**
Necrotic area	% necrotic area from total soleus area
Macrophage infiltration	% $F4/80^+$ area from total soleus area
Neutrophil infiltration	Number of NIMPR-14^+ cells by mm^2
M2 polarization	Number of double stained $F4/80^+$ and $MRC1^+$ cells in total $F4/80^+$ area
Vessel and arteriole density	Number of $CD31^+$ and SMA^+ vessels, respectively by mm^2
Collagen deposition	% red staining area from total soleus area
Myocyte density	Number of myocytes by mm2
Myocyte cross sectional area	Mean of all $laminin^+$-surrounded areas
Regenerating myocytes	% Centrally-nucleated myofibers from total number of myofibers
Cxcl1 expression	% of $Cxcl1^+$ staining from total soleus area in respect to WT staining

3.1.3 Western blot analysis

CXCL1 and MMP-10 protein levels were measured by Western blot in WT and *Mmp10*$^{-/-}$ mice tissues (crural muscles). Briefly, tissues were collected and frozen in order to grind them into powder. Proteins were extracted by adding RIPA buffer (Sigma-Aldrich) plus protease inhibitors (Roche), homogenized with polytron (Kinematika AG PT3000) and centrifuged to get rid of cellular debris (10 min, 13.000 rpm, 4ºC).

Protein concentration was determined by the Bradford assay (BioRad Laboratories) and 60 µg total homogenate were loaded into a gel for SDS-PAGE (4-12% Bis-Tris gel, Invitrogen). Proteins were then transferred to a nitrocellulose membrane (iBLOT transfer stacks, Invitrogen), blocked with 10% milk in TBST (5 mM Tris, 34.25 mM NaCl, 0.1 % Tween 20, pH7.6). The membrane was then cut at the level of 25 KDa. The upper part was incubated overnight at 4ºC for the detectionof MMP-10 with a rabbit anti-human antibody (Acris) followed by 1 hour incubation at room temperature (RT) with a HRP-conjugated goat anti-rabbit antibody (Dako). Then the membrane was stripped (30 min, 37ºC with Restore Western blot tripping buffer, Thermo Scientific) for the detection of loading control by 4ºC overnight incubation with a monoclonal antibody anti-GADPH (Sigma) followed by 1h RT incubation with a HRP-conjugated goat anti-mouse antibody (Santa Cruz). The lower part of the membrane was incubated overnight for the detection of KC, which is the murine homologue for human CXCL1 protein, with a rabbit anti-mouse KC antibody (Fitzgerald) followed by 1 hour incubation at RT with a peroxidase-conjugated goat anti-rabbit antibody (Dako).

Protein detection was developed by a chemiluminiscent substrate, ECL Advance, for KC and MMP-10 and ECL Prime for GAPDH. (Western Blotting Detection Kit, Life Technologies). Images were captured with the Odyssey imaging system (Li-cor Biosciences) and its quantification was performed with Image Studio Lite (Li-cor Biosciences).

3.1.4 Thioglycollate-induced peritonitis model

Twelve week-old WT and *Mmp10*$^{-/-}$ mice were intraperitoneally injected with thioglycollate into the peritoneal cavity using a $26G_{1/2}$ needle. 48 hours later mice were sacrifized and then, 8 mL of ice-cold PBS were injected into the peritoneal cavity. After gently massaging the belly, 5 mL of ascitic liquid were retrieved from the belly using an $18G_{1/2}$ needle. Cells were then counted and stained for F4/80-fluorescein isothiocyanate (FITC) (Biolegend), Ly6G-phycoerythrin (PE), and CD11b-allophycocyanin (APC) (BD Pharmigen) with the antibodies diluted 1:400, 15 min at

4ºC for flow cytometry analysis (FACs Calibur, BD Bioscience) for the analysis of neutrophil and macrophage recruitment.

3.1.5 Gene expression analysis

RNA from tissues and cells was extracted using a semi-automated system for the isolation and purification of nucleic acids (Abi Prism 6100, Applied Biosystems) and 1 µg was reverse transcribed with random primers and Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (Invitrogen).

200 ng of cDNA were taken for real-time PCR (RT-PCR) using TaqMan® Low Density Custom Arrays (LDA, Applied Biosystems) (Table 7) and ABI PRISM®7900HT Sequence Detection System (Applied Biosystems). Customized 384-well LDAs for PCR amplification were designed using individual primers for genes of interest, chosen and purchased from the assays on demand gene-expression products. A total of 100 µL mastermix containing 200 ng cDNA were loaded into each of the eight ports. The distribution into 48 reaction cavities per port was carried out by two short centrifugation steps (1 min 1200 rpm in a swinging bucket rotor, Rotina 35R, Hettich). *β-actin* was used as housekeeping gene.

Table 7. Low-density array probes for gene expression analysis.

Gene name	**Gene name**	**APPLIED BIOSYSTEMS CODE**
18S rRNA	*RNA18S*	Hs99999901-s1
Vascular endothelial growth factor A	*VEGFA*	Mm00437304_m1
Serine (or cysteine) peptidase inhibitor, clade E, member 1	*SERPINE1*	Mm00435860_m1
Tissue inhibitor of metalloproteinase 4	*TIMP4*	Mm00446568_m1
Matrix metalloproteinase 14 (membrane-inserted)	*MMP14*	Mm00485054_m1
Fatty acid binding protein 4, adipocyte	*FABP4*	Mm00445880_m1
Matrix metalloproteinase 8	*MMP8*	Mm00772335_m1
Matrix metalloproteinase 3	*MMP3*	Mm00440295_m1
Interleukin 5	*IL5*	Mm00439646_m1
Actin, beta, cytoplasmic	*ACTB*	Mm00607939_s1
Tissue inhibitor of metalloproteinase 3	*TIMP3*	Mm00441826_m1
Prostaglandin-endoperoxide synthase 2	*PTGS2*	Mm00478374_m1
Mast cell protease 6	*MCPT6*	Mm00487645_m1

Tumor necrosis factor	*TNF*	Mm00443258_m1
Interleukin 1 beta	*IL1B*	Mm00434228_m1
Matrix metalloproteinase 9	*MMP9*	Mm00442991_m1
Matrix metalloproteinase 13	*MMP13*	Mm00439491_m1
Prostaglandin E receptor 4 (subtype EP4)	*PTGER4*	Mm00436053_m1
Peroxisome proliferator activated receptor gamma	*PPARG*	Mm00440945_m1
Matrix metalloproteinase 7	*MMP7*	Mm00487724_m1
Matrix metalloproteinase 11	*MMP11*	Mm00485048_m1
Tenascin C	*TNC*	Mm00495662_m1
Tissue inhibitor of metalloproteinase 1	*TIMP1*	Mm00441818_m1
Chemokine (C-X-C motif) ligand 1	*CXCL1*	Mm00433859_m1
Colony stimulating factor 1 (macrophage)	*CSF1*	Mm00432688_m1
FMS-like tyrosine kinase 1	*FLT1*	Mm00438980_m1
Matrix metalloproteinase 2	*MMP2*	Mm00439508_m1
Tissue inhibitor of metalloproteinase 2	*TIMP2*	Mm00441825_m1
Matrix metalloproteinase 10	*MMP10*	Mm00444630_m1
Interleukin 10	*IL10*	Mm00439616_m1
Colony stimulating factor 1 receptor	*CSF1R*	Mm00432689_m1
Interleukin 6	*IL6*	Mm00446190_m1

Real-time PCR was performed on an ABI PRISM®7900HT sequence detector (Applied Biosystems) using TaqMan™ gene expression Assays-on-demand (Applied Biosystems) for *Mmp10* (Mm00444630_m1) and *Cxcl1* (Mm00433859_m1). *β-actin* (Mm.PT.49.9990212.g, IDT) was used as housekeeping gene.

3.2 *In vitro* Studies

3.2.1 Isolation of peritoneal leukocytes

Ice-cold PBS was injected into the peritoneal cavity of WT and *Mmp10*$^{-/-}$ mice after sacrifize. Peritoneal lavages were retrieved as previously described (section 3.1.4). Cells were then centrifuged at 1200 rpm, at 4ºC for 5 min. For RNA assays, the resulting pellet was resuspended in red blood cell lysis buffer and stored at -80ºC for RNA isolation. For cell culture experiments cells were immediately used, as described below.

3.2.2 CXCL1 cleavage by MMP-10

To elucidate whether CXCL1 is a substrate for MMP-10, human recombinant CXCL1 (R&D Systems) was incubated with active rhMMP-10 (5:1 substrate/enzyme molar ratio) for 24 hours at 37 ºC in assay buffer (100 mmol/L NaCl, 5 mmol/L $CaCl_2$, 20 mmol/L Tris-HCl, pH 7.5). Digestion products were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with GelCode Blue stain reagent (Pierce). MMP activity inhibitor GM6001 (Ryss Laboratory) was used as negative control (1 µM).

3.2.3 Isolation of lung endothelial cells

Mouse lung ECs from WT and *Mmp10*$^{-/-}$ mice were isolated by selection with ICAM-2-coated magnetic beads. In short, mice were sacrificed by CO_2 inhalation, and lungs were excised and finely minced prior to collagenase A (0.1% v/v, Gibco) digestion at 37ºC for 1 hour under shaking. The digested tissue was homogenized with a 14G syringe and centrifuged at 200 g for 5 min. Then, the cell pellet was extensively washed and seeded in a 1% gelatin-coated flask. After 4 days in culture (5% CO_2, 37ºC), ECs were recovered using Dynabeads® Sheep anti Rat IgG (Invitrogen) coupled to a rat anti-mouse ICAM-2 antibody (BD Pharmingen) and selected by magnetic binding to MACS LS columns (Miltenyi Biotech). ECs were cultured in DMEM:F12 medium (Sigma-Aldrich) supplemented with 20% fetal calf serum (Sigma-Aldrich), 30 mg/mL endothelial cell growth supplement (ECGS, Sigma-Aldrich), 100 mg/mL heparin (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco).

3.2.3.1 In vitro regulation of CXCL1 by MMP-10

ECs stimulation

To determine *Cxcl1* expression in ECs we used WT cells as follows: when WT ECs reached confluence they were subjected to serum deprivation for 15 hours, then stimulated with 2 nM active rhMMP-10 and their RNA collected in lysis buffer 12 hours after. The RNA was reverse transcribed and analyzed by RT-PCR, as previously described.

For the studies regarding Cxcl1 regulation by MMP-10, actinomycin D (Act D) was used. WT ECs were serum deprived overnight and then pre-incubated with Act D (0.5 µM, Sigma-Aldrich) for 1 hour, and stimulated with active rhMMP-10 (2 nM) for additional 12 hours and harvested for CXCL1 expression analysis. To study the stability of CXCL1 mRNA after serum starvation cells were stimulated with rhMMP-10 for 12 hours and then treated with ActD for 9 hours.

Leukocyte transendothelial migration

100.000 murine lung ECs from WT and *Mmp10*$^{-/-}$ mice (isolated and cultured as described above) in 100 µL of complete medium (see above) were plated in the upper chamber of gelatin precoated 3-µm-pore-size Boyden chambers (Transwell® Permeable support, Costar) and 600 µL of complete medium were poured onto the lower chamber. One day after, cells were serum starved by replacing complete medium by serum free medium in both chambers. Twenty-four hours after, ECs were stimulated with 20 ng/mL of TNFα (Sigma) in serum free medium for 2 hours. In the meantime, intraperitoneal leukocytes were isolated from WT and *Mmp10*$^{-/-}$ mice (as explained above), resuspended in RPMI (0.5 % bovine serum albumin (BSA), 1% penicillin/streptomycin) and stained with 0.2 µM of calcein (Molecular Probes) for 30 min at 37ºC. Then, the medium of lower chamber was replaced by stimulation medium (RPMI 0.5 % bovine serum albumin, 1% penicillin/streptomycin, 100 ng/mL MCP-1 (Sigma) and the medium of the upper chamber by 150.000 calcein-stained leukocytes in 100 µL of RPMI (0.5 % BSA, 1 % penicillin/streptomycin). Cells were incubated during 2 hours at 37ºC. Migrated monocytes were dettached from the lower chamber by incubation with 5 mM EDTA in cold PBS during 5 min at 4ºC. The resulting supernatant was centrifuged at 1500 rpm during 5 min and stained for CD45 (1:250, APC anti mouse CD45, BD Pharmingen) 20 min at 4ºC, for flow cytometry analysis (FACs Calibur, BD Bioscience).

3.3 Extracellular vesicles study

All EV studies were carried out in platelet-poor plasma (PPP) from WT and Mmp10-/- mice which were sacrifized 3, 15 and 28 days after ischemia. Briefly, blood was collected by heart puncture after sacrifizing the animals and kept in ice. PPP was prepared by two sequential centrifugations (10 min, 2500 rpm, 4ºC, followed by 2 min, 13000 rpm, 4ºC) and kept at -80ºC until its analysis.

3.3.1 Nanoparticle tracking analysis (NTA)

To measure particles/EVs number and size distribution an NS500 (NanoSight Limited) equipped with an electron multiplying charge coupled device camera (Andor Technology) and a 405 nm laser were used. During measurements, temperature was kept at 22°C. The viscosity of water at 22°C (0.95 mPa s) was used, as samples were diluted in PBS buffer. Silica beads with a diameter of 100 nm and known concentration were used to adjust the focus height of the objective [166,167]. NTA v2.3.0.17 software (NanoSight Limited) was used for data analysis. Before the measurement, samples were diluted 2000-8000 times with 0.05 μm-filtered PBS buffer depending on the sample concentration. Ten videos of 30 seconds were captured per measurement at camera level 14. The detection threshold was set at pixel value 10 throughout all the measurements.

3.3.2 Flow cytometry

Cellular origin of EVs was measured by flow cytometry. 45 μl of 10 times diluted plasma samples were stained by the addition of 5 μL (4 μg/mL) FITC-labelled lactadherin for PS detection (Haematologic Technologies) combined with another 5 μl (4 μg/mL) of one of the following rat anti-mouse antibodies: CD41-PE (clone MWReg30; BD Pharmigen), CD62E-PE (clone 10E9.6, BD Pharmigen), F4/80-APC (clone BM8, eBioscience) or Ly6G APC (1A8, eBioscience) for the detection of MPs derived from platelets, activated ECs, macrophages and neutrophils, respectively. The dilution of the antibodies and the plasma samples was carried out in 0.32% citrate in PBS buffer 0.05 μm filtered, pH 7.4 in order to avoid clotting of the plasma. Unlabelled sample was used as a negative control for lactadherin-labelled samples. As negative controls of antibody stainings the following rat isotype controls were used at the same concentration as the antibodies: IgG1,κ PE (clone eBGR1, BD Pharmigen), IgG2a,κ PE (clone eBR2a, eBioscience) and IgG2a,κ APC (clone R35-95, eBioscience). Samples

were incubated for 15 min in darkness at room temperature and diluted with 400 µL 0.05 µm-filtered PBS before being measured on Apogee A50-Micro (Apogee Flow Systems, Hemel Hempstead) for 60 seconds or $5x10^5$ total events. FlowJo software was used for data analysis. Concentration values were obtained by referring the counts obtained to the volume measured for each sample.

3.4 Statistical analysis

Results from *in vivo* and *in vitro* studies are expressed as mean±SEM. The statistical analysis for comparisons among more that two groups was performed by Kruskal-Wallis (non-parametric) or 2-way ANOVA (parametric), followed by Mann-Whitney U-test or Bonferroni for comparisons between two groups, respectively. Paired samples were analyzed using Fridman's test followed by Wilcoxon to compare between two related samples. Statistical significance was established as $P<0.05$. For the analysis of particle concentration of different sizes and different genotypes, 2-way ANOVA was performed followed by t-test. The statistical analysis was performed with SPSS for Windows software package version 15.0.

4- RESULTS

Acute ischemia triggers the activation of different overlapping processes in order to enable tissue repair. Muscle regeneration should be studied as a continuous process where the correct resolution of the degenerative phase is indispensable for the proper activation and resolution of the regenerative phase. Bearing this in mind, but in order to give a clearer overview on the involvement of MMP-10 on the skeletal muscle remodeling after injury, the disclosure of the results obtained in this research will be divided into two stages with different end points.

The first stage will comprise the degenerative phase (3 days post-ischemia), in which necrosis and inflammation of the skeletal muscle can be observed. The second stage of the reparative processes will be named as the regenerative phase (15 or 28 days after ischemia). In this stage, the resolution of the activation of satellite cells, ECs and smooth muscle can be seen [35,39]. The completion of this processes takes longer, thus the final results should be studied at later time points.

Since the project was focused on the study of different grades of ischemia, the analysis of the tissues was performed in mild ischemia, with about 50% drop in perfusion after ligation, and severe ischemia (total excision model), in which more than 95% of the perfusion was shortened after the excision of the femoral artery.

4.1 DEGENERATIVE PHASE OF ISCHEMIA

4.1.1 MMP-10 expression is up-regulated in skeletal muscle after ischemia

To determine whether ischemia could trigger the expression of MMP-10 in the skeletal muscle, we used the most severe model of ischemia to analyze the expression of MMP-10 in soleus muscles of WT mice. Mice were subjected to total excision of the femoral artery [165] and their crural muscles harvested at different time points. *Mmp10* mRNA was barely detectable at baseline and increased drastically within the 3 days following femoral artery excision (Figure 13A, $P<0.05$). This was confirmed by Western blot 3 days after surgery (Figure 13B). At the regenerative phase (day 15 and 28 post-excision) MMP-10 levels were similar to those at baseline (Figure 13A).

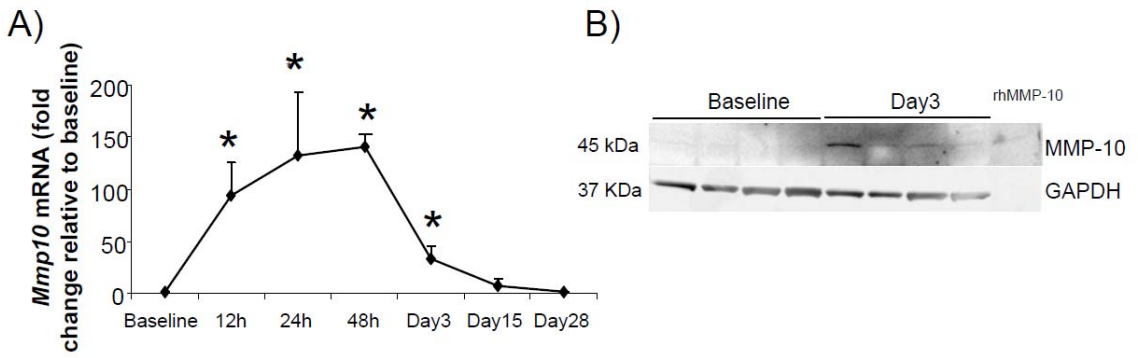


Figure 13. A) Time course for *Mmp10* mRNA levels in skeletal muscle after femoral artery excision in WT mice. Fold change relative to baseline (n=5). *P<0.05 vs. baseline. B) Western blot for MMP-10 and GAPDH (loading control) in crural muscles at baseline and day 3 after severe ischemia. Active rhMMP-10 (5 ng) was used as positive control (n=4).

In order to determine the *in-situ* expression of MMP-10 we performed immunohistochemistry in soleus muscles of WT mice at baseline, day 3 and 28 after femoral artery excision (total excision model). As shown in Figure 14A, at baseline, MMP-10 expression was observed in large and small vessels and in myocytes as a diffuse cytoplasmatic staining. Three days post-ischemia, when the immune response is taking place, MMP-10 signal increased drastically. However, at day 28 when the regenerative phase is resolving, MMP-10 expression could barely be detected (Figure 14A).

To further analyze the observed upregulation of MMP-10 three days after femoral artery excision, we performed double immunofluorescence for different markers to determine its cellular origin. We concluded that the increased expression of MMP-10 was restricted to ECs and the interstitial tissue (Figure 14B).

In summary, MMP-10 is overexpressed in the skeletal muscle in response to ischemia only during the degeneration phase and its expression is localized in the interstitial tissue and in ECs.

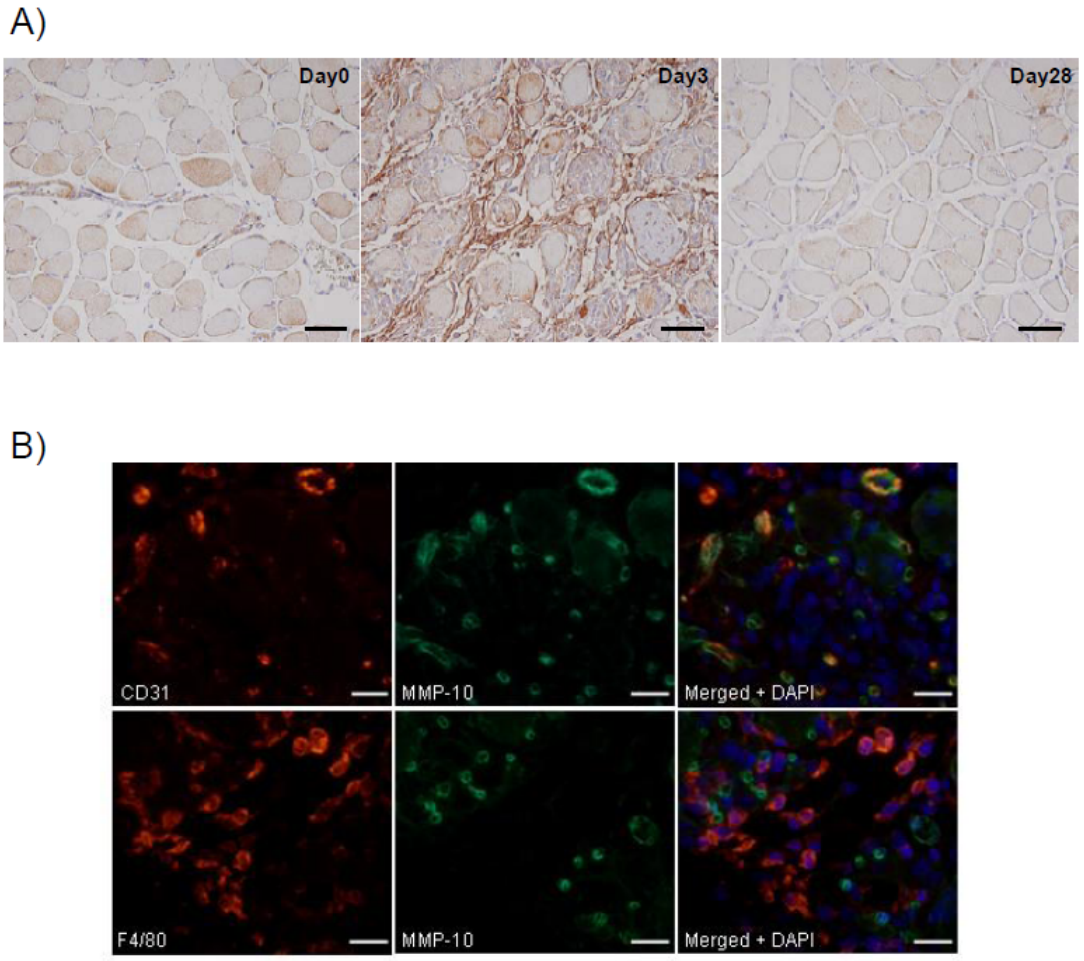


Figure 14. A) Immunohistochemistry for MMP-10 in WT solei at baseline, 3 and 28 days post-ischemia. Scale bar denotes 10 µm. B) Double immunofluorecence for MMP-10 and the endothelial marker CD31 (upper line), and the macrophage marker F4/80 (lower line) in WT solei 3 days post-excision. Scale bar denotes 25 µm.

Since MMP-10 is indeed upregulated after ischemia, we performed the mild ischemia and the total excision models in WT and *Mmp10*$^{-/-}$ mice in order to asses its role in tissue remodeling.

4.1.2 Delayed reperfusion and increased necrosis in *Mmp10*$^{-/-}$ mice

To study whether MMP-10 absence could affect blood supply recovery after ischemia, we measured the perfusion rate to the hind limbs of WT and *Mmp10*$^{-/-}$ mice in both models.

In mild ischemic conditions, tissue perfusion dropped by 50% immediately after ischemia in both genotypes (Figure 15A). Two days post-ischemia blood flow started to recover in WT but not in *Mmp10*$^{-/-}$ mice (perfusion % of non-ligated limb: 73±11 WT vs

58±12 *Mmp10*$^{-/-}$; *P*<0.05). The latter presented a delayed but fast recuperation rate and by day 4 caught up with WT mice (perfusion % of non-ligated limb: 84±6 WT vs 89±6 *Mmp10*$^{-/-}$). Animals subjected to the total excision model presented a more severe phenotype and slower recovery. Twenty-four hours post-excision, perfusion was reduced by 70% in excised versus non-excised limbs from WT mice. As shown in Figure 15B, tissue oxygenation increased gradually and was almost normalized 24 days after ischemia. *Mmp10*$^{-/-}$ mice presented reduced reperfusion 24 hours and 3 days post-excision (*P*<0.05), and reached WT values by day 7. Our results show that MMP-10 inactivation results in delayed tissue reperfusion early after arterial ischemia.

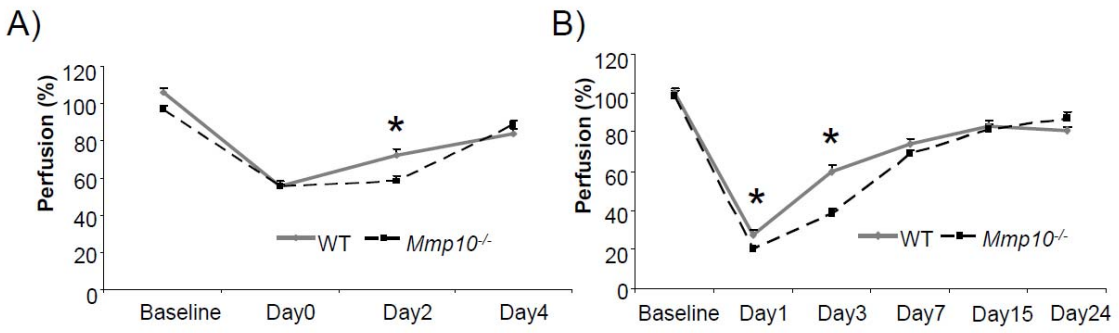


Figure 15. A) Tissue perfusion (laser Doppler) in *Mmp10*$^{-/-}$ mice 2 days post-ligation in mild ischemia model (n=6/genotype). *P<0.05 vs. WT. B) Perfusion assessed by microPET analysis in total excision model in *Mmp10*$^{-/-}$ mice 1 and 3 days after excision. n=10-15/genotype. *P<0.05 vs. WT.

To examine whether differences observed in tissue reperfusion could influence muscle degeneration, morphological analysis on soleus muscles were performed. To determine the necrosis rate, H&E staining was assessed in soleus sections 3 days post-ischemia. In mild ischemic conditions, the necrotic area was about 30% and comparable between WT and *Mmp10*$^{-/-}$ mice (Figure 16A). In total excision model, however, the necrotic area in *Mmp10*$^{-/-}$ mice was larger than WT mice (*P*<0.001; Figure 16B), indicating that the lower perfusion to the limbs in *Mmp10*$^{-/-}$ mice is accompanied by greater necrosis under severe ischemic conditions.

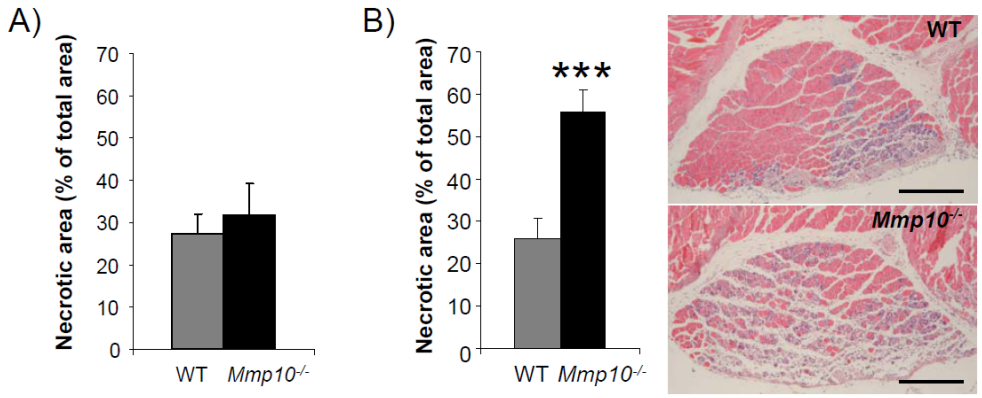


Figure 16. Tissue necrosis 3 days after mild ischemia (n=6) (A) and in total excision model (B) (n=28, ***P<0.001 vs. WT). Representative micrographs (H&E) of WT and *Mmp10*$^{-/-}$ solei 3 days post-ischemia are shown. Scale bar denotes 500 µm.

4.1.3 Genetic inactivation of MMP-10 regulates leukocyte recruitment *in vivo* and *in vitro*

To determine whether MMP-10 could modulate the inflammatory response triggered by ischemia, soleus sections were immunostained for neutrophils (NIMP-R14), and macrophages (F4/80) 3 days post-ischemia. As shown in Figures 17A and B, in the mild ischemia model *Mmp10*$^{-/-}$ mice presented increased macrophage infiltration compared to WT ($p<0.05$) though no differences in neutrophils were observed.

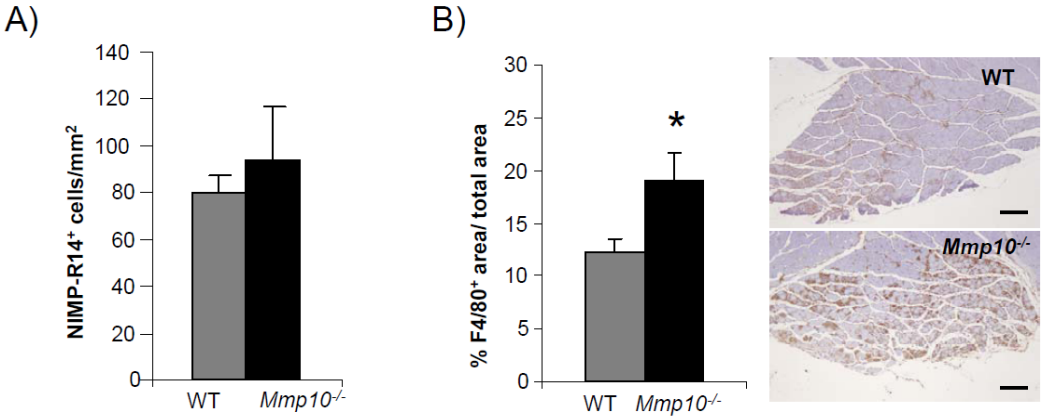


Figure 17. Neutrophil (A) and macrophage (B) infiltration in WT and *Mmp10*$^{-/-}$ mice 3 days post-ligation in mild ischemia model. Representative micrographs for F4/80 immunohistochemistry are shown (B). Scale bar denotes 250 µm (n=6/genotype, P<0.05).

However, in the total excision model, *Mmp10*$^{-/-}$ solei showed increased number of neutrophils (Figure 18A, *P*<0.001) and macrophages (Figure 18B, *P*<0.05). Besides, we also studied whether the exacerbated muscle degeneration observed in MMP-10 deficient mice after severe ischemia could be related not only to differences in macrophage numbers but also to their subtypes. Therefore, the number of wound healing/pro-angiogenic macrophages (M2-like) from the total excision model was measured in soleus sections 3 days post ischemia by the expression of the MRC1 mannose receptor. No statistical difference in the number of MRC1^{+}F4/80^{+} cells was detected between the genotypes, although *Mmp10*$^{-/-}$ mice presented a 50% reduction compared to WT (Figure 18C).

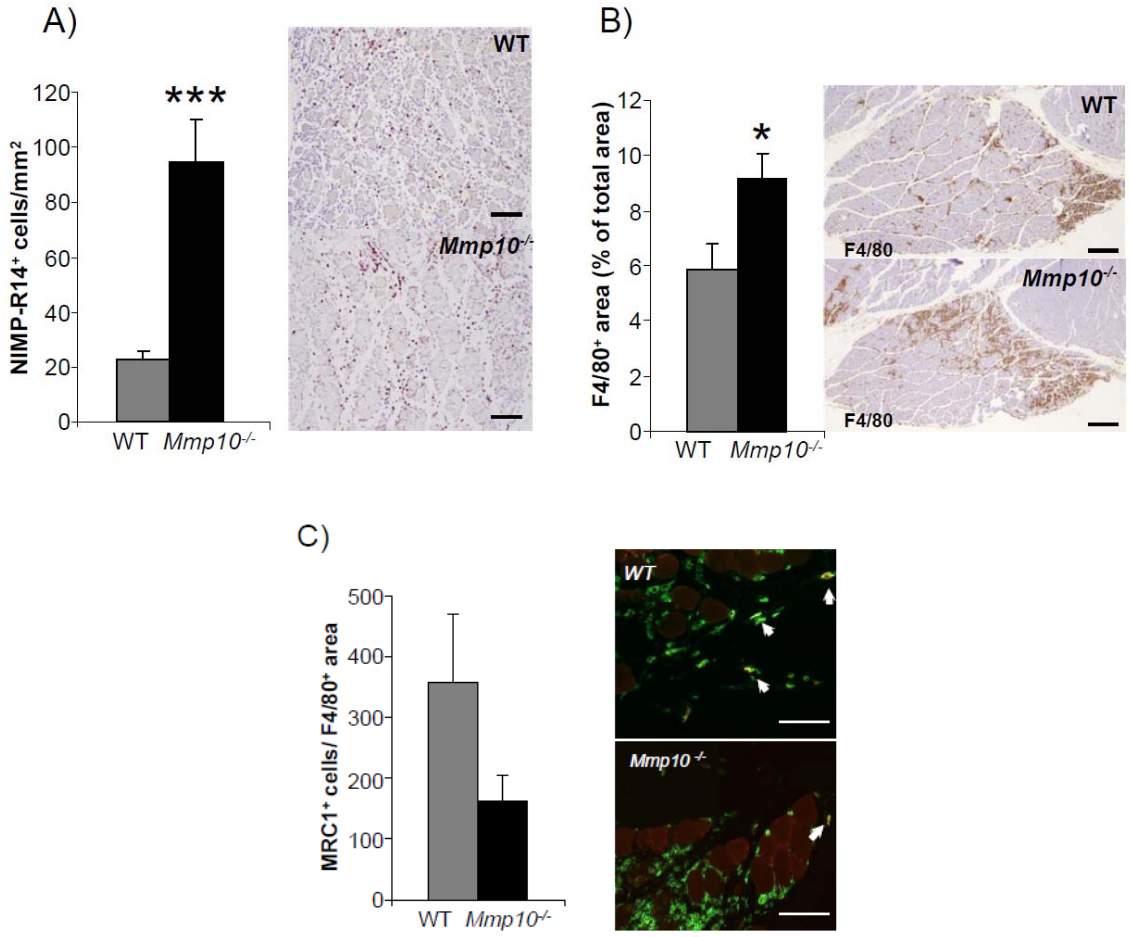


Figure 18. Inflammatory profile in total excision model. Representative micropgraphs and quantification of NIMP-R14^{+} (A) and F4/80^{+} (B) density at day 3 (n=27). *P<0.05 and ***P<0.001 vs. WT. Scale bar denoting 10 and 250 µm, respectively. C) Density of M2-like macrophages (F4/80^{+} MRC1^{+}) at day 3 in soleus sections (n=8/genotype). Representative micrographs of MRC1^{+} macrophages are shown. Scale bar denotes 100 µm. Green corresponds to F4/80^{+} cells, red to MRC1^{+} cells and yellow to double immunostained cells indicated by arrowheads.

In order to obtain deeper insight into the involvement of MMP-10 in leukocyte recruitment, we measured leukocyte transmigration through an endothelial monolayer *in vitro*. We isolated ECs from murine lungs from WT and *Mmp10*$^{-/-}$ mice to build up the endothelial layer and peritoneal leukocytes from both genotypes for the transmigration assay. As shown in Figure 19, leukocyte transmigration significantly increased when ECs were MMP-10 deficient, and further incremented when both endothelium and leukocytes were null for MMP-10 (P<0.01).

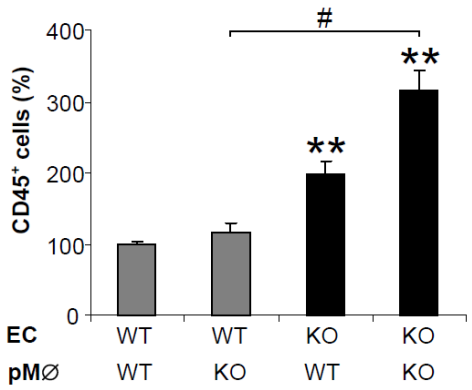


Figure 19. Leukocyte transendothelial migration *in vitro* of WT and *Mmp10*$^{-/-}$ (KO) cells. The absence of endothelial MMP-10 increases leukocyte transmigration, which is further augmented when leukocytes also lack this protease. (5 independent experiments, n=6/experiment) **P< 0.01 vs. WT/WT. #P< 0.01 vs. WT/KO.

Our data showed an exaggerated inflammatory response after ischemia in absence of MMP-10 manifesting a role for MMP-10 in leukocyte trafficking. To confirm these results we used an *in vivo* inflammation model of thioglycollate-induced peritonitis in WT and *Mmp10*$^{-/-}$ mice and analyzed the recruitment of neutrophils and macrophages. Forty-eight hours after thioglycollate injection no differences in total number of cells were detected between WT and *Mmp10*$^{-/-}$ mice (number of recruited cells: $20.8x10^6$ $\pm 2.8x10^6$ WT vs $20.2x10^6$ $\pm 3.7x10^6$ *Mmp10*$^{-/-}$). However, a shift in leukocyte populations between the genotypes was observed, as MMP-10 inactivation increased neutrophil recruitment (Figure 20A, P<0.05), while reducing the number of recruited macrophages compared to WT (Figure 20B, P<0.05).

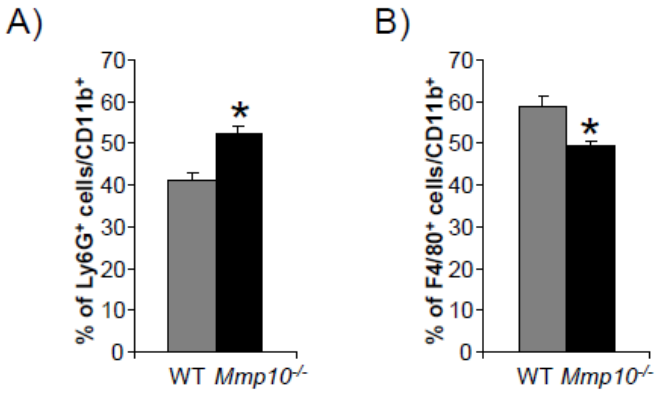


Figure 20. Percentage of $Ly6G^+$ neutrophils (A) and $F4/80^+$ macrophages (B) in peritoneal lavages after thioglycollate injection in WT and *Mmp10*$^{-/-}$ mice. *P<0.01 vs. WT (n=6).

In summary, we concluded that during the degeneration phase *Mmp10*$^{-/-}$ mice present lower perfusion rates accompanied by greater necrosis and greater leukocyte infiltration after severe ischemia when compared to WT mice. We believe that this phenotype could be related to a modulation of the inflammatory response by MMP-10.

4.2 REGENERATIVE PHASE OF ISCHEMIA

4.2.1 Altered collateralization and neovascularization in *Mmp10*$^{-/-}$ mice in response to ischemia

To address whether reduced perfusion in *Mmp10*$^{-/-}$ mice early after ischemia was related to a decreased vascularization or collateralization at baseline, we performed immunohistochemistry for CD31 and αSMA, respectively, in soleus sections of non-ligated (control) and ligated limbs (Figure 21 and 22A) but no differences were found between the genotypes at that time point.

As expected according to the angiogenic process, both genotypes increased the number of $CD31^+$ vessels at day 14 in mild ischemic conditions, although there were no differences between genotypes throughout the whole angiogenic response (Figure 21).

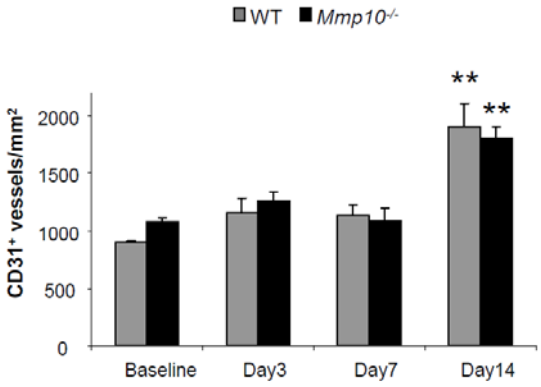


Figure 21. Vessel density ($CD31^+$ vessels) measurements at day 3, 7 and 14 post- femoral artery ligation in mild ischemic conditions (n=6). **P<0.01 vs. baseline.

At more severe ischemic conditions, WT mice presented a gradual increase in vessel density from day 3 to 15, while at day 28 the number of $CD31^+$ capillaries drops to baseline. In *Mmp10*$^{-/-}$ mice however, although the angiogenic response was similar to WT at day 3 and 15, vessel density remained elevated 28 days after femoral excision (Figure 22A), suggesting a slower vessel regression when MMP-10 is not functional or a slower recovery of the tissue after a greater damage. The analysis of αSMA positive arterioles shows a similar pattern, with an undistinguishable increase in arteriole density 15 days post ischemia between the genotypes, and a different vessel regression at day 28 (*P*<0.05, Figure 22B).

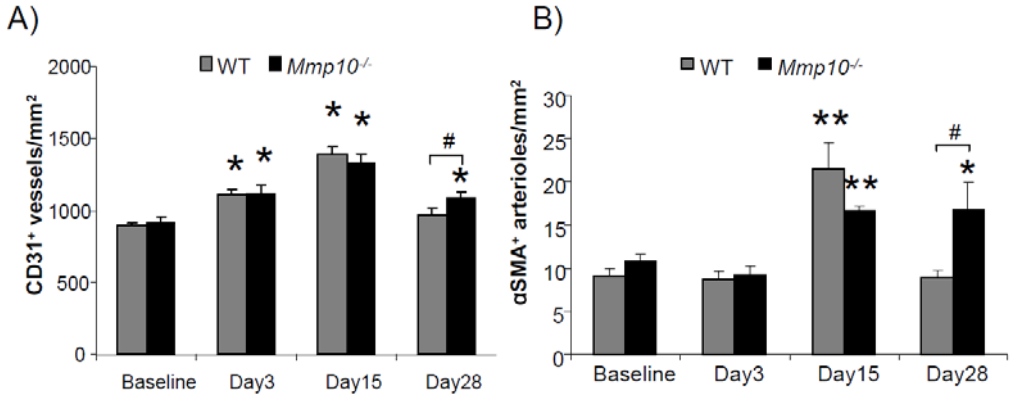


Figure 22. Morphometric analysis of vessel ($CD31^+$, A) and arteriole (αSMA^+ B) density in soleus sections of WT and *Mmp10*$^{-/-}$ excised limbs 3, 15 and 28 days post-ischemia in the total excision model (n=10). Grey bars correspond to WT mice, black bars correspond to *Mmp10*$^{-/-}$. *P<0.05 and **P<0.01 vs. baseline; #P<0.05 vs. WT.

4.2.2 MMP-10 does not influence total collagen content in soleus after ischemia

In the degenerative phase, a provisional matrix is deposited to maintain muscle integrity. During the regenerative phase, the scar must be reorganized and contracted to enable the repair process [112]. MMPs, as primary ECM proteolytic enzymes are key molecules in the degradation process of the scar [168]. Therefore, we analyzed whether MMP-10 inactivation could affect total collagen content in the excised limbs (Sirius red staining). As shown in Figure 23, mild and severe ischemia increased collagen fraction in the ligated limbs compared to their controls without differences between genotypes, suggesting no role for MMP-10 in overall muscle fibrosis.

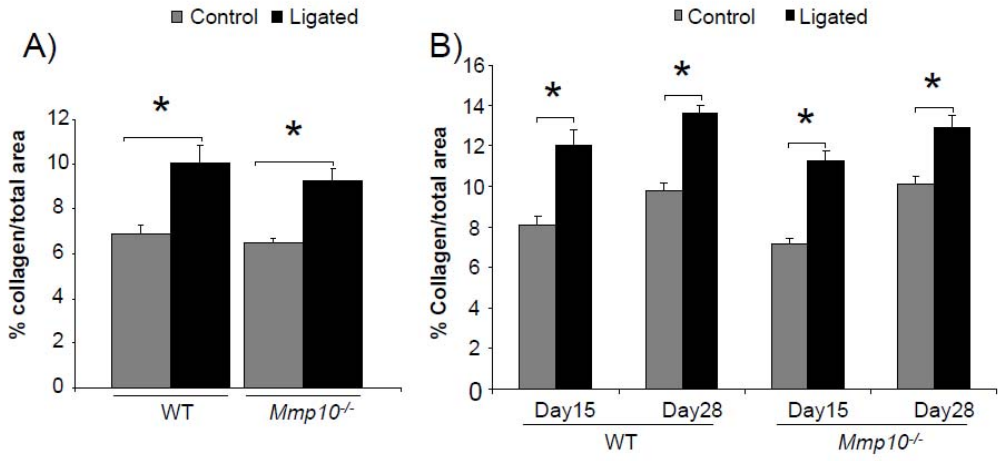


Figure 23. A) Total collagen content measurement (Sirius red) in mild ischemia model 14 days post-ligation (n=6; *P<0.05 vs non-ligated limb) and in the total excision model (B) 15 and 28 days after ischemia (n=10, *P<0.05 vs. non-ligated limb).

4.2.3 Genetic deletion of MMP-10 delays muscle regeneration after ischemia

Skeletal muscle repair is a highly synchronized process that triggers well-characterized morphological changes. Newly formed myofibers present small caliber and central located myonuclei. When the fusion of myogenic cells is completed, myofibers increase in size and myonuclei move to the periphery, making the regenerated muscle morphologically and functionally indistinguishable from the undamaged tissue [43]. To assess the role of MMP-10 in this process, the myocyte density and the number of centrally nucleated myofibers were measured in soleus sections. After mild ischemia

no differences between WT and *Mmp10*$^{-/-}$ tissues were observed at any timepoint (Figure 24).

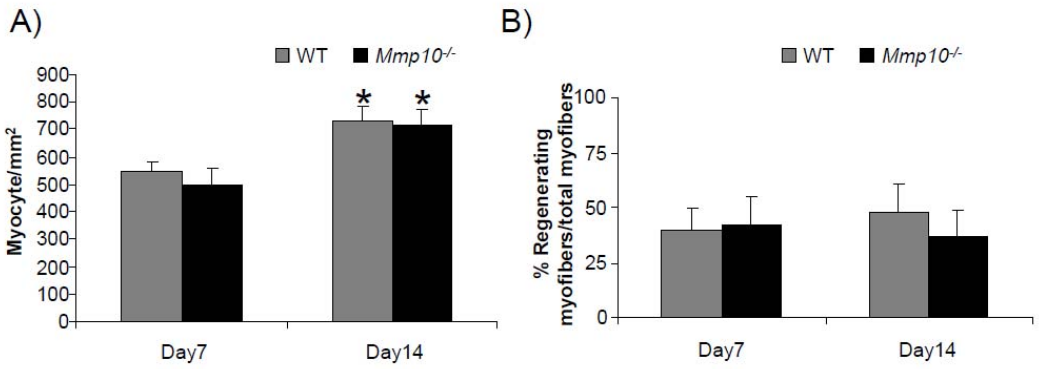


Figure 24.Muscle regeneration in mild ischemia model. A) Myocyte density 14 days post ligation. *P<0.05 vs. day 7. B) Percentage of regenerating myofibers with central nuclei in mild ischemia model 7 and 14 days after femoral artery ligation (n=6).

However, the total excision of the femoral artery induced a deep regeneration of the skeletal muscle. As a result, WT mice presented increased number of smaller and centrally-nucleated myocytes 15 days after excision compared to baseline, and less but bigger perinucleated fibers, resembling the morphology of non-injured muscle, 28 days after ischemia (Figure 25A-C). In *Mmp10*$^{-/-}$ solei however, myocyte density was lower and fiber size bigger than WT mice 15 days post-ischemia (Figure 25A, B). At day 28, when the majority of the fibers should be already perinucleated, only 30% of them presented that phenotype in the *Mmp10*$^{-/-}$ mice (Figure 25C), indicating a delayed muscle repair. These results suggest the requirement of MMP-10 in muscle regeneration after ischemia.

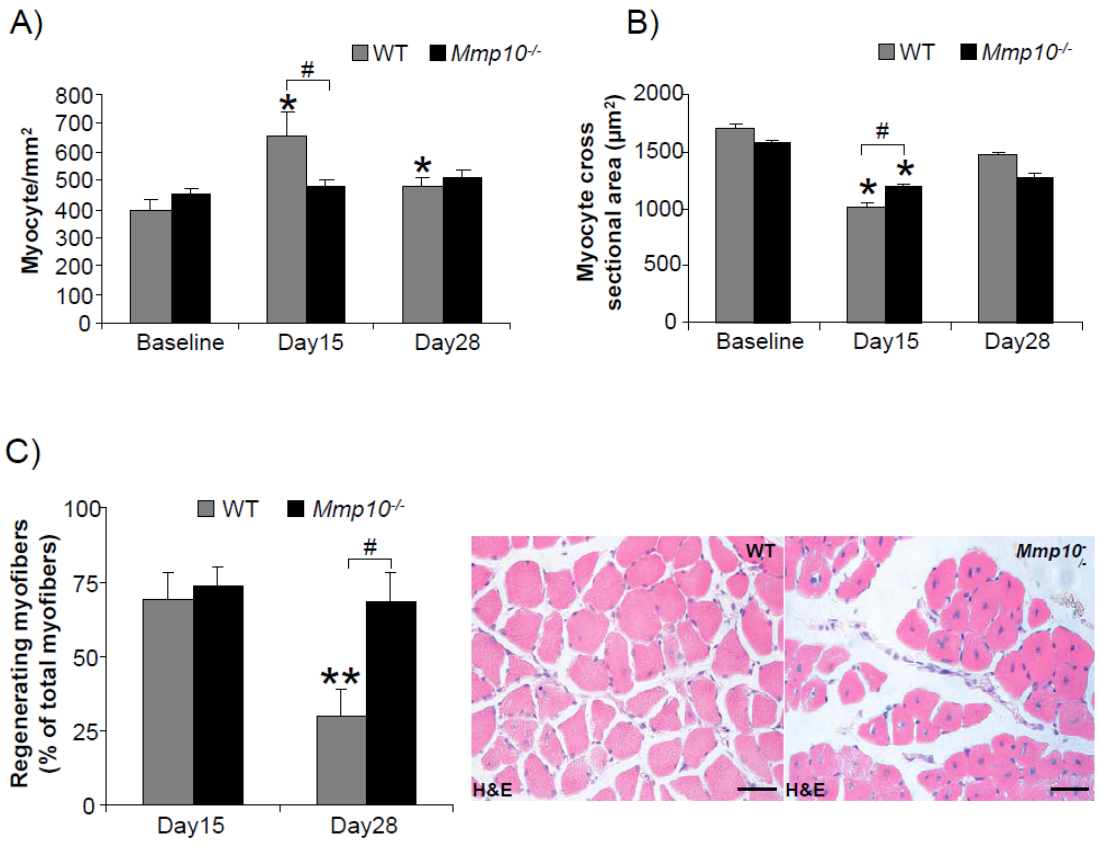


Figure 25. Muscle regeneration in total excision model. A) Myocyte density in soleus sections (H&E) at baseline, day 15 and 28 after femoral artery excision (n=10) *P<0.05 vs. control; #P<0.05 vs. WT excised. B) Myocyte cross-sectional area 15 days post-excision. *P<0.05 vs. control; #P<0.05 vs. WT. C) Number of regenerating myocytes 28 days after femoral artery excision (n=10) **P<0.01 vs. day15; #P<0.01 vs. WT. Representative micrographs at day 28 are shown. Scale bar denotes 50 µm.

Finally, we determined the fiber-size distribution in laminin stained soleus sections. At baseline, 70% of the fibers presented sizes between 1000 and 2000 µm^2 in both genotypes (Figure 26A). As expected, 15 days post-excision, fiber-cross sectional area was reduced in WT mice compared to baseline conditions. In contrast, *Mmp10*$^{-/-}$ mice showed less small fibers and increased frequency of bigger myocytes compared to WT (P<0.05, Figure 26B). At day 28, fiber-size distribution was similar to controls, though the amount of small-size myocytes was slightly increased in *Mmp10*$^{-/-}$ solei (Figure 26C).

Taken together, our studies concluded that *Mmp10*$^{-/-}$ muscles present a slower regeneration rate when compared to WT ones.

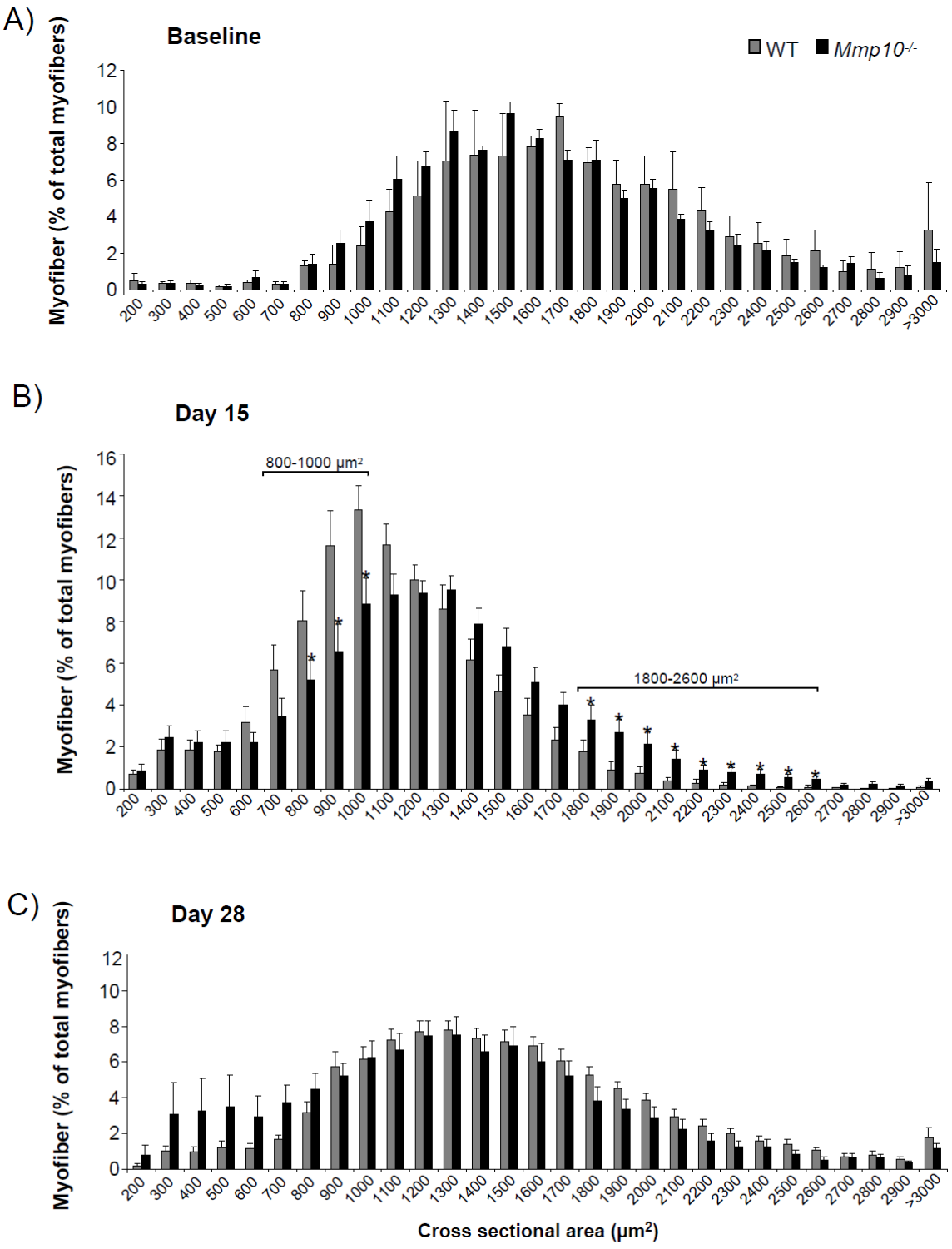


Figure 26. Fiber size distribution measured in laminin stained soleus sections at baseline (A), day 15 (B) and day 28 (C) after artery excision. *P<0.05 vs. WT (n=10). Grey bar refers to WT and black bar to *Mmp10*$^{-/-}$.

4.3 RECOVERY EXPERIMENTS

4.3.1 rhMMP-10 administration to *Mmp10*$^{-/-}$ mice improves skeletal muscle regeneration

The total excision model showed a clear involvement of MMP-10 in the overall regeneration of the skeletal muscle. Therefore, we used this model for the assessment of the direct implication of MMP-10 in muscle repair. *Mmp10*$^{-/-}$ mice were treated with rhMMP-10 after ischemia to perform immunohistochemical analysis of soleus muscles. Two groups of *Mmp10*$^{-/-}$ mice were i.v. injected with 6.5 µg/kg of rhMMP-10 or vehicle at different time points. Group 1 was treated with the protein right after excision to evaluate its effect at the degenerative phase (3 days post-ischemia). Group 2 was injected one day post-ischemia to study the outcome of rhMMP-10 administration at the regenerative phase (28 days after excision). We used vehicle-injected WT mice as control.

Mmp10$^{-/-}$ animals receiving rhMMP-10 right after excision (group 1) recovered the WT phenotype regarding necrosis and number of infiltrating macrophages three days post-ischemia, although neutrophil recruitment remained similar to that observed in untreated *Mmp10*$^{-/-}$ mice (Figure 27A-C). Regarding the regeneration rate, group 2 showed a similar percentage of regenerating myofibers to that observed for WT animals in rhMMP-10-treated *Mmp10*$^{-/-}$ mice 28 days post-ischemia (Figure 27D). These results confirm the direct involvement of MMP-10 in muscle repair after ischemia.

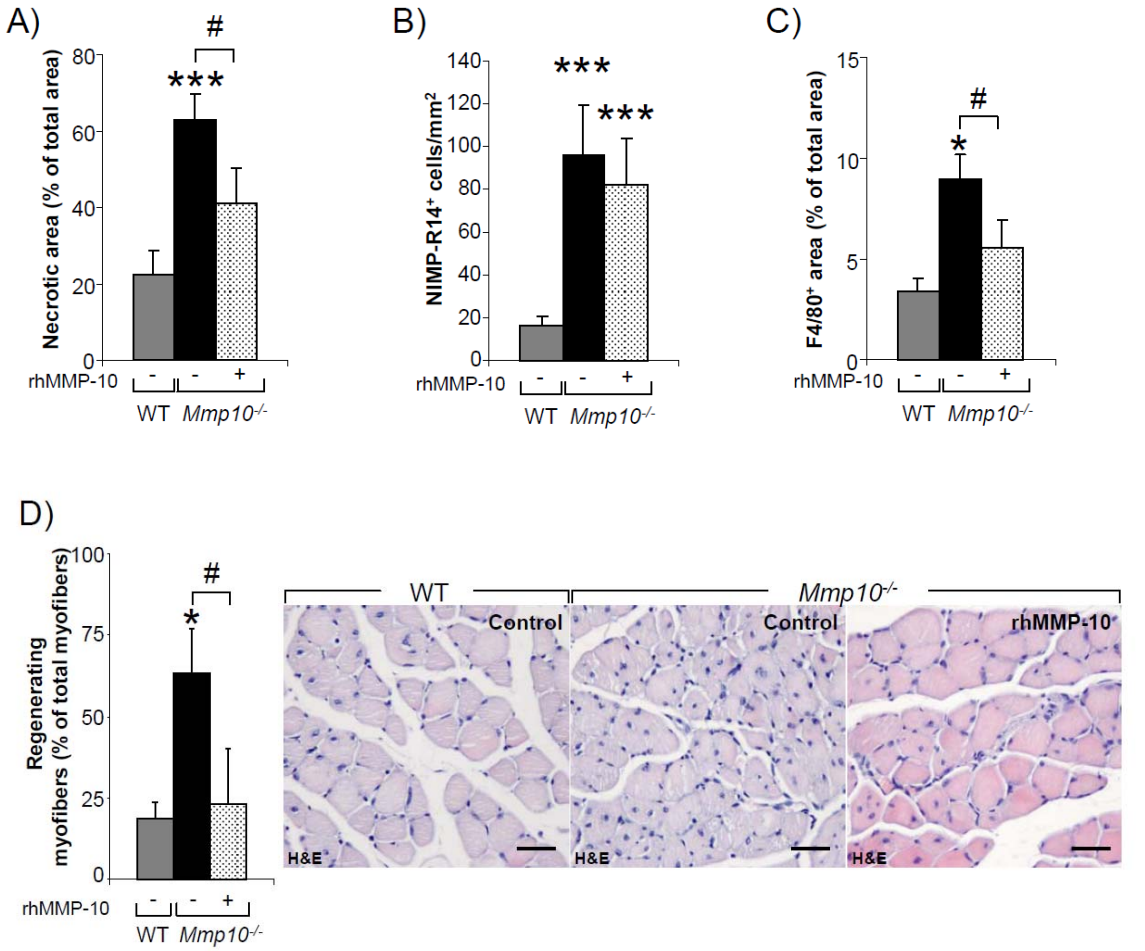


Figure 27. The administration of rhMMP-10 increases muscle recovery in *Mmp10*$^{-/-}$ mice. A-C) Percentage of necrotic area (A), neutrophil (B) and macrophage infiltration (C) in soleus sections of rhMMP-10-injected animals (group 1) 3 days post-femoral artery excision. Vehicle-injected WT (grey bar), *Mmp10*$^{-/-}$ (black bar) and rhMMP-10-injected *Mmp10*$^{-/-}$ mice (doted bar). *P<0.05 and ***P<0.001 vs. vehicle-injected WT mice (n=15). #P<0.05 vs. vehicle-injected *Mmp10*$^{-/-}$ mice. D) Number of regenerating myocytes in rhMMP-10-treated Mmp10$^{-/-}$ mice 28 days post-excision (group 2). *P<0.05 vs. vehicle-injected WT mice. #P<0.05 vs. vehicle-injected *Mmp10*$^{-/-}$ mice (n=5). Representative micrographs show increased number of centrally-nucleated cells in *Mmp10*$^{-/-}$ mice (central panel) compared to WT (left panel) and rhMMP-10-treated null mice (right panel). Scale bar denotes 50 µm.

4.4 UNDERLYING MECHANISMS OF MMP-10 ACTIVITY IN ISCHEMIA

4.4.1 CXCL1 expression is increased in *Mmp10*$^{-/-}$ skeletal muscle

To study the molecular mechanism underlying the increased inflammatory and necrotic rate in the absence of MMP-10, we isolated lung ECs and leukocytes from the peritoneum of WT and *Mmp10*$^{-/-}$ mice for gene expression analysis by using low

density arrays (LDA, Table 6). Interestingly *Mmp10*$^{-/-}$ ECs and leukocytes presented increased expression (~2 and ~3 fold) of the leukocyte chemoattractant *Cxcl1* (*Gro*α) compared to WT cells (not shown), that was further confirmed by real time-PCR (Figure 28A, B).

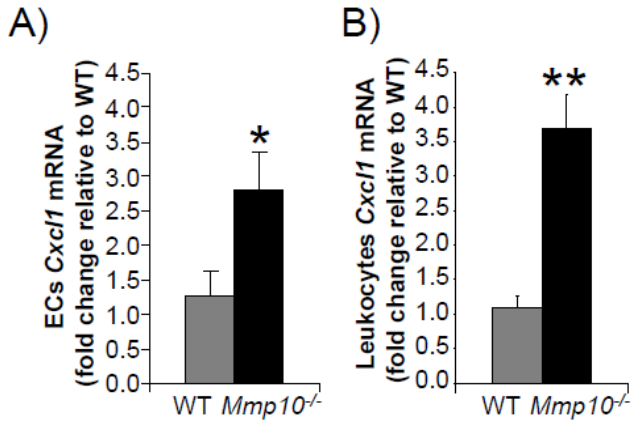


Figure 28. *Cxcl1* (*Gro*α) expression in *Mmp10*$^{-/-}$ mouse lung ECs (A) and leukocytes (B) *in vitro* (n=6). *P<0.05 and **P<0.01 vs. WT.

The results obtained *in vitro* were confirmed *in vivo* in the total excision model by measuring *Cxcl1* mRNA expression in the soleus and gastrocnemius crural muscles of WT and *Mmp10*$^{-/-}$ mice at baseline and different time points after femoral artery excision. As shown in Figure 29A, *Cxcl1* expression was maximal at 12 hours and decreased gradually 24 and 48 hours after excision. In absence of functional MMP10, *Cxcl1* was strongly up-regulated at 12, 24 and 48 hours post-ischemia compared to WT conditions (P<0.05). At later time points its expression was almost undetectable in both, WT and *Mmp10*$^{-/-}$ mice. To assess if the increased in mRNA levels was accompanied by an increased in CXCL1 protein, Western Blot of soleus and gastrocnemius muscles was performed in the muscles obtained 12 hours after ischemia. No statistical difference was observed although there was a tendency towards increased expression in *Mmp10*$^{-/-}$ mice (Figure 29B).

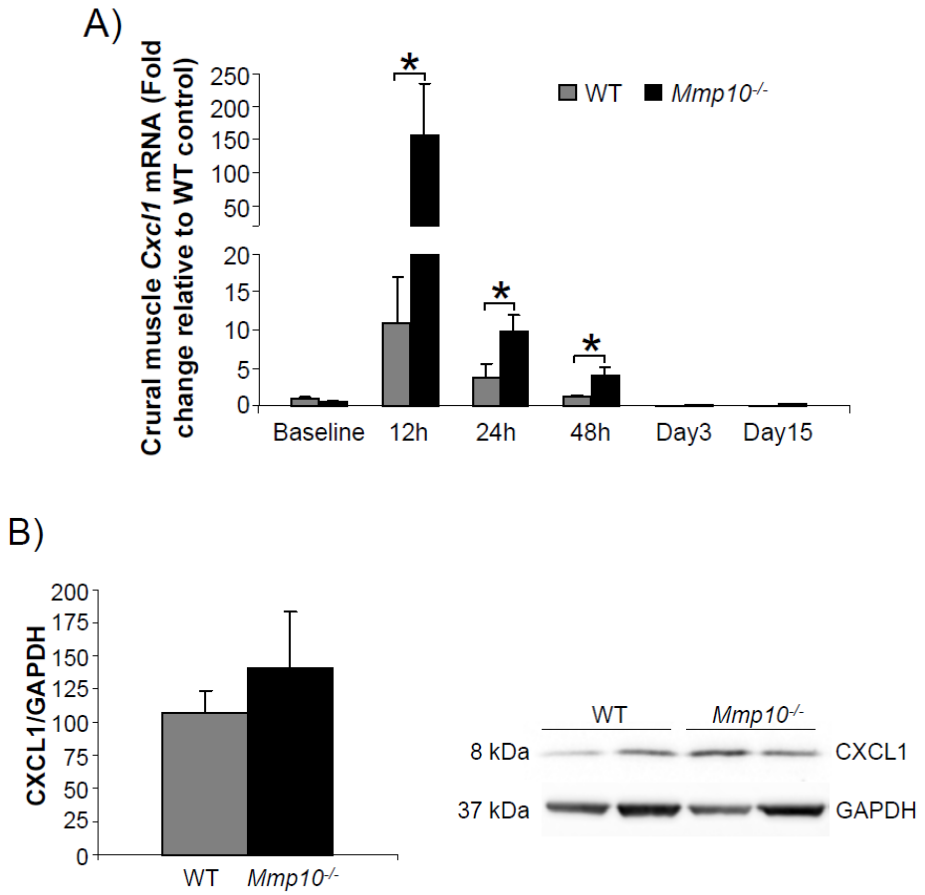


Figure 29. A) *Cxcl1* mRNA levels in WT and *Mmp10*$^{-/-}$ skeletal muscle after femoral artery excision (n=4-5, *P<0.05 vs. WT). B) Western blot for CXCL1 in crural muscles 12 hours post-excision. GAPDH was used as loading control (n=4).

In order to observe the *in situ* expression of CXCL1 and its regulation in absence of functional MMP-10, we performed immunofluorescence for CXCL1 in baseline, day 3 and day 28 soleus sections of WT and *Mmp10*$^{-/-}$ mice (Figure 30). Control solei showed a similar CXCL1 expression in large vessels and at the periphery of the myofibers in WT and *Mmp10*$^{-/-}$ mice (Figure 30A). Three days post-ischemia CXCL1 was localized in myocytes and inflammatory cells and increased by 30% in *Mmp10*$^{-/-}$ mice compared to WT (Figure 30B, *P*<0.05). Twenty eight days post-ischemia CXCL1 signal was similar to that found at baseline with no differences between the genotypes (Figure 30C).

Our *in vitro* and *in vivo* results show the overexpression of Cxcl1, both at mRNA and protein levels, in absence of MMP-10, suggesting the involvement of MMP-10 in CXCL1 regulation in ischemic conditions during the degeneration phase.

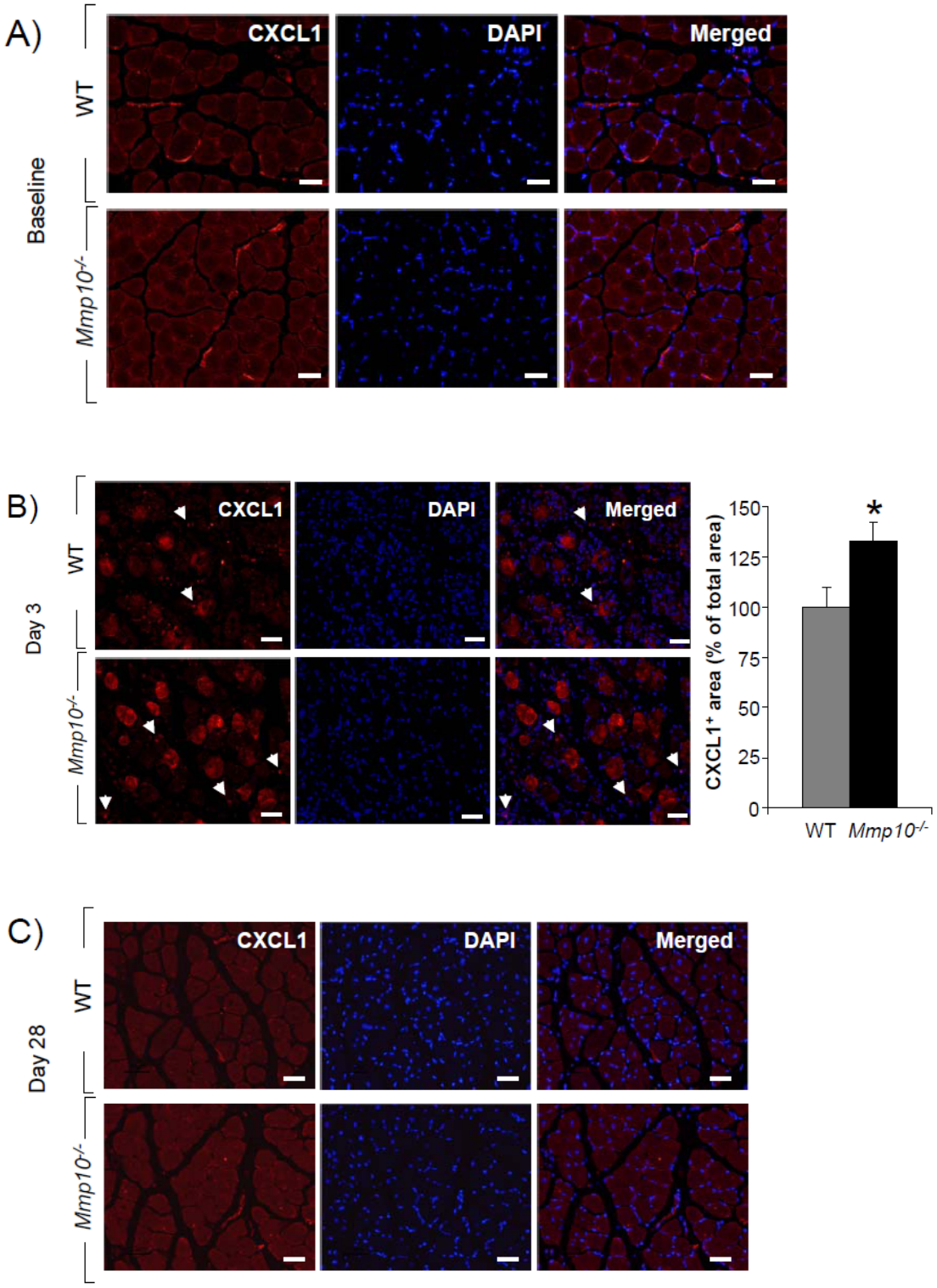


Figure 30. Immunofluorescence for CXCL1 in soleus sections at baseline (A), 3 days post-ischemia and its quantification (B) and 28 days post ischemia (C). *P<0.05 vs. WT (n=10). Arrowheads point to inflammatory cells. Scale bar denotes 50 µm.

4.4.2 MMP-10 regulates *Cxcl1* expression *in vitro*

To determine if MMP-10 could be regulating *Cxcl1* expression *in vitro,* experiments were carried out on mouse lung endothelial cells. ECs from WT mice were serum starved for 15 hours and then stimulated during 12 hours with active rhMMP-10. As shown in Figure 31A, *Cxcl1* mRNA expression was significantly decreased upon rhMMP-10 stimulation in WT ECs. The preincubation of WT ECs with ActD increased *Cxcl1* expression in WT. This increase was not reverted with the later addition of rhMMP-10 (Figure 31B), pointing to a regulation at the transcription level of *Cxcl1* by MMP-10. The addition of ActD after rhMMP-10 stimulation did not further modify the stability of *Cxcl1* mRNA (Figure 31C). These data suggest a transcriptional regulation of *Cxcl1* by MMP-10 rather than a direct involvement in the stability of *Cxcl1* transcript.

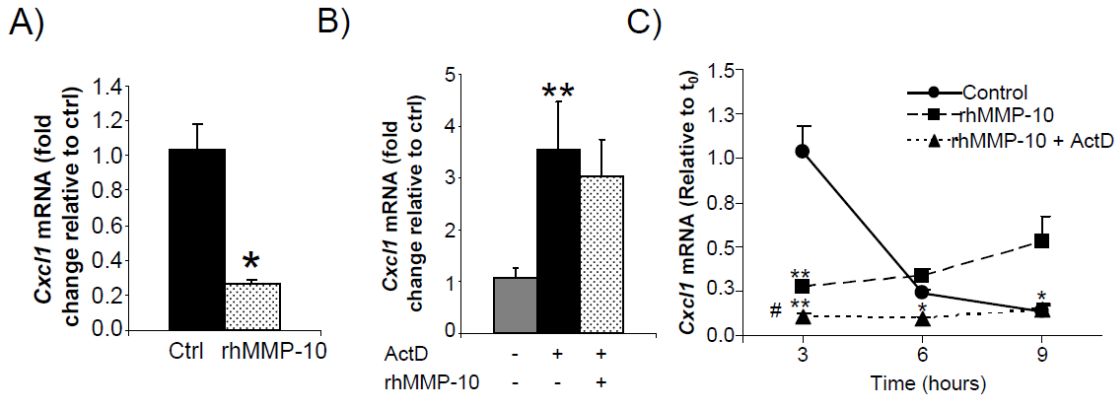


Figure 31. CXCL1 expression is increased in absence of functional MMP-10 *in vitro.* A) *Cxcl1* mRNA expression in rhMMP-10-treated WT ECs 15 hours post stimulation (n=3 experiment/2 independent experiments). **P*<0.05 *vs.* vehicle. B) Preincubation of WT ECs with ActD (1 hour, 0.5 µM) increased *Cxcl1* mRNA levels that was not reverted by the posterior addition of rhMMP-10 (12 hours, 2nM) ***P*<0.01 *vs.* control (n=6). C) WT ECs were stimulated with rhMMP-10 for 12 hours and then treated with Act D for additional 9 hours. Results were plotted as a function of time (relative to time 0 [t0]). rhMMP-10 reduced CXCL1 expression compared to control conditions, and remained steady during the experiment ***P*<0.01 *vs.* control,# *P*<0.01 *vs.* rhMMP-10 (n=6).

To address whether MMP-10 could modify CXCL1 activity by proteolytic cleavage, recombinant human CXCL1 (rhCXCL1) was incubated for 24 hours with active rhMMP-10 and the resulting products analyzed by SDS-PAGE. GM6001, a broad MMP inhibitor was used to block MMP-10 activity. As shown in Figure 32, no proteolytic effect was observed in our experimental conditions.

Our results show a transcriptional regulation of *Cxcl1* by MMP-10, although the exact mechanism has to be further determined in future studies.

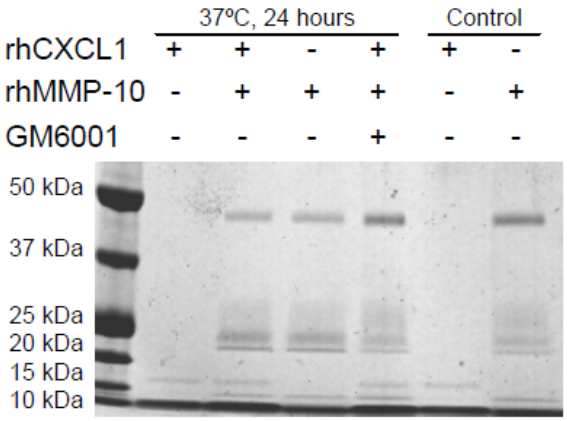


Figure 32. SDS-Page for recombinant human CXCL1 (rhCXCL1, 15 kDa) after incubation with active rhMMP-10 (45 kDa). No proteolytical cleavage of CXCL1 was observed when incubating with rhMMP-10 for 24 hours at 37ºC. GM6001 was used to inhibit MMP activity. Controls refer to untreated CXCL1 and rhMMP-10, respectively.

4.5 CHARACTERIZATION OF EXTRACELLULAR VESICLES AFTER ISCHEMIA.

Different disease states, like ischemia, result in the release of EVs that have been involved in intercellular signalling. To analyze whether MMP-10 could modulate EV release after ischemia and how EVs could influence muscle recuperation, we decided to study their circulating levels and cellular origin in hindlimb ischemia. In order to characterize EVs we conducted two different studies on mice platelet-free plasma using the most accurate methodology currently available.

To study EV concentration we used NTA, a method capable to detect a wide range of particle diameters, with the lower detection limit at 50 nm. For the characterization of the cellular origin of the vesicles, we used an Apogee flow cytometer, which is able to detect biological vesicles already as small as 180 nm [169].

The data obtained from the study of baseline plasmas is not included in the analysis since the samples were highly haemolytic. Haemolytic samples contain fragments of

erythrocytes that affect the measurements of total particle concentration. Furthermore, the rupture of red blood cells could trigger the activation of different cell types after blood collection and influence EV release.

4.5.1 Different inflammatory states do not influence total particle concentration and size in mouse plasma.

We measured total particle concentration of whole plasma samples of WT and *Mmp10*$^{-/-}$ mice 3 and 28 days after ischemia. By using NTA we determined that the peak concentration of circulating particles ranged from 50 to 80 nm, although no differences between genotypes could be observed at any time point (Figure 33). Moreover, the number of total particles on plasma remained the same at the degeneration (day 3) and the regeneration phase (day 28).

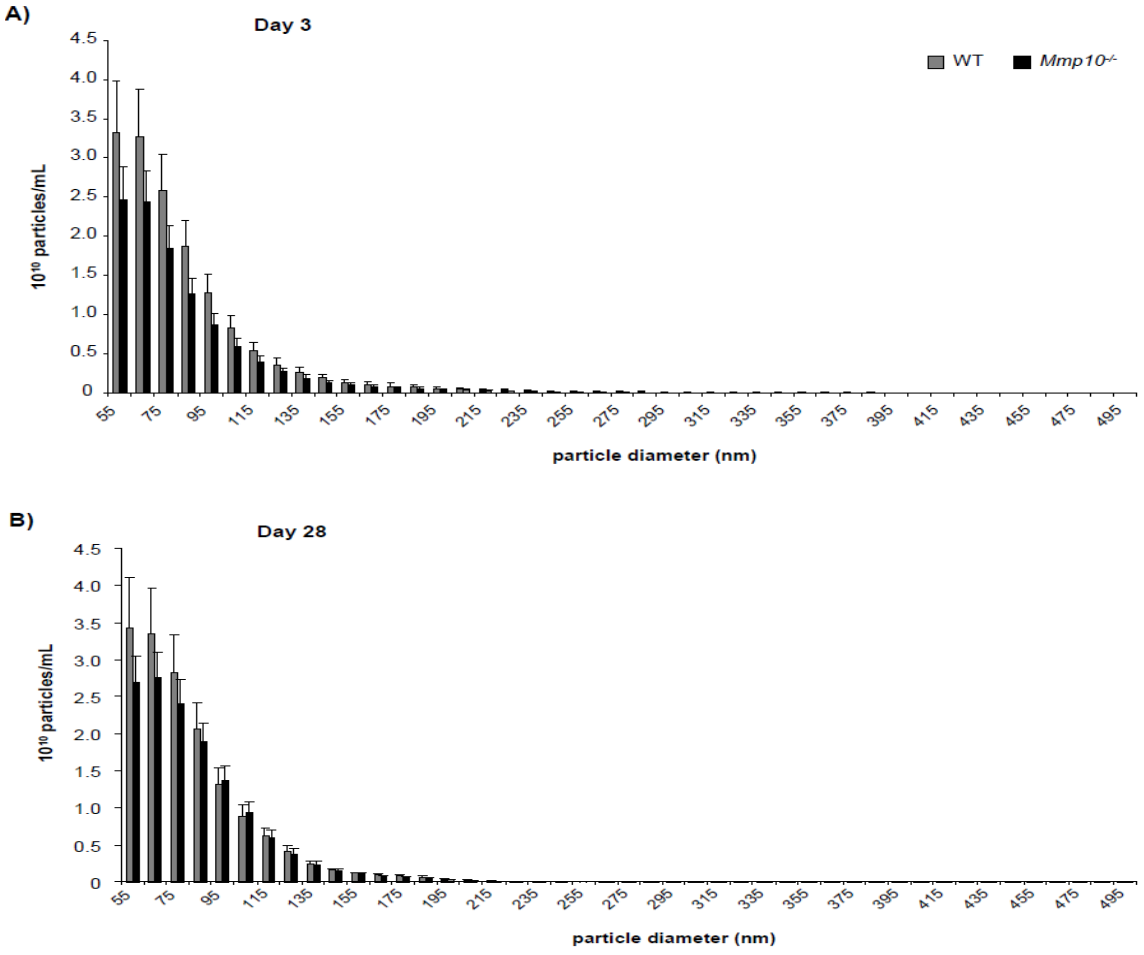
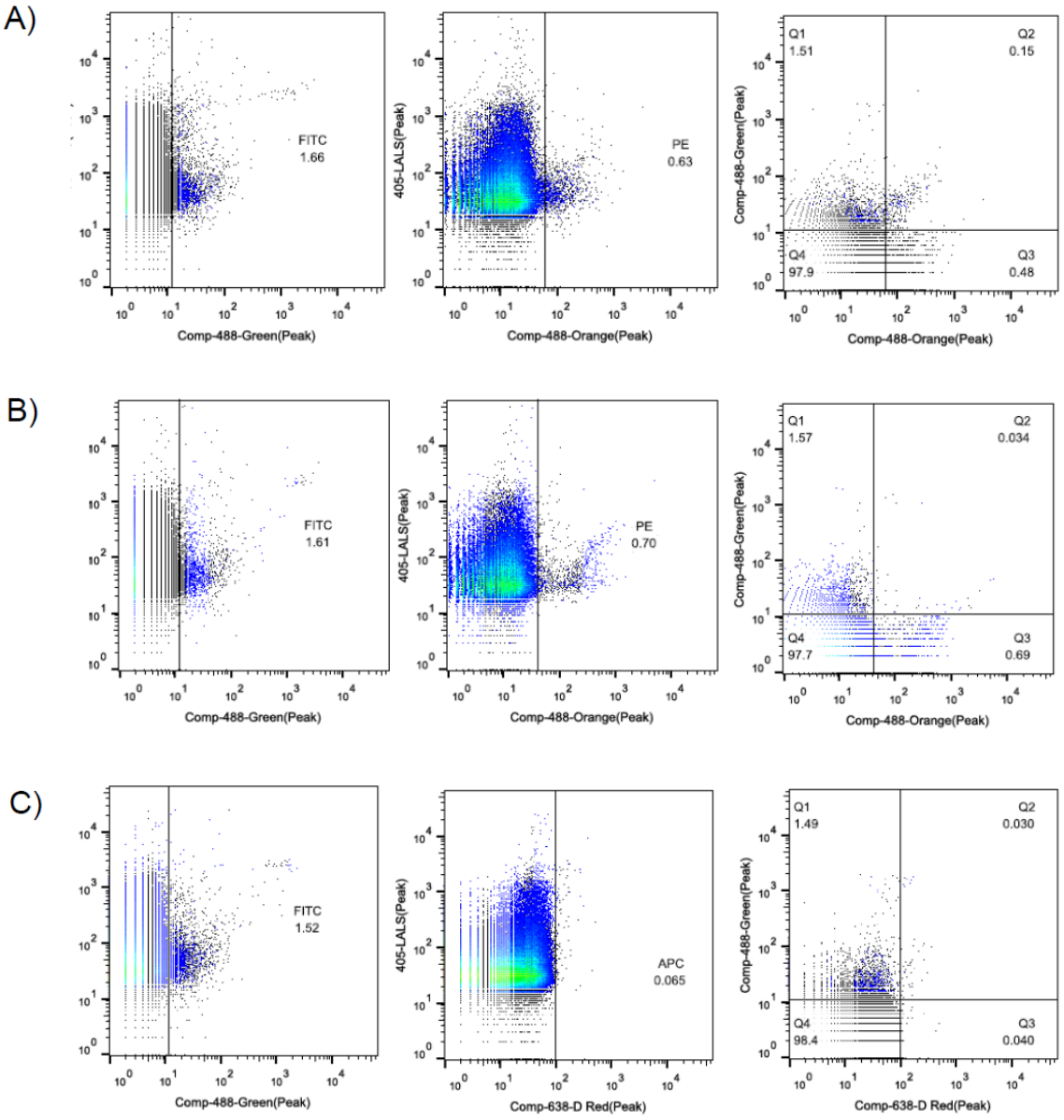


Figure 33. Size-distribution histogram of total particle concentration in WT and *Mmp10*$^{-/-}$ mice 3 (A) and 28 days (B) after ischemia measured with NTA. Grey bar correspond to WT, black bar corresponds to *Mmp10*$^{-/-}$. Mean+SEM (n=15/genotype).

4.5.2 Characterization of cellular origin of the extracellular vesicles present in plasma.

Flow cytometry was used to characterize the population of extracellular vesicles larger than 180 nm present in WT and *Mmp10*$^{-/-}$ mice plasma 3, 15 and 28 days after ischemia. We analyzed the presence of PS, by the binding of lactadherin, and the cellular origin of the vesicles by the use of cell specific antibodies (Figure 34). Isotype control antibodies were analyzed and subtracted from cell-specific antibody measurements.



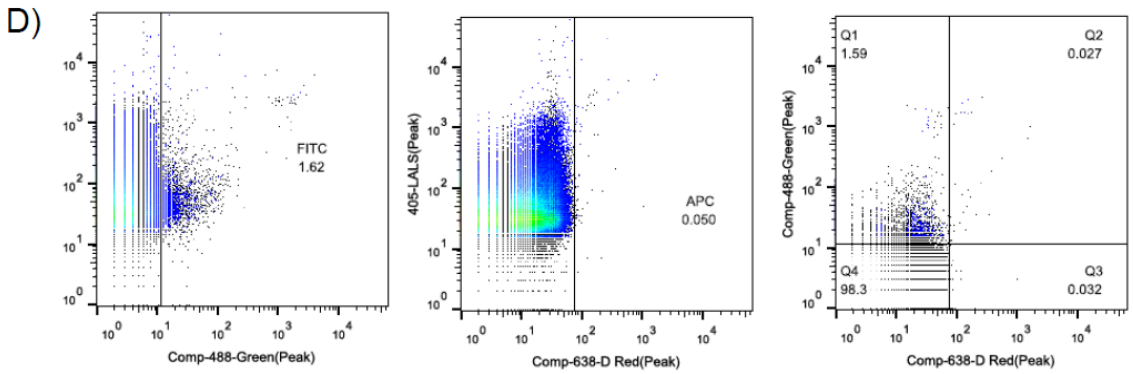


Figure 34. Representative dot-plots of WT vesicles after ischemia by flow cytometry. A) Double staining for platelet marker (CD41-PE) and lactadherin-FITC. B) Double staining for activated EC marker (CD62E-PE) and lactadherin-FITC. C) Double staining for monocyte/macrophages marker (F4/80-APC) and lactadherin-FITC. D) Double staining for neutrophil marker (Ly6G-APC) and lactadherin-FITC.

To have an idea of the presence of PS on the different vesicle populations we analyzed the co-expression of the cellular marker and PS on the vesicles. The analysis revealed that the majority of vesicles did not expose PS. When analyzing PS^+ vesicles in different populations, data showed that less than 50% all monocyte/macrophage and neutrophil-derived vesicles exposed PS on their surface, 30% of platelet-derived vesicles were PS^+ and only 5% of vesicles from activated ECs (Figure 35).

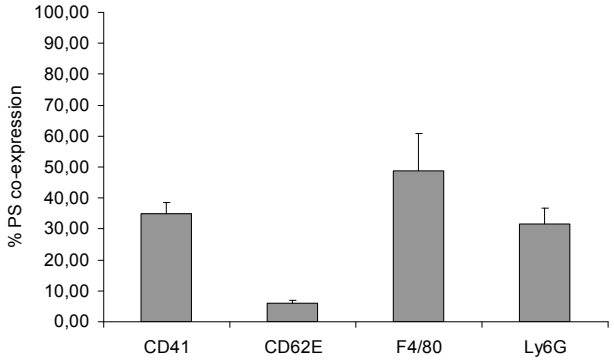


Figure 35. Representative plot of the quantification of PS^+ vesicles of each cellular origin in WT mice. CD41 for platelets, CD62E for activated ECs, F4/80 for monocyte/macrophages and Ly6G for neutrophils vesicles.

When it came to the total number of vesicles (PS positive and negative) in each subpopulation, the majority of them were platelet and EC derived (Figure 36), while the concentration of vesicles derived from monocyte/macrophages and neutrophils was

extremely low. No differences on vesicles concentration from different cell origins between genotypes were observed at day 3, 15 or 28.

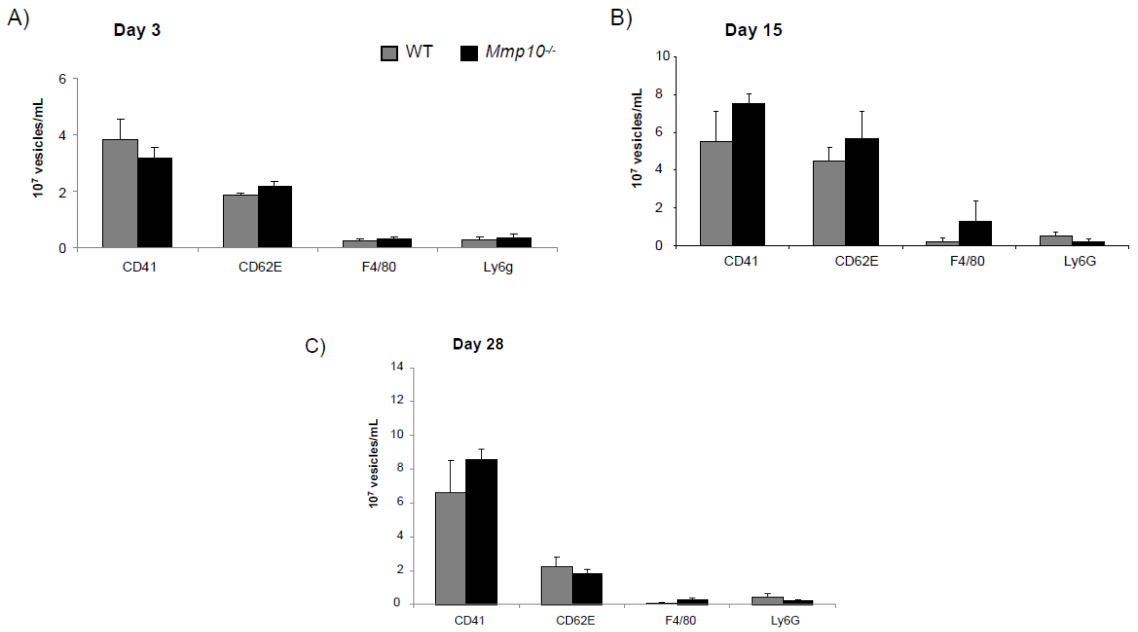


Figure 36. Overview of EVs concentrations and their cellular origin in plasma at day 3 (A), day 15 (B) and day 28 (C) after ischemia. The predominant population of vesicles corresponds to platelet and activated EC-derived. Leukocyte-derived vesicles concentrations are almost neglectable. Cellular origin markers: CD41 for platelets, CD62E for activated ECs, F4/80 for monocytes/macrophages and Ly6G for neutrophils. Day 3, n=15 mice/genotype; day 15, n=3 mice/genotype and day 28, n=5 mice/genotype.

For a better understanding of the data provided in Figure 36, the time course of each population of EVs was plotted (Figure 37). The results showed a tendency of increasing platelet-derived vesicles after ischemia, that in the case of *Mmp10*$^{-/-}$ mice reached significant levels at day 28 (Figure 37 A). At day 15, when the angiogenic process is occurring, we observed a peak of vesicles released from activated ECs that achieved significant levels in *Mmp10*$^{-/-}$ mice (Figure 37 B). No differences in the concentration of leukocyte-derived vesicles were found among day 3, 15 and 28 (Figure 37 C and D).

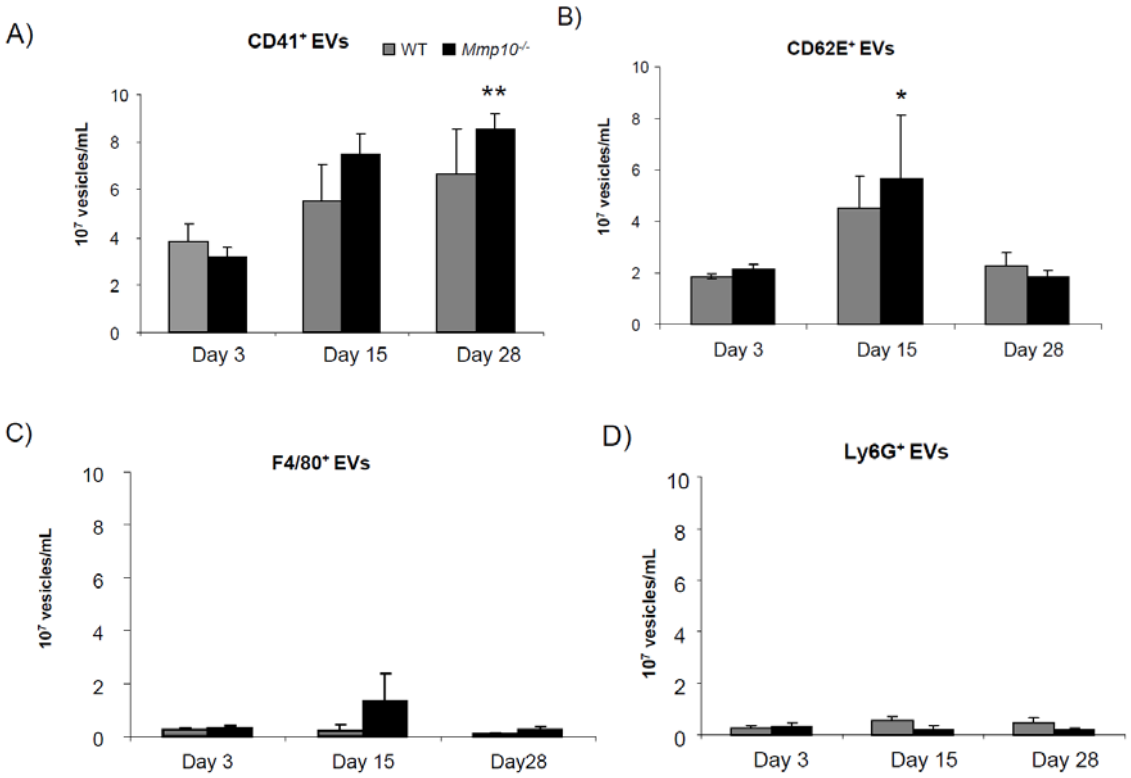


Figure 37. Time course of vesicle concentration. The concentration of vesicles derived from platelets increases overtime (A). Vesicles derived from activated ECs peaked at day 15 (B), while leukocyte-derived vesicles do not vary throughout the different time points (F4/80, C and Ly6G, D). **P<0.01 vs day 3, *P<0.05 vs day 28. Day 3, n=15 mice/genotype; day 15, n=3 mice/genotype and day 28, n=5 mice/genotype

In conclusion, the different inflammatory and regenerating phenotypes showed by WT and *Mmp10*$^{-/-}$ mice are not accompanied by different concentrations of platelet-, endothelial- or leukocyte-derived vesicles. NTA measurements could also not reveal differences in total particle concentration or size-distributions profiles between he genotypes at any timepoint studied.

5-DISCUSSION

5.1 Role of MMP-10 in PAD

The main finding of our study is that MMP-10 is neccesary for skeletal muscle repair after hypoxia, as the absence of MMP-10 results in delayed reperfusion, increased necrosis and inflammation during the degenerative phase, which is associated later with delayed vessel regression and myocyte regeneration. This study underscores, for the first time, the relevance of MMP-10 after ischemia as a new pathophysiological mechanism in diseases characterized by a shortage of oxygen and nutrient delivery to the skeletal muscle such as PAD.

PAD is a manifestation of atherosclerotic vascular disease that represents a spectrum of disease severity, encompassing both asymptomatic and symptomatic disorders that may manifest as either intermittent claudication (IC) or critical limb ischemia (CLI). PAD affects more than 200 million individuals. The increase in the prevalence of PAD by 24% between 2000 and 2010, suggests that in the absence of preventive efforts, the disease burden will rise over time [2]. Only 10% of the patients present the classical IC symptoms, and most of the times musculoskeletal disease or neuropathy coexist with PAD. CLI affects 8 to 12 million individuals in the United States and is increasingly prevalent in Europe and Asia [170]. Even though CLI is the initial manifestation in roughly 1-2% of all patients, its mortality rate goes up to 25% at 1 year [2].

Inflammation plays an important role in the development and progression of PAD [4]. Moreover, PAD leads to broad adaptive changes in the arterial wall and the ischemic muscle in response to atherosclerosis and blood flow impairment, respectively. MMPs contribute extensively to tissue remodelling by degrading ECM components in diverse vascular pathologies [1,6–9], including PAD, although there are scarce available reports regarding MMP presence and activity in this disease. The available data evidence an increase in circulating MMP-9 levels in diabetic PAD patients and in those with CLI [100,104]. Another study correlates the IMT index in the femoral artery with total and active circulating MMP-9 levels. The results showed a correlation between MMP-9 and the presence of moderate and severe plaques [104]. In Aorto-Occlusive disease, another degenerative arterial disease like PAD, MMP-8 has also been found augmented compared to healthy volunteers [68]. Regarding MMP-10, it has been shown that its circulating levels are associated with severity and poor outcome in PAD [171]. The study of MMPs in experimental models of muscle ischemia shows activity of MT1-MMP, MMP-2 and -9 after injury [172], suggesting their participation in tissue recovery. Concomitantly, the genetic deficiency of the two gelatinases impairs neovascularisation

and reduces leukocyte infiltration in the ischemic lower limbs [122,123], supporting the need of MMP activity for the correct repair of the skeletal muscle.

Ischemia of the lower limbs increases the degree of necrosis and degeneration of the muscles in PAD patients, characterized by increased proteolytic activity [100]. Based on our previous studies showing a key role for MMP-10 in liver tissue repair after injury [127]; and in skeletal muscle regeneration in models of toxin-induced damage and muscular dystrophy [117], we investigated the function of MMP-10 in muscle degeneration and repair after ischemia. We used two models of femoral artery ligation: a mild model with a 50% reduction in blood flow, and a more aggressive one, where the blood flow to the crural muscles was completely interrupted. Our findings, showing a relevant role of MMP-10 after severe ischemia, but not in mild conditions, are in line with other studies reporting a major role for MMP-10 in severe pathological conditions, such as cancer [126,173–175], and led us to focus the discussion on the results obtained after total excision of the femoral artery as a more prominent phenotype.

5.2 The role of MMP-10 in necrosis and inflammation

The initial stage of muscle repair after injury is characterized by necrosis of the damaged tissue and activation of the inflammatory response. MMPs have been related to inflammation due to their classical ability to degrade the ECM which favours the migration of leukocytes towards the injured site [44]. As previously reported, the role of MMPs should be independently studied because the different members of the family may present different and even opposite functions in the same process [176]. We have found that in absence of MMP-10, tissue necrosis and inflammatory cell infiltration are increased, suggesting a role of MMP-10 in the regulation of the inflammatory response, whereas in similar rodent models of hindlib ischemia MMP-2 and MMP-9 deficient mice present reduced macrophage infiltration to the damaged muscle [123,177]. We provide further evidence on the role of MMP-10 as regulator of inflammatory cell trafficking by using an *in vitro* model of transmigration, in which the absence of functional MMP-10 led to an increase in leukocyte transmigration; and an experimental model of peritonitis, where we observed increased number of neutrophils in *Mmp10*$^{-/-}$ mice. Our results are consistent with previous *in vivo* studies showing augmented inflammation in models of lung infection [176], experimental colitis [178] and muscular dystrophy [117] in *Mmp10*$^{-/-}$ mice, supporting a role for MMP-10 in inflammatory cell

trafficking. Phenotypic improvement of *Mmp10*$^{-/-}$ mice, in terms of necrosis and inflammation, after systemic rhMMP-10 administration further confirmed the direct role of this protease in muscle degeneration.

We hypothesize that the exacerbated inflammatory response observed in absence of functional MMP-10 might be responsible for the sustained necrosis observed in *Mmp10*$^{-/-}$ mice. Even though inflammation is essential to activate the molecular pathways responsible for muscle repair, in excess it might promote tissue deterioration. Neutrophils are the first to infiltrate into the damaged area to elicit the early phagocytic response, and to promote macrophage recruitment [112]. These cells are later replaced by monocytes/macrophages. Prolonged presence of neutrophils and classically activated M1 macrophages, markedly proinflammatory, can propagate the degenerative response by releasing proinflammatory cytokines and cause further tissue damage through the release of free radicals like nitric oxide and superoxide, which are cytotoxic molecules [179,180].

Depending on the local environment, macrophages undergo specific differentiation [181]. Until now, two distinct states of polarized activation for macrophages have been defined: the classical pro-inflammatory phagocytic macrophages (M1) and a subset of macrophages (M2) which present a phenotype mostly directed to immunosuppression and wound healing/tissue repair [182]. Although the exact mechanism is unknown, some studies indicate that the removal of cellular debris after injury is what causes the shift in macrophages from M1 to M2 phenotype [182,183]. M2 macrophages play a major role in promoting growth and regeneration, since their absence inhibits muscle regeneration [182]. The variety of macrophages subsets present different MMPs expression profiles. Some MMPs are mostly expressed by M1 macrophages, e.g., MMP-1, -3 and -10, whereas others are predominantly expressed in the M2 subpopulations as it is the case of MMP-12 [184]. *In vivo*, we found a tendency to a decreased number of anti-inflammatory M2 macrophages in *Mmp10*$^{-/-}$ muscles that could be partially responsible for the delayed muscle regeneration of later stages compared to WT mice. Whether this delayed polarization towards M2 is directly mediated by the absence of MMP-10 or consequence of the sustained inflammatory response observed upon the genetic deletion of MMP-10 will need to be further studied.

5.3 The role of MMP-10 in the arteriogenic and angiogenic response after hindlimb ischemia

Ischemia triggers the activation of arteriogenesis and angiogenesis to restore blood flow. MMPs contribute to these processes by degrading ECM components, and promoting the release of different growth factors [185], thus facilitating SMC and EC migration and proliferation. *In vitro* studies have pointed out the proangiogenic profile of MMP-2, -7, -9 and MT1-MMP [119,186–189] and also how other MMPs are regulated during vessel formation [190]. In addition, *in vivo* ischemic models have broadly demonstrated the requirement of the gelatinolytic activity in neovascularization of the skeletal muscle and have given light into the possible underlying mechanisms [191,192]. MMP-2 and -9 have been proposed as modulators of bone marrow-derived endothelial progenitor cell recruitment [122,123]. In addition, MMP-9 is suggested to be responsible of proper capillary branching after ischemia [177] although the list of mechanisms for MMP-9 contribution to angiogenesis keeps expanding. [192]

We first determined whether the delayed reperfusion observed in *Mmp10*$^{-/-}$ mice early after femoral artery excision could be the result of decreased collateralization at baseline, as recently reported for the tibialis anterior (TA) muscle [128]. However, our results show no differences in the collateralization profile in the soleus between the genotypes. Additional studies addressing whether the differences in vascularization between the TA and the soleus muscle are due to the distinct fiber types/metabolic demands of the different muscles will be important to understand the basis for the discrepancy between these studies.

In contrast to previous reports, where *Mmp9*$^{-/-}$ and *Mmp2*$^{-/-}$ mice showed impaired neovascularization after ischemia [122,123], we described a similar arteriogenic and angiogenic response to hypoxia in WT and *Mmp10*$^{-/-}$ mice. At the latter steps of fiber repair process, when muscle perfusion is restored and regeneration is about to be completed, collateral and capillary density return to baseline conditions in WT mice. *Mmp10*$^{-/-}$ animals, however, present sustained arteriole and capillary density at this time point, suggesting a maintained angio- and arteriogenesis due to a previous worsen degenerative state and/or to the requirement of MMP-10 for vessel regression. Other studies have shown that the over-expression of MMP-10 induces capillary tube

collapse *in vitro* [120] and vascular rupture *in vivo* [193], supporting the involvement of MMP-10 in vessel relapse rather than in vessel growth.

Whether MMP-10 is involved in vessel relapse through the activation of EC apoptosis in ischemia remains unknown, although it could constitute one possible mechanism to explain our phenotype. It has been described that MMP activity can affect apoptosis at different levels. Likewise, the cleavage of different adhesion molecules by MMPs is a prerequisite for apoptosis to occur in ECs [194]. Degradation of laminin by MMP-3 is another example of enhanced apoptosis in mammary epithelial cells [195]. In addition, there are also available data on how MMP-7 can induce or inhibit apoptosis depending on the process under study. By promoting the release of Fas ligand from the membrane, MMP-7 induces apoptosis of neighboring cells, while by activating tyrosine kinase-mediated pathways decreases cancer-cell apoptosis [196]. MMP-10 has also been related to apoptosis in cancer models via ECM degradation, which can result in the release of IGF-1, a molecule involved in the protection of tumor cells against proapoptotic signals [197].

5.4 MMP-10 in muscle fibrosis after ischemia

After injury, the production of collagen should be carefully regulated in order to achieve muscle repair and avoid excessive scaring. Fibrous scar tissue includes collagen types I and III. MMPs have proteolytic activity against a wide range of extracellular proteins and thus, are essential for the correct remodeling of the ECM [56]. Several MMPs, such as MMP-1, -2 and -9 are involved in ECM remodeling of skeletal muscle at different stages of muscle repair, facilitating myogenesis [114], angiogenesis [123,198–200] and reducing muscle scaring [201].

MMP-1 aids the regeneration process by denaturing collagen resulting in reduced scaring, as proven in a laceration model in which MMP-1 was injected into the damaged muscles 33 days post injury [202]. Contrary to MMP-1, the specific inhibition of MMP-9 by doxycycline ameliorates the development of excessive fibrosis [203]. It has been shown that *Mmp10*$^{-/-}$ mice present a different ECM composition in the TA muscles at baseline when compared to WT mice, resulting in reduced levels of collagen I, III and IV [117]. However, our results indicate that overall fibrosis of the skeletal muscle is

not affected by MMP-10 after ischemia suggesting that MMP-10 might not play a critical role in the regulation of global fibrosis in our experimental conditions, although a deeper analysis of the different components of the ECM should be performed in order to compare soleus matrix composition to the reported results in other hindlimb muscles.

5.5 The role of MMP-10 in myofiber regeneration after hindlimb ischemia

The inflammatory/damage signals produced in response to tissue injury trigger the activation of satellite cells to proliferate, differentiate and fuse, leading to new myofiber formation and tissue repair [43,114,204]. The analisys of the skeletal muscle at the regenerative stage after ischemia showed that in absence of MMP-10, muscle regeneration is delayed as previously described in other skeletal muscle injury models [117]. Moreover, MMP-10 has been shown to induce proliferation of satellite cells [117]. Therefore, we could speculate that the exacerbated inflammatory response observed in *Mmp10*$^{-/-}$ mice early after ischemia could be affecting satellite cells activation. However, a deeper analysis should be performed in order to clarify this issue.

For the migration of satellite cells, the basement membrane of the myocytes must be degraded [205]. Gelatinases have been described to be augmented and their activity increased in the periphery of muscle fibers after hindlimb ischemia accompanied by dramatic basement membrane component alteration [200]. Type IV collagen, a major component of the basement membrane is one of the main MMP-10 substrates, suggesting a possible role of MMP-10 in satellite cell migration. In addition, the role of MMP-10 in muscle regeneration has been further studied *in vitro* where it has been found in contact with the laminin layer of satellite cells and proven to be essential for proper myocyte fusion during myotube formation [117].

Regarding the role of MMPs in muscle physiopathology, the regulation of these proteases should be independently studied and can result in different phenotypes. The importance of MMP activity in skeletal muscle physiology can already be observed in basal conditions, as MT1-MMP deficient mice have smaller and heterogeneous myofibers and some centrally nucleated cells, leading to the hypothesis that MT1-MMP would contribute to maintain myofiber integrity [206]. Moreover, when it comes to injury

models, some MMPs facilitate the proper resolution of the damage, while others might be detrimental. The local injection of MMP-1 promotes muscle regeneration in two models of tissue injury, a mdx mice model and a laceration one in gastrocnemius muscles [116,202]. However, the role of other MMPs like MMP-9 is not clear since there is evidence that the genetic deficiency of this metalloproteinase alleviates skeletal muscle pathogenesis by augmenting regeneration in dystrophic (mdx) mice [207], and after crush induced damage [203], while another study shows how increased levels of MMP-9 are needed for proper muscle regeneration in the soleus muscles and how the presence of MMP-9 during all stages of myoblast differentiation is required for proper muscle repair [208].

In our study, we corroborate the requirement of MMP-10 in muscle regeneration after ischemia, since delayed myogenic differentiation in absence of MMP-10 could be restored back to WT levels by rhMMP-10 administration. Whether this could be the result of inappropriate satellite cell activation/migration due to impaired degradation of the components of the basal lamina by MMP-10 [128], or by missing signals that limit the damaged tissue to efficiently activate the regenerative program remains an open question.

5.6 Molecular mechanisms underlying MMP-10 involvement in muscle repair

To study the molecular mechanisms governed by MMP-10 in ischemia, we conducted gene expression analysis and found upregulation of the gene encoding for the chemoattractant protein *Cxcl1* (*Groα*) in absence of MMP-10 *in vivo* and *in vitro*. Experiments conducted to determine how MMP-10 could modulate CXCL1 suggested a transcriptional regulation of *Cxcl1* by MMP-10, although the exact mechanism has to be further explored in future studies. To determine whether MMP-10 could also regulate CXCL1 protein activity, we examined CXCL1 proteolytical processing by MMP-10 as described for other MMPs and chemokines [209–211], but no evidence for cleavage involvement was found.

CXCL1 is a member of the CXC chemokine family that has been traditionally related to neutrophil, T-lymphocyte and macrophage chemotaxis [212], essential cell types in the inflammatory response required after injury. In addition, Cxcl1 has been shown to favour neutrophil recruitment in different models of inflammation like acute lung injury

[213] or LPS infection [214]. An excess of CXCL1 produced in the absence of MMP-10 could be one of the mechanisms involved in the exacerbated inflammatory response observed in *Mmp10*$^{-/-}$ mice. In our animal model of severe ischemia, we found increased levels of *Cxcl1* throughout the whole degenerative phase compared to the WT, peaking at 12h coinciding with the summit of neutrophil infiltration between 6 and 24h after injury [182]. These *in vivo* results are in agreement with the *in vitro* studies we have performed, that showed increased leukocyte recruitment in the absence of MMP-10 activity.

Besides its pro-inflammatory activity, chemokines have been described to also influence other processes like myogenesis [215]. Among many chemokines studied, one member of the CXC family and its receptor, CXCL12 (SDF1α) and CXCR4, respectively, are shown to be key regulators of the migration of proliferating and terminally differentiated muscle cells *in vitro* and *in vivo* [215,216]. Moreover, SDF1α /CXCR4 system has been involved in muscle repair after toxin-induced damaged in a MMP-10 dependent mechanism. The study indicates that the pro-regenerative effect of this axis is inhibited in muscles from *Mmp10*$^{-/-}$ mice although the exact mechanism remains unclear [128]. A recent study reports that CXCL1 is expressed in murine muscle cells early during *in vitro* myogenesis, and repressed when the maturation of the myotubes is reached [215]. Our and other studies support a role of chemokines in muscle repair after injury, and argue towards a strong regulation, either at the RNA or at the protein level, of chemokines by MMPs [217]. SDF1α is a chemokine related to stem cell mobilization, homing and activation, and CXCL1 is associated to inflammatory cell recruitment. MMP-10 has the ability to modulate both chemokines of the same family, further supporting the requirement of MMP-10 at the different processes of muscle repair.

In summary, skeletal muscle repair after injury is a highly synchronized process that comprises two interdependent processes; inflammation and myogenesis, being the proper activation and resolution of the former, essential for the correct initiation of the later. We propose that functional MMP-10 is required for skeletal muscle repair following ischemia by influencing the inflammatory response during the degenerative phase. The molecular mechanism underlying this process could be partially explained by the regulation of CXCL1.

5.6 Extracellular vesicles after ischemia

The discovery of EVs has yielded a new perspective into the study of different disease states as a novel communication network for intercellular signalling [218]. EVs are released in physiological and pathological conditions [151] although they have mainly been studied in disease states. These vesicles are involved in many vital processes such as coagulation, inflammation and endothelial function presenting not only deleterious but also beneficial effects [139]. Ciculating levels of EVs are indicators of cell activation or apoptosis [162]. Therefore, many studies proposed them as biomarkers for health and disease. EVs levels have been reported to increase in patients with uncontrolled risk factors, atherosclerosis and also in a wide range of inflammatory and cardiovascular diseases such as heart failure, arrhythmias and stroke [162]. In addition, EVs have recently been linked to early development of CVDs as demonstrated in an analysis of the Framingham Heart Study, which revealed that circulating endothelial-derived MPs are associated with the presence of cardiometabolic risk factors, mainly dyslipidemia [219].

Regarding ischemia, different *in vivo* and *in vitro* models support the involvement of these vesicles in the underlying mechanisms triggered by oxygen and nutrient depletion. Thus, we measured total particle/EV concentration in WT and *Mmp10*$^{-/-}$ plasma after ischemia to understand whether differences in EVs concentrations could be related to MMP-10 activity. No differences were observed between the genotypes suggesting no involvement of MMP-10, or of the exacerbated inflammatory response derived from its absence, in EVs release after limb ischemia. Previous studies report increased levels of EVs upon inflammatory conditions compared to baseline conditions [151,162,220]. Unexpectedly, we did not see differences in the total number of particles by NTA between very inflammatory stages (day 3 post-ischemia) and baseline-like conditions (day 28 post-ischemia). One possible explanation could be that the samples obtained for the analysis were collected at non-fasting conditions, increasing the concentration of lipoproteins in blood. High levels of lipoproteins could be masking the EVs population (see “technical challenges” section).

Characterization of the different subpopulations of EVs in plasma

We characterized the concentration of different subpopulations of EVs based on their cellular origin in both genotypes and at different time points after ischemia by flow

cytometry. No differences could be addressed between the genotypes possible due to the experimental settings used since, in contrast to most studies where the characterization of vesicles is done on concentrates of plasma vesicles after isolation, we conducted our studies in whole platelet-poor plasma, thus low concentrations of vesicles could not be detected.

Nevertheless, we observed a low number of platelet-derived EVs at the degenerative phase that gradually increased after ischemia in both genotypes. Decreased number of EVs might be related to a decrease concentration of platelets after surgery, like in inflammatory conditions (e.g.: sepsis [221]). Platelet consumption, related to small blood losses during surgery or to the clots formed at ligation sites during femoral artery occlusion, is typical during or after surgical procedures. The increase in $CD41^{+}$-EVs at the regenerative phase could be regarded to the restored platelet levels at that time point.

According to our results showing the same angiogenic response to hypoxia in both genotypes, our data report no differences in the concentration of EC derived vesicles between WT and *Mmp10*$^{-/-}$ mice. However, at day 15, when angiogenesis is at its maximum level, EC derived EVs peaked, suggesting the need of EC derived EV for proper angiogenesis reponse after ischemia. Our results would be in agreement with other studies, which have pointed out the relevant role of these vesicles at the angiogenic process. It has been shown that the addition of EC-derived EVs *in vitro* increases EC tube formation. The regulation of the levels of these vesicles seems to be a key factor to promote vessel formation since high levels show an inhibitory effect [222]. The role of EVs in angiogenesis has also been shown in a *in vivo* model of hindlimb ischemia, where the administration of EVs after femoral artery ligation increased neovascularisation of the calves [223].

In respect to the leukocyte-derived vesicles, we could not find any clear vesicle population throughout the whole study. The use of more appropriate markers for monocyte detection may lead to different results since the marker used, F4/80, is present in low concentration in monocytes but high in tissue macrophages. In plasma, only monocyte-derived vesicles will be found, thus markers such as CD14 would be more appropriate for the determination of this subpopulation of EVs. Regarding neutrophil vesicles, we also detected very low number of particles. Concentration of the

EVs by different centrifugation steps would increase the number of EVs to be detected, but it would also lead to clumping of the vesicles impairing true vesicle concentration measurement.

The most described and reviewed feature of circulating EVs is the procoagulant phenotype they can exhibit. The presence of PS and TF can initiate the coagulation cascade and, since in human atherosclerotic plaques TF-bearing EVs have been found, the study of these vesicles has opened a new window for the understanding of atherothrombosis [139,162]. However, recent studies have shown that sample handling may induce PS exposure on vesicles accounting as false positive events. Vortexing, centrifugation steps and freezing of the samples may vary the concentration of vesicles and induce PS exposure [224]. Therefore, the presence of PS on EVs as procoagulant agent in the body fluids should be carefully studied [225] and consequently, we did not associate the presence of PS in the plasma samples to a procoagulant state in our study.

There is increased evidence of non-PS harbouring MPs. In platelet-poor-plasma from healthy donors, 80% of MPs do not expose PS [226]. Several authors describe that platelets do only release PS-harbouring EVs when activated, and that the PS exposure rates also depend on the stimuli [138,147,226,227]. Our results are in agreement with these studies since the majority of EVs detected from the analyzed cellular origins did not expose PS on their surface.

A deeper study of the characterization of the role of EVs in tissue regeneration after ischemia should be performed. It is expected that the acute ischemia presented in this model would involve a greater number of locally released EVs from different cellular origins instead of the systemic populations we found in plasma [223] and therefore the study of tissue derived EVs would be more informative than the study of the systemically released EVs.

Technical challenges in EVs studies

Classically, the protocols for the study of EVs encouraged the isolation of these vesicles by repeated centrifugation and washing steps. Nevertheless, for the present study, EVs were measured directly in plasma because washing steps involve a great

loss in total EVs, and pelleting of the vesicles induces their clumping, appearing as one single event on the flow cytometers.

The classical method to study total concentration of EVs is flow cytometry, which involves the use of synthetic beads of known diameters to compare and gate the EV populations. This approach has been proven wrong since the refractive index of the commercially available beads widely differs from the refractive index of EVs [169]. Thus, the light scattered by beads of a certain diameter differs from the scatter of EVs (roughly, a 140 nm polystyrene bead and a 400 nm-vesicle will scatter the same light on the side scatter channel of a flow cytometer [169]) which makes the comparison between beads and EVs unreliable. Consequently, we did not quantify total concentration of EVs by flow cytometry. Improvement of the current methodologies for the determination of the total EV population should be done in order to measure their total concentration.

Instead of flow cytometry for quantification of total EV concentration, we used NTA, which measures total particles present in the samples. However, this method detects not only EVs but also lipoproteins (especially VLDL) and protein aggregates present in plasma. Fluorescence NTA devices could help visualising determined EVs populations by the use of specific antibodies [228]. Other more precise methodologies would be also recommended, like resistive pulse sense (RPS). RPS presents a higher lower detection limit (approx. 100 nm with the NP100 pore) but is much more accurate in determining concentration. We have performed some measurements with RPS in the plasma of the ischemic mice, and with this technique we were able to detect greater number of particles in the more inflammatory conditions (*Mmp10*$^{-/-}$) compared to the WT situation, thus suggesting that more sensitive methodologies could be more adequate to identify differences in EV populations in whole plasma.

The concentration of the different populations that can be detected by flow cytometers is directly related to their lower detection limit. Conventional flow cytometers detect normally vesicles larger than 300-800 nm. For the characterization of the different EV populations after ischemia, we utilized the flow cytometer Apogee with the lowest detection limit commercially available. This flow cytometer allows for the detection of single EVs larger than 180 nm [169], allowing the detection of a totally new undescribed population of small EVs. A comparison between a conventional flow cytometer (FACs Calibur) and Apogee in one freshly measured plasma indicated that Apogee detects almost 10000 times more total counts. From them, Apogee revealed more than 9 times

more platelet- derived vesicles than Calibur, 57 times more PS^+ vesicles and 21 times more RBC-derived ones (personal communication from A. Böing).

5.7 Study limitations

5.7.1 Animal models

We are aware of the limitations of the current study. In the first place, although commonly assumed, hindlimb ischemia model should not strictly be considered as a model of PAD [229]. The mechanisms underlying acute and chronic ischemia differ widely. In humans, chronic limb ischemia results mainly from atherosclerosis gradually occluding arteries over a time span of months to years. The gradual onset of the ischemia allows the downstream muscles to adjust to a lowered supply of oxygen and nutrients. Moreover, increases on shear stress occur gradually instead of acutely, allowing a longer period of collateral vessel formation [39]. In order to mimic the human pathology more accurately, the study should be performed in a model in which an ameroid constrictor would gradually decrease the artery lumen [230]. Besides the type of ischemia, the presence of cardiovascular risk factors, i.e. age, chronic inflammation and dyslipidemia, should also be taken into account in the animal model.

5.7.2. Gene expression analysis

We focus our study on CXCL1 after analyzing data obtained from gene expression screening by quantitative PCR using low density arrays (Applied Biosystems). With the limitation of the reduced set of genes, we found Cxcl1 to be significantly upregulated in absence of MMP-10 and later confirmed this result by qPCR in independent experiments, *in vitro* and *in vivo*. We acknowledge the limitation of this technique and believe that a broader study of chemokine profile by using microarray analysis would help to clarify the involvement of MMP-10 in inflammation.

5.7.3 Microparticle study

In respect to MPs analysis, as previously mentioned, the concentration values obtained by NTA should be carefully analyzed. This method allows the detection of particles ranging from 50 to 1000 nm, although some EVs present smaller diameters. In addition, the measurements obtained with this technique are not absolute since the presence of the few larger particles can mask the smallest ones. Regarding the characterization of the cellular origin of the particles, the use of flow cytometry also presents limitations mainly due to the lower detection limits of the cytometers. Apogee cytometers present the lowest detection limit in the state of the art, but it is still

insufficient to characterize most MPs present in the samples since the mode size of the particles is approximately 70 nm diameter and the cytometer only detects vesicles normally bigger than 180 nm [231].

6-CONCLUSIONS

1. Ischemia induces MMP-10 expression in the injured skeletal muscle.
2. The absence of MMP-10 activity results in delayed reperfusion followed by increased necrosis and inflammation during the degenerative phase of muscle repair, while it induces a slower regeneration rate with delayed vessel regression at the reparative phase post-ischemia. This results in delayed muscle regeneration in *Mmp10*$^{-/-}$ mice.
3. The absence of functional MMP-10 does not affect angiogenesis, collateralization and overall fibrosis in the experimental model of hindlimb ischemia.
4. The injection of rhMMP-10 into *Mmp10*$^{-/-}$ ischemic mice recovers the WT phenotype confirming a direct involvement of MMP-10 in muscle repair after ischemia.
5. The role of MMP-10 in skeletal muscle repair would be partially explained by the regulation of at least one chemokine, CXCL1.
6. No differences in EVs concentration could be demonstrated both during the degenerative and regenerative phases after ischemia between genotypes.

In conclusion, our results confirm the direct involvement of MMP-10 in muscle repair after ischemia by influencing the inflammatory response during the degenerative phase, that later will modulate myogenic differentiation during the regenerative phase.

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APPENDIX

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