# Dual Role of Matrix Metalloproteinases in Brain Injury and Neurorepair after Cerebral Ischemia

**Feifei Ma**

**DOCTORAL THESIS**

Barcelona

Vall d'Hebron
Institut de Recerca
VHIR

UAB
Universitat Autònoma
de Barcelona

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This thesis is submitted by Feifei Ma to apply for her doctoral degree at Universitat Autònoma de Barcelona.
This work has been directed by Dr. Anna Rosell and Dr. Joan Montaner from Vall d'Hebron Institut de Recerca


**Directors:** **Doctoral student:**

Dr. Anna Rosell Novel Feifei Ma

Dr. Joan Montaner Villalonga


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Molt Gracies!

# Abstract

Ischemic stroke is a leading cause of mortality, long-time disability and morbidity in the world. Currently, the only effective treatments are the intravenous thrombolytic therapy with tissue plasminogen activator (tPA) and the new emerging intra-arterial thrombectomies, which require to be administered in the hyperacute phase of stroke (<4.5 hours for tPA treatment and <6 hours for endovascular therapy). Therefore, it is necessary to investigate new therapies that could be used to treat a large number of patients in a later phase to repair and rewire the damaged tissue. One approach to achieve these objectives is to enhance vascular remodeling and neurogenesis in the ischemic brain.

The aim of this thesis is to study the modulation of extracellular matrix metalloproteinase-13 (MMP-13) in brain injury and neurorepair and to investigate several MMPs as serving biomarkers to monitor motor and functional improvement during neurorehabilitation therapy.

Using a distal middle cerebral artery occlusion model of ischemia in mice, we found that the modulation of MMP-13 is involved in ischemic damage and repair mechanisms. After ischemia-reperfusion, MMP-13 deficiency reduces infarct size, improves functional outcome and protects from hemorrhagic transformation, but impairs cortical tissue recovery by reducing proliferating neuroblast migration and vessel remodelling in a microenvironment with reduced trophic factors. At the same time MMP-13-silenced endothelial progenitor cells present an aberrant i*n vitro* tubulogenesis function.

As MMP-13 deficiency protects brain injury acutely and participates in post-stroke tissue recovery, this thesis also studies the temporal profile of plasma MMP-3, MMP-12 and MMP-13 during rehabilitation therapy to investigate their potential role as biomarkers of tissue recovery. Our results show that high levels of plasma MMP-13, as well as MMP-12, are strongly associated with stroke severity acutely, but not MMP-3. Interestingly, those patients with a better motor outcome during rehabilitation present elevated MMP-3 and decreased MMP-12/-13 levels. MMP-3 is closely associated with the better post-stroke motor recovery, indicating the potential as a biomarker of final functional improvement. These are important results that envision the use of biomarkers that could help to adjust, personalize and modulate neurorehabilitation therapies according to patients' needs.

In conclusion this thesis shows the role of MMPs in tissue injury and repair after stroke and the importance to monitor and modulate these proteases in favor of patients' recovery.

# Resum

L'ictus isquèmic és una de les principals causes de mortalitat, discapacitat de llarg termini i de morbiditat al món. Actualment, els únics tractaments eficaços són el tractament trombolític intravenós amb activador del plasminogen tissular (tPA) i les novedoses trombectomies intra-arterials, que requereixen ser administrades en la fase hiperaguda de la malaltia (<4,5 hores per al tractament amb tPA i <6 hores per les emergents la teràpies endovasculars). Per tant, és necessari investigar noves teràpies que podrien ser utilitzades per tractar un gran nombre de pacients en una fase posterior de la malaltia per reparar el teixit danyat. Alguns enfocaments terapèutics apunten que per assolir aquest objectiu és necessari potenciar la remodelació vascular i la neurogènesi en el cervell isquèmic.

L'objectiu d'aquesta tesi és l'estudi de la modulació de la metaloproteinasa de matriu-13 (MMP-13) en la neurorreparación i el d'investigar diverses MMPs com a biomarcadors que serveixin per monitoritzar la millora de la funció motora durant la teràpia neuro-rehabilitadora. Utilitzant un model d'isquèmia cerebral per oclusió de l'arteria cerebral mitja en ratolins, es va trobar que expressió de la MMP-13 està implicada en el dany i en mecanismes de reparació post-isquèmia. Després de la isquèmia-reperfusió, la deficiència en MMP-13 redueix la mida de l'infart, millora l'estatus funcional en la fase més aguda i protegeix de l'aparició de transformacions hemorràgiques, però interfereix negativament en la recuperació del teixit cortical mitjançant la reducció en la migració neuroblast en proliferació, en el remodelat vascular i en un microambient amb factors tròfics reduïts. Al mateix temps les cèl·lules endotelials progenitores amb un silenciament transitori de l'expressió de MMP-13 presenten una funció aberrant en assajos de tubulogènesis, *in vitro*.

Com que la deficiència de MMP-13 protegia del dany cerebral en el model animal d'isquèmia al temps que participava en la recuperació del teixit en fases de reparació, aquesta tesi també estudia el perfil temporal dels nivells plasmàtics de MMP-3, MMP-12 i MMP-13 durant la teràpia neuro-rehabilitadora per investigar el seu paper com a potencials biomarcadors del teixit en recuperació. Els nostres resultats mostren que alts nivells de plasma MMP-13, així com MMP-12, estan fortament associats amb la severitat i extensió de la isquèmia, però no els de MMP-3. Curiosament, els pacients amb un millor status en la funció motora durant la rehabilitació presenten els nivells més elevats de MMP-3 i reduïts de MMP-12 / -13. També, els valors de MMP-3 estan estretament associats amb la millor recuperació motora després de l'accident cerebrovascular, el que indica el seu potencial com a biomarcador de la millora en la funció motora. Aquests resultats són importants per imaginar el futur ús d'un biomarcador per tal de poder ajustar, personalitzar i modular teràpies de neuro-rehabilitació d'acord a les necessitats dels pacients.

En conclusió aquesta tesi mostra el paper de les MMPs en el dany i reparació de teixits després de l'ictus isquèmic i ressalta la importància de monitoritzar i modular aquestes proteases en favor de la recuperació dels pacients.

# Index

# Abbreviations

| | |
|---|---|
| **ADAMTS** | ADAmalysin-like Metalloproteinase with ThromboSpondin motifs |
| **Ang** | Angiopoietin |
| **BBB** | Blood Brain Barrier |
| **BI** | Barthel Index |
| **CAHAI** | Chedoke Arm and Hand Activity Inventory |
| **CNS** | Central Nervous System |
| **CXCL12** | C-X-C motif chemokine 12 |
| **CXCR-4** | C-X-C chemokine receptor type 4 |
| **DNA** | DesoxyriboNucleic Acid |
| **DWI** | Diffusion-Weighted Imaging |
| **EPCs** | Endothelial Progenitor Cells |
| **FAC** | Functional Ambulation Categories |
| **FGF** | Fibroblast Growth Factor |
| **$FeCl_3$** | Ferric Chloride |
| **FMA** | Fugl-Meyer Assessment |
| **GABA** | γ-Aminobutyric acid |
| **HT** | Hemorrhagic Transformation |
| **HI** | Hemorrhagic Infarctions |
| **IL** | InterLeukin |
| **KO** | Knock-Out |
| **MCA** | Middle Cerebral Artery |
| **MCAo** | Middle Cerebral Artery occlusion |
| **MMP** | Matrix MetalloProteinase |
| **MRC** | Medical Research Council |
| **MRI** | Magnetic Resonance Imaging |
| **NIHSS** | the National Institutes of Health Stroke Scale |
| **NO** | Nitrogen Oxygen |
| **OECs** | Outgrowth Endothelial Cells |
| **PDGF** | Platelet-Derived Growth Factor |
| **PH** | Parenchymal Hematoma |
| **PWI** | Perfusion-Weighted Imaging |
| **rCBF** | residual Cerebral Blood Flow |
| **RNS** | Reactive Nitrogen Species |
| **ROS** | Reactive Oxygen Species |
| **SDF-1** | Stromal Derived Factor-1 |

| | |
|---|---|
| **SS-QOL** | Stroke Specific Quality of Life |
| **SVZ** | SubVentricular Zone |
| **SGZ** | SubGranular Zone |
| **TIA** | Transient Ischemia Attack |
| **TIMP** | Tissue Inhibitors of MetalloProteinase |
| **t-PA** | tissue Plasminogen Activator |
| **TNF-alpha** | Tumor Necrosis Factor-alpha |
| **TTC** | 2,3,5-TriphenylTetrazolium Chloride |
| **VEGF** | Vascular Endothelial Growth Factor |
| **WHO** | World Health Organization |
| **8-OHdG** | 8-HydrOxy-2'-deoxyGuanosine |

# 1. Introduction

## 1.1 Ischemic Stroke

Stroke is one of the leading causes of death and long-term disabilities worldwide. Every year, according to the World Health Organization (WHO), 15 million people suffer a stroke: nearly 5 million die and another 5 million are permanently disabled (www.who.org). Stroke occurs due to alterations with the blood supply to the brain: either the blood supply is blocked or a blood vessel within the brain ruptures. As a consequence of the sudden reduction of the physiological levels of blood flow to the brain, it results in a loss of oxygen and glucose followed by cell death, brain injury and the loss of neurological functions.

This pathology is currently one of the most important health problems because its annual incidence is about 200 cases per 100,000 inhabitants and is the leading cause of death in women and second in men in Spain[1], in addition to the major cause of long-term disability due to motor and sensory limitations. The main risk factors associated with stroke are hypertension, diabetes, atrial fibrillation, smoking and high cholesterol levels, being more prevalent after the age of 65[2].

There are two types of stroke: ischemic strokes and hemorrhagic strokes (**Figure 1**), plus the transient ischemic attacks (TIAs) which last a relatively short time and usually causes no permanent injury to the brain (www.strokeassociation.org). In numbers, around 80% of all strokes belong to the ischemic subtype and the other 20% are caused by a hemorrhage.

During an ischemic stroke, the blood supply to the brain decreases dramatically which usually happens when an artery is blocked by a blood clot or by the narrowing of the arteries. The part of brain that depends on the blood supply of the affected artery cannot sustain the normal cell metabolism due to the limited amount of oxygen and glucose, and the so called ischemic cascade is activated.

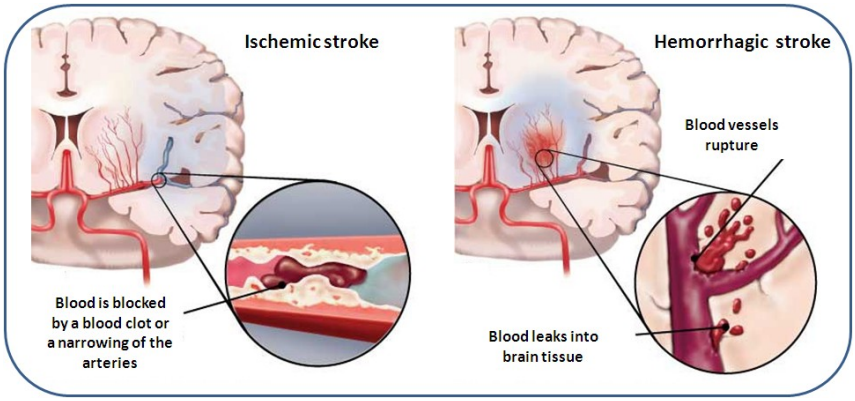


**Figure 1. Ischemic and hemorrhagic stroke in human brain. The figure is adapted from Heart & Stroke Foundation (www.heartandstroke.com)**

In a hemorrhagic stroke, an artery in the brain bursts: blood vessels inside the brain leak causing an intra-cerebral hemorrhage, while blooding under the outer meningeal coverings of the brain and into the fluid-filled space surrounding the brain leading to a subarachnoid hemorrhage.

The TIA, also called as “mini-stroke", is often characterized as a “warning stroke” as a stroke finally occurs with most of those patients presenting a TIA symptoms in the following hours to months[3]. The reduction of blood flow in TIA is temporary which might not cause real cellular death and clinical symptoms usually last for a few minutes. However, patients that have suffered a TIA have more vascular events than those who did not fulfill the criteria of TIA at either 30 days or 90 days after the initial events[4].

The work presented in this thesis focuses on human ischemic stroke and an experimental model of this disease, therefore the remaining introduction text on mechanisms, treatments and molecular pathways of injury and repair will focus on this type of stroke.

### 1.1.1 Ischemic Cascade and Neuroinflammation

In ischemic stroke, the loss of blood supply into the brain results in a rapid loss of oxygen and glucose for the tissue, followed by the reduced cell viability or cell death accompanied by a destructured extracellular matrix that limits cell-cell interactions and the normal function of neuronal networks.

As described below, all the process occurs in a cascade of pathological events: once the residual cerebral blood flow (rCBF) decreases below the 30% of the baseline

blood flow, neuronal dysfunction occurs. As the electrical function of the brain is intimately related to metabolism, a biochemical cascade is initiated, involving excitotoxicity, oxidative stress, inflammation and apoptosis[5,6]. Physiologically, the brain has a very high consumption of oxygen and glucose but little capability to store energy; hence the mitochondria play a key role during the metabolism in the brain. Once ischemia occurs, insufficient energy leads to membrane evoked potential cessation and cells depolarization, activating voltage/acid-sensing ion channels, leading to an imbalance in sodium and calcium levels (**Figure 2**). Calcium is thought to initiate a series of cytoplasmatic and nuclear events including targeting the intrinsic mechanical apoptosis[7]. Activation of calpains by increased calcium results in the cleavage of cell death related pro-apoptotic proteins. Actually, cells in ischemic core undergo necrosis and apoptosis: the initiation in ischemic core is thought to occur by an apoptotic pathway, but the severe decrease in energy level causes a shift from apoptosis toward secondary necrosis. At the same time, the energy-dependent processes such as presynaptic re-uptake of excitatory amino acids are impeded, which increase the accumulation of glutamate in the extracellular space and subsequent excitotoxicity. Induced cell damage occurs through necrotic and apoptotic cell death pathway after the triggering of glutamate receptors[7]. In parallel, mitochondria oxidative phosphorylation is also impaired due to the low levels of oxygen and glucose during ischemia, leading to the release of reactive oxygen radicals (or reactive oxygen species, ROS) and reactive nitrogen species (RNS) as well as increased anaerobic glycolysis[6]. ROS are considered as the main cause of membrane and fundamental cellular components damage[8]; and RNS act together with ROS forming highly reactive products which are capable of damaging lipids, proteins and DNA of the cells[9]. These direct damages on cellular membrane and other compartments, together with the indirect injuries from free radicals stimulating various cellular molecular pathways, lead to an abnormal accumulation of fluid within the brain parenchyma, which is called brain edema[10]. In addition, the overproduction of lactic acid from anaerobic glycolysis of the brain tissue causes neuron-toxic lactate accumulation, increasing the acute excitotoxicity phenomena which is also related to neuronal cell death[11].

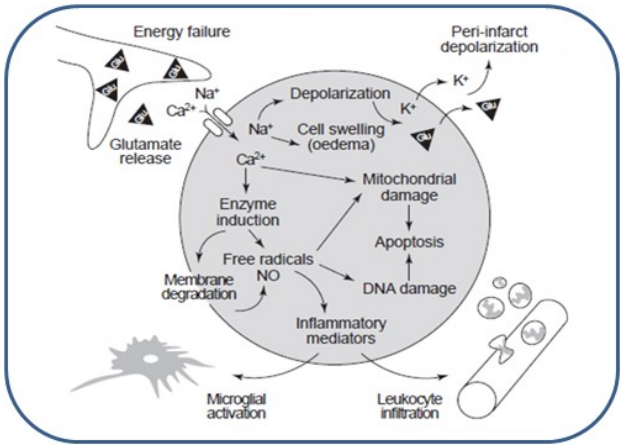


**Figure 2. Summary of pathological mechanisms in the ischemic brain. The figure is adapted from Dirnagl *et al*. 1999.**

However, the immediate permanent cell damage/death after the severe reduction of blood flow occurs mainly within the center of the ischemic territory, which is called the "ischemic core". The peripheral hypoperfused tissue, where the blood flow is inadequate to support neuronal function, but just adequate to maintain cell viability, might be salvageable and is called "ischemic penumbra"[12]. If functional hypoperfusion is not confirmed by neuroimaging techniques, the territory surrounding the infarct core should be named peri-infarct boundary/tissue. With time going on, without effective vessel reperfusion, the ischemic threshold increases and the infarct core expands into the penumbra which might be accompanied by neurological deterioration of the patient. Brain cells, particularly the vulnerable neurons, undergo apoptosis which dominates cell death during this expansion[13]. As shown in the **Figure 3**, the initial ischemic infarct lesion expands to the ischemic hypoperfused penumbra leading an extensive damage after several hours.

Timely effective vessel perfusion or potential neuroprotective interventions can reduce the spreading of depolarization and decrease the final infarct size[7,14]. The penumbra therefore is still the target for any hyperacute stroke therapy, and to identify the penumbra where the tissue is at risk and to apply in-time treatments would be of great importance[15]. In accordance with this dynamics of infarct expansion, the only effective treatments to avoid infarct expansion are the thrombolytic therapies with recombinant tissue plasminogen activator (t-PA) that should be administered within the first few hours[16], and the new endovascular treatments which are also required to be performed within the first hours after stroke onset[17,18,19].

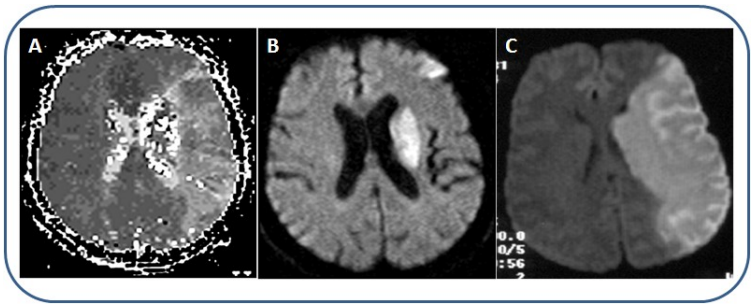


**Figure3. Magnetic resonance imaging (MRI) from an ischemic stroke patient. Perfusion-weighted imaging (PWI) indicating the large area of hypoperfusion at <6 hours after stroke onset (A). Diffusion-weighted imaging (DWI) showing the ischemic core at the same level of PWI at <6 hours after stroke onset (B) and at 48 hours (C).**

Finally, other mechanisms are also involved in the process of ischemic stroke at later time-points, such as inflammation. The calcium activated second-messenger systems, the increased free radicals as well as hypoxia itself trigger the expression of numbers of pro-inflammatory genes and activation of these molecules[7]. A rapid activation of resident inflammatory cells followed by the infiltration of circulating inflammatory cells in ischemic brain region has been documented[20]. Besides, the presence of necrotic cells in the tissue is also highly immunostimulatory. As a consequence, a large amount of inflammatory factors including cytokines, chemokines, matrix metalloproteinases (MMPs), adhesion molecules and ROS are released from injured tissue.

Increased evidences indicate that inflammation contribute to ischemic brain damage and predominantly is deleterious in the early phase after ischemic stroke[20]; but growing evidence suggests that several neurological repair mechanisms might be coupled to the neuro-inflammation stimuli[21,22]. Major steps in the repair process include removal of dead cells, development of an anti-inflammatory micro environment, and generation of pro-survival factors fostering tissue reconstruction and repair[23,24]. Very few knowledge regarding to the participation of inflammation in brain tissue recovery is established, and even less in the long-term effects. This thesis will explore the role of some matrix metalloproteinases and growth factors in the delayed phase of ischemic damage.

### 1.1.2 Ischemic Reperfusion Damage

The reperfusion of vascular supply to an area temporarily deprived of blood flow is not always related to a final recovery of the tissue and good functional outcome, and

after stroke, there is usually some degree of reperfusion related to spontaneously reperfusion or treatment-induced recanalization of the occluded artery. Administration of t-PA 3 hours after clot-induced ischemia leads to effective reperfusion without affecting infarct volume or functional outcome at 24 hours after MCAo in mice[25]. Insufficient reperfusion or delayed recanalization is frequently associated with an exacerbation of tissue injury and profound tissue damage as explained in this section. During reperfusion after ischemia, both the innate and adaptive immune systems as well as the complement system, platelets and coagulation factors are involved[26]. Particularly during the early phase of reperfusion, innate immune cells dominate the cellular compositions of infiltrates such as resident microglia cells (the main innate immune cells in the brain), which response firstly to brain injury. Microglia exerting neurotoxic functions enhances the blood brain barrier (BBB) permeability by preceding the leukocyte infiltration. The BBB is constituted by vascular endothelial cells, basement membrane and pericytes, as well as the end-feet of astrocytes, by providing to the central nervous system a highly controlled cerebral homeostasis[27]. Both ischemia and reperfusion can destabilize and destroy the balance of this microenvironment by inducing the disruption of BBB integrity as proved in experimental models occluding the middle cerebral artery (MCA)[28]. But in transient ischemia models, the BBB disruption is typically more severe than that in permanent models, related to the sudden increased blood volume during reperfusion[29].

A major phenomenon during arterial reperfusion is the recirculation of oxygen which induces the overproduction of ROS and nitric oxide (NO) resulting in additional oxidative stress. On one hand cell death occurs through a number of mechanisms including both necrosis and apoptosis by mediating lipid peroxidation and desoxyribonucleic acid (DNA) damage induced by oxidative stress[26]. On the other hand impaired endothelial cell function occurs as a result of the excessive pericytes contraction and the exposure of the sub-endothelial extracellular matrix to blood flow, altering the permeability of BBB[10].

Subsequent hemorrhagic transformation (HT) of ischemic infarction is a well-recognized life-threatening consequence of ischemia followed by reperfusion. This phenomena occurs in around 10%-40% of ischemic stroke patients during ischemia-reperfusion, being the main complication of t-PA treatment[30]. Either early or delayed administration of t-PA cause different degrees of hemorrhage. From animal models of cerebral ischemia, the administration of t-PA 20 minutes after injury significantly increases the small HT within the infracted tissue (named hemorrhagic infarctions, HI), while 3 hours post-ischemia treatment increases the

occurrence of severe HT including type II HI and massive and expanding HT (named parenchymal hematomas, PH)[25]. In stroke patients, the presence of mild HI might not cause major neurological deterioration of the patient and it is considered a sign of benign reperfusion of the artery, whereas PH is usually associated with a worsening of the neurological status of the patient (especially those causing clinical symptoms in patients which represent 3-7 % of all HT events[31]).

As mentioned, the disruption of the BBB and the continued endothelial damage mediated by free radicals contribute to the appearance of HT in ischemic-reperfusion tissue. However, the mechanism of early HT (within 18-24 hours after stroke onset) is different from the delayed HT (beyond 18-24 hours). The early HT is thought to relate to leukocytes-derived MMPs damaging the BBB, while the delayed HT associated with ischemia activated brain derived proteases, neuroinflammation and other factors[30]. Other consequences of the disruption of BBB are the aggravation of tissue swelling, capillary plugging, platelet adhesion and thrombosis, being those correlated with enhanced stroke damage by the communication within neurovascular units[32].

### 1.1.3 Animal Models for Ischemic Stroke

Animal models of cerebral ischemia are effective experimental tools to better understand the pathological mechanisms of ischemic injury and to test potential therapeutic interventions in preclinical trials. As different types of stroke occur in human associated to different mechanisms, etiologies and outcomes, there is no single universal animal model for all forms of human stroke[33].

There are multiple animal models of focal cerebral ischemia involving both permanent and transient middle cerebral artery occlusion (MCAo) mostly applied in rodents due to ethical and economic reasons. Intra-luminal filament models are well-established methods and widely considered adequate to reproduce primary ischemic insult and subsequent cell death, glial activation, blood brain barrier damage, among other pathological mechanisms[34,35]. But this model presents high premature mortality rates due to the large ischemic lesion affecting both the striatum and the cortex (**Figure 4**), produced by a proximal occlusion of the MCA, which might be not suitable for long-term neurorepair or safety studies. On the other hand, a different size of infarct lesion between young and aged mice is found only in distal MCAo models but not proximal, indicating that small infarct lesions are more willing to be modulated by neurogenesis-involved mechanisms[36]. Besides, many

human strokes are small in size and the occlusion of distal MCA is the most common reason for human ischemic stroke[37,38]. In experimental models, distal MCAo can be induced by permanent electrocoagulation, vessel contraction (endothelin injection) or by a transient ligation/compression of the distal branch of the MCA generating a cortical infarction. Among other described methods[39], the embolic and thromboembolic models are ideal methods to mimic the clinical ischemic stroke, and offer the potential to evaluate thrombolytic agents or combination therapies[40,41]. However, these models induce occlusions without control of exact reperfusion time and degree. A focal cerebral ischemia model produced by ferric chloride ($FeCl_3$)-induced thrombus formation has been described in a previous publication[42]. This model holds the advantage of low mortality, easy to perform and no additional trauma to the brain tissue. However, reperfusion is still hard to achieve and to control. Finally, a new reproducible method for distal focal cerebral ischemia in mice with reperfusion by the distal MCA compression was developed in our lab, based on the previous mechanical methods[43]. The present model holds the advantages of minimal damage to the vessel and underlying tissue, high reproducibility and low mortality, at the same time that allows controlled tissue reperfusion. Mortality is a critical parameter in experimental studies as low mortality rates are important to reduce the number of animals and avoid selective bias of the obtained results. This model together with the distal electro-coagulation of the MCA has been used as the experimental ischemia model in this thesis.

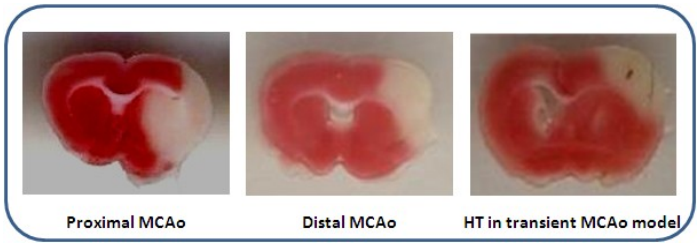


**Figure 4. 2,3,5-Triphenyltetrazolium chloride (TTC) stained brain sections non-viable tissue (white areas) after different experimental models of stroke in rodents.**

### 1.1.4 The Neurovascular Unit

Although the mechanisms of single cell death have been studied deeply, the mechanism by which the whole BBB responds to ischemia-reperfusion in the injury and recovery phases of stroke are still under investigation. The concept of neurovascular unit was suggested more than one decade ago, and refers to the

interconnection among cerebral endothelial cells, astrocytes, adjacent neurons and the extracellular matrix together with the circulating elements in the blood stream[2]. It is a functional unit revealing the complex inter-relationships among all the brain cells and tissue environment related to cerebral diseases. Neurons, as the core functional cell in the brain tissue, play a dominant role during the development of neurological dysfunction in cerebral ischemia; however, neurons are not the only target influencing tissue outcome in ischemia-reperfusion damage. The neurovascular unit emphasizes the dynamics communication of vascular, cellular and matrix signaling in the brain (**Figure 5**).

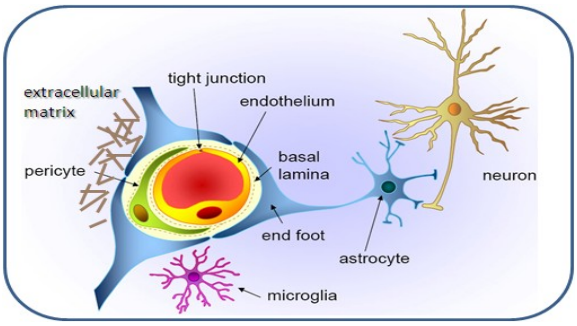


**Figure 5. Organization of the capillary neurovascular unit. The figure is adapted from Abbott *et al*. 2010.**

An example is the disruption of the BBB which is crucially dependent on endothelial-astrocyte-matrix interactions which are related to the tight junctions and basal lamina integrity. Endothelial cells, the main source of reactive radical species after ischemia-reperfusion, contribute to the BBB connection via adherent and tight junctions to form a physical barrier together with pericytes. Astrocytic end-feed wraps around capillary endothelial cells regulate cerebral capillary blood flow as well as the permeability. We now know that the endothelial cells and astrocytes engage in a complex cross talk to regulate each other's function[44]. Extracellular matrix proteins contribute to the structural formation of basal lamina that connects to endothelial cells by the cell-matrix interaction. When the BBB is disrupted by ischemia, injury to the microvasculature can lead to injuries to neuron in the same territory[45]. In this context, ischemic brain damage is not the only trigger of neuronal cell death, also the local environment which influences surrounding cells, allowing neurons to survive or to die.

The function of this neurovascular unit in the cerebral micro environment is also related to the "cross-talk" among cells[46]. Increased studies have documented that various factors mediate the interaction both in health and disease including neurotransmitters and inflammatory mediators[47]. For example, during synaptic

activities, being initiated by neurons, both neurons and astrocytes release neurotransmitters, including acetylcholine, glutamate and γ-Aminobutyric acid (GABA) directly or indirectly stimulating the production of vasodilators to control the vasodilatation of arteries at the site of activation[44].

But the homeostasis of the neurovascular unit is also important during stroke recovery. The expression of reactive astroglia expressed glutamate transporter is positive related with the neurological score during day 1-3 after ischemia-reperfusion in rats[48]. Post-stroke injection of antagonist of glia-specific glutamate transporter significantly changes the number of neurons responding to forelimb stimulation after stroke in mice[49].

### 1.1.5 Angio-neurogenesis

In spite of the neurological dysfunctions caused by stroke are a consequence of tissue and cell damage, surviving patients undergo some spontaneous recovery by triggering self-repair processes (**Figure 6**)[50,51]. However these are limited in terms of tissue repair.

Recovery could therefore occur by multiple mechanisms[52,53]:

1) Restoration of damaged neurons and their function;

2) Remodeling the structure or function of the remained undamaged neural cells (so called “neuroplasticity”);

3) Formation of new functional neurons from pre-existing progenitors.

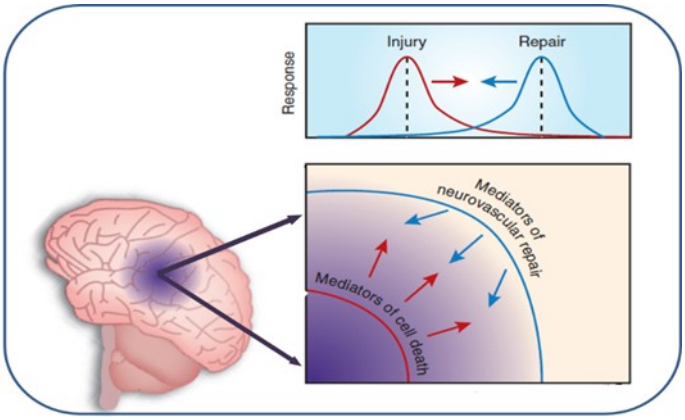


**Figure 6. Representation of injury and spontaneous repair mechanisms triggered by stroke. The figure is adapted from E Lo *et al*, 2008.**

The formation of new functional neurons is called neurogenesis.

Physiologically, newborn neurons generated from the subventricular zone (SVZ) migrate to olfactory regions whereas those neurons generated from subgranular zone (SGZ) migrate to hippocampus[54]. It has been reported that after cerebral ischemia, SVZ-derived neural precursor cells have the capability to respond to the insult and migrate toward to the lesion area to produce new cells replacing the damaged cells in rodents[55,56], and this migration and proliferation was also observed in human in response to ischemic stroke[57]. This generation of newborn neurons after stroke is proven associated with functional recovery by integrating into the existed neuronal network[58]. However, the sustained functional improvement is obviously earlier than the maturation of these new generated neurons[59]. Besides, the majority of these newborn cells die from apoptosis in a few days, and the rate of differentiation is low[55,60,61]. This mismatch supports the hypothesis that neurogenesis induced-recovery is mediated by mechanisms other than directly neuronal replacement, possibly through local growth factors mediated functional restoration in the peri-infarct tissue[59].

As detailed in previous sections, ischemia initiates inflammation, increases microvascular permeability and might cause hemorrhage, which does not only influence neurons in the first stage but also other glial cells supporting neurons and the microvessels that supply oxygen and glucose. The concept of the neurovascular unit proposes that, injury to any part of the unit affects the other components unbalancing the neurovascular homeostasis that allows the physiological environment of the brain. In this regard, the most classical view of neurorepair as the replacement of dying neurons has changed in the last decades for a more global understanding of the brain, including other cell types (glial cells, inflammatory cells and stem/progenitor cells) but also the extracellular matrix and their interactions. Therefore endogenous mechanisms of neurorepair include angio-vasculogenesis (formation of new blood vessels), gliogenesis (formation of new glial cells), neurogenesis (formation of new neurons), re-myelination (new myelin sheaths on demyelinated axons), among others. Part of this thesis focuses on angiogenesis as a key mechanism to modulate during stroke repair.

Classically, the formation of new blood vessels was thought to be mediated exclusively by embryogenic vasculogenesis, followed by the sprouting of endothelial cells from pre-existing vessels during angiogenesis[62]. However, this dogma was overturned with the discovery of bone marrow-derived CD34+ cells with endothelial characteristics and circulating in adult human blood[63]. These cells, referred as

endothelial progenitor cells (EPCs), are capable of differentiating *ex vivo* into endothelial-phenotyped cells, and represent a new model for endothelial regeneration and vessel repair. In this context, EPCs have been shown to be highly migratory to ischemic areas and a powerful therapeutic potential by directly enhancing angiogenesis or by forming new vessels through vasculogenesis[64,65]. In addition to the therapeutic interest of endothelial progenitors for vascular regeneration, it is extremely important to know more on the role played by the growth factors secreted by these cells which enrich the so called neurovascular niche.

It is known that angiogenesis-related genes such as vascular endothelial growth factor (VEGF) are up-regulated as soon as 1 hour after ischemia onset; and the post-stroke vascular formation in the peri-infarct area is also involved in the process of tissue recovery, involving angiopoietins (Ang) up to 21 days after ischemia[60,66,67]. But angiogenesis activation after cerebral ischemia does not occur alone, it is actually coupled to neuroblast cell migration from the SVZ[58]. In this context, the role might be as important as the one played by neural precursor cells.

The so called neurovascular niche defines these complex mechanisms of crosstalk between cerebral endothelium and neural precursors under ongoing neurogenesis. Stroke changes the cellular environment in the peri-infarct area by forming a unique neurovascular niche, in which angiogenesis and neurogenesis are linked through specific vascular factors and chemokins[59]. Apart from the VEGF, the cytokine stromal derived factor-1 (SDF-1), also named as C-X-C motif chemokine 12 (CXCL12), is another important molecular playing a central role in the post-stroke neurovascular niche. The SDF-1 and its receptor C-X-C motif chemokine receptor type 4 (CXCR-4) participate in a variety of physiological and pathological processes including angiogenesis and neurogenesis through mediating the proliferation of neurogenitors, regulating the migration, differentiation, as well as functional integration of newborn neurons into existing networks[68]. We have also learnt that cerebral vessels express SDF-1 days after cerebral ischemia in peri-infarct areas where neurogenesis occurs, and the specific blockage of angiogenesis by the administration of specific inhibitors (such as endostatin) also blocks endogenous post-stroke neurogenesis from the SVZ[59].

As we will see in this thesis, several therapeutic approaches under investigation have their target on the modulation and enhancement of these mechanisms that participate in neurological repair after stroke.

## 1.2 Matrix Metalloproteinases

The degradation of extracellular matrix substrates plays a crucial role in both ischemia induced disruptions of the BBB as well as in cell proliferation and cell migration during tissue repair. Together with adamalysin-like metalloproteinases with thrombospondin motifs (ADAMTSs), MMPs are capable to degrade the extracellular matrix, plating important roles in many physiopathological processes[69,70]. MMPs are a family of zinc-dependent proteinases, involved in the degradation and remodeling of extravascular matrix and vascular compartment due to their capability to degradation of basal lamina and extracellular matrix components. This protease expression and activation occurs in physiological conditions during the normal homeostasis of the extracellular matrix but the dysregulation of some MMPs also participates in processes of tissue injury and repair[71]. Despite MMPs have been considered for a long time to be the prototypic matrix-degrading proteases, in recent years and with the application of engineered genetic models, they have been shown functions more as processing enzymes that control numerous, diverse cell processes. Indeed, via cleavage of cytokines, chemokines, antimicrobial peptides, and cell surface proteins, MMP proteolysis affects critical regulatory functions related to immunity, tissue repair, differentiation, and cell transformation[72].

There are four primary subgroups of MMPs on the premise of the structure domain: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11) and others not belonging to any of them[73]. Physiologically, most of the MMPs are synthesized as inactive forms (also named pro-forms), and their activity is strictly controlled by gene transcription which is mediated by variety of inflammatory cytokines, hormones and growth factors[74]. However, MMPs are also regulated at post-transcriptional level through an activation mechanism: the activated MMPs can participate in processing the activation of other MMPs, and the irreversible MMPs' activation can be blocked by tissue inhibitors of metalloproteinase (TIMP)[75]. Uncontrolled MMP activity has been described in several diseases and many pathological processes including cerebral ischemia are associated to an increase in MMP levels and activity related to the degradation of proteins such as laminin, fibronectin, collagen, proteoglycans, and others.

Different investigators have suggested that the upregulation of MMPs such as MMP-2, MMP-3 and MMP-9 contribute to infarct extension and BBB breakdown after stroke[76,77,78], and blood levels of some MMPs can also be easily monitored as potential biomarkers of brain tissue damage and/or neurological outcome[79,80]. Also,

brain MMP-9 is up-regulated in ischemic brain tissue related to vessel disruption and blood extravasation in human samples[81,82,83], and the gene or pharmacologic inihibition of MMP-9 has demonstrated to protect the brain from cerebral ischemia in preclinical models[84,85,86]. A list of the putative role of most relevant MMPs related to ischemic stroke has already been summarized by some researchers (**Table 1**)[87].

**Table 1. MMPs and their putative roles in ischemic stroke. (The table is adapted from Lakhan *et al*. 2013)**

| MMP | Putative roles in ischemic stroke |
|---|---|
| MMP-2 (gelatinase A) | Attacks major components of the basal lamina around the cerebral blood vessels including collagen IV, lamina and fibronectin. May contribute to infarction and hemorrhagic volume. |
| MMP-3 (stromelysin-1) | Degrades the extracellular matrix proteins fibronectin, denatures collagen, lamina and proteoglycans. Plays a key role in the initial opening of the BBB after stroke and the development of hemorrhagic transformation, particularly with tPA treatment. Promotes remyelination and the chemokines induced neural progenitor cells' differentiation and migration. |
| MMP-8 (collagenase-2) | Preferentially cleaves collagen I. Unregulated in infracted tissue but studies are limited. |
| MMP-9 (gelatinase B) | Attacks major components of the basal lamina including collagen IV, lamina and fibronectin. Plays a key role in the delayed opening of the BBB especially in the states of systematic inflammation, related to hemorrhagic transformation particularly with tPA treatment. Is involved in the neurovascular remodeling by mobilizing VEGF, myelin reformation and EPCs angiogenic function; promotes the chemokines induced neural progenitor cells' differentiation and migration. |
| MMP-12 (macrophage metalloelastase) | Degrades soluble and insoluble elastin. Contributes to the acute brain damage may related to the activation of MMP-9. |
| MMP-13 (collagenase-3) | Preferentially cleaves collagen II. Upregulated in infracted tissue but its study is limited. |

At the beginning of this section we have pointed out that these proteases might also contribute to beneficial remodeling during stroke recovery. In this regard, early studies already shown that MMPs are expressed during development and contribute

to morphogenesis of the CNS[88]. They are also related to neurogenesis mediating the differentiation and chemokine-induced cell migration of adult neural progenitor cells[89]. MMPs may also modulate bioavailable levels of various growth factors (such as VEGF by MMP-9 actions) by processing pro-form precursors or by liberating active molecules from matrix-hidden compartments[90]. After stroke, newly born neuroblasts migrate from the SVZ to peri-infarct cortex, and increased vascular remodeling is also found in this area[91,92]. At 2 weeks after cerebral ischemia in mice, MMP-9 is enhanced in the SVZ and co-localized with BrdU-labeled cells and neuroblasts whereas the pharmacological inhibition of MMPs reduces the extension of neuroblast signals that extend from the SVZ into the damaged striatum and post-stroke angiogenesis impairing functional recovery[93,94]. As we will better describe latter, our group has recently described the central role of MMP-9 in vascular remodeling mediated by endothelial progenitor cells in the context of cerebral ischemia both in vitro and *in vivo*[95,96].

Regarding MMP-13 (collagenase-3), the dominant collagenase, the current knowledge comes from cancer, bone-related diseases and re-epithelialazation of the skin during wound healing[97,98,99], but limited studies have been published related to the role of this protease in stroke disease. A few studies from our group and others have shown an increased expression of MMP-13 in infarct areas compared to healthy control human tissue[81], located in neurons as well as in glial cells in ischemic models[100,101]. Also a high level of MMP-13 in blood is related to the early lesion expansion in stroke patients who received thrombolytic therapy[102]. The modulation of MMP-13 during the delayed phases of stroke is still unknown.

## 1.3 Treatments for Ischemic beyond t-PA

Although the understanding of post-ischemic injury is still limited, new insights of therapeutic approaches are accumulating. Being the blockage of a cerebral artery by a blood clot, the pathological cause of ischemic stroke, the recanalization therapy to reperfuse the artery and recover the cerebral blood flow is still the only effective treatment to reduce tissue injury and improve neurological outcome. The intravenous thrombolysis with t-PA is still the mainstay of acute ischemic stroke management for the patients within 4.5 hours from symptom onset, being a standard therapy for those patients with moderately severe symptoms younger than 80 years old and without contraindications for the medication[16,103]. The new emerging effective intra-arterial therapy using stent retrievers also requires to be applied within the first 6 hours[17,103]. As both of these two successful treatments require rapid intervention, only 5% to 7% of patients are treated with t-PA along with 1% to 2% benefit from intra-arterial therapy, whereas the majority of patients do not meet criteria to receive these available reperfusion therapies. In this context, the development of neurorestorative and neurorepair therapies available for the vast majority of patients to improve neurological deficits after ischemic stroke is a great challenge.

As mentioned, the spontaneous brain neurorepair is limited in efficacy and heterogeneous among patients. Strategies to enhance the endogenous restorative mechanisms of recovery are sought as stroke treatments. The central goal of these potential therapies should be to prevent further damage during the delayed phases of ischemia minimizing the damaging molecular events and cell death, at the same time that new opportunities of tissue recovery should be stimulated to remodel and recover the affected tissue **(Figure 6)**.

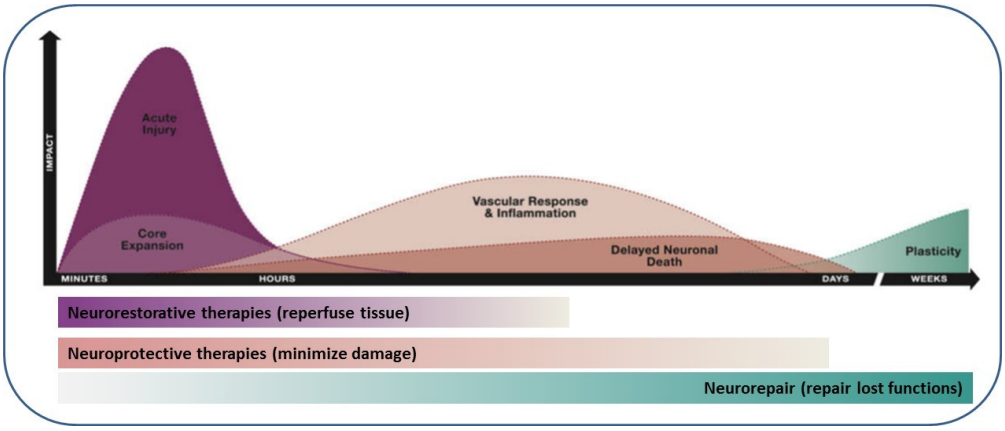


**Figure 6. Spectrum of therapies for ischemic stroke according to the chronical sequence of**

**mechanisms of injury and repair. The figure is adapted from Zaleska *et al*. 2009.**

### 1.3.1 Neurorehabilitation after Stroke

Despite important advances in acute stroke management in the last two decades (the management of patients in stroke units, the thrombolytic therapy or the new emerging endovascular treatments), each year about five million people survive a stroke worldwide with some disabilities that limit their independence for daily activities (www.who.int). This fact also carries an enormous socio-economic burden estimated at €196 billion every year for the countries of the European Union (www.ehnheart.org). For those patients the only approved treatment during the sub-acute and chronic phases of the disease is neurorehabilitation to recover the impaired or lost neurological functions with the objective of improving their independency status and quality of life[104,105].

During rehabilitation, the improved outcome in patients is the result of early mobilization, rehabilitation intensity and other measures to prevent/treat medical and psychological complications, altogether being performed by an inter-disciplinary team, and also according to spontaneous individual and heterogeneous response to therapy[106,107]. And the ultimate goal of any rehabilitation therapy is to reach the maximum recovery of the impaired function in an effort to reduce disability and achieve functional independence for daily activities of the patient. However, the recovery of other non-motor functions (the executive function, attention, memory, etc.) is also the subject for rehabilitation therapies.

Importantly, for the overall rehabilitation process it is very important to assess patient's neurological function, independence and quality of life after stroke. The impact of the disease can be measured using certain scales which also allow to monitor and to evaluate the impact of the received therapies. As there is no consensus on the use of a single scale that can measure all the affected aspects of disability after stroke, many scales are commonly used in the clinical practice and in clinical trials to evaluate the therapeutic potential of a particular therapy[105,108] despite certain limitations in their sensitivity, specificity and reliability among observers. It is therefore common to use a battery of scales to assess neurological deterioration or improvement of the patient's function in rehabilitation therapy[104,109,110]. **Table 2** shows a summary of the most used scales during rehabilitation therapy after stroke.

**Table 2. Summary of the most used tests/ scales to measure the outcome in stroke rehabilitation.**

| Measures | Description |
|---|---|
| NIH Stroke Scale (NIHSS) | A quantitative measure of the severity of symptoms/ neurological deficit associated with cerebral infarcts. |
| Fugl-Meyer Assessment (FMA) | A disease-specific impairment index to assess motor function, balance, sensation qualities and joint function. |
| Medical Research Council (MRC) | To exam the peripheral nerve lesions by testing the muscle strength. |
| mRankin | A global outcomes rating scale for daily activity/disability. |
| Barthel Index (BI) | The most widely used measure of functional disability. |
| Functional Ambulation Categories (FAC) | To assess ambulation capability. |
| Chedoke Arm and Hand Activity Inventory (CAHAI) | A functional assessment for the upper-limb recovery. |
| 10 meters' Walking | A sub maximal test to assess functional capability. |
| Stroke Specific Quality of Life Scale (SS-QOL) | To assess health-related quality of life. |

### 1.3.2 Neurorehabilitation and Tissue Repair

From a biological perspective, we know that neurorehabilitation enhances different aspects of brain plasticity such as axonal remodeling, cell genesis, angiogenesis or synaptogenesis[111], which could be patient-dependent, and the biological mechanisms responsible for these individual responses to treatment are still being investigated.

Fortunately, in some patients the recovery of the neurological function occurs spontaneously after stroke and in others it is achieved with some types of rehabilitation therapy up to 6 months or 1 year after stroke[109]. The spontaneous recovery is related to brain plasticity and is also observed in animal models of stroke being particularly significant in mice[112]. In this sense, several mechanisms have been

described to be related to functional recovery such as: the activation, proliferation and migration of endothelial/neural progenitor cells, changes in existing neural pathways, the compensation phenomena and also an increased neuroanatomical plasticity leading to the formation of new neuronal connections[111]. Today we know that the brain repair/regeneration process occurs from outlying areas of the infarct core, where these stem/progenitor cells arrive to replace or to provide trophic support to the pre-existing cells traffic[113].

Another important aspect during rehabilitation is the convenience to have biomarkers that could support an accurate and individual prognosis of stroke recovery during the rehabilitation program, which would help to decide on the intensity, type or duration of the rehabilitation programs[108].

However, very few studies have investigated the use of biomarkers during rehabilitation therapy for post-stroke recovery linked the progress of functional rehabilitation (determined by specific scales). In this regard the levels of certain cytokines such as interleukins (IL), tumor necrosis factor alpha (TNF-alpha) and adhesion molecules have been monitored in patients with a first-ever stroke under rehabilitation therapy compared with a control population showing an increase in IL-6 and TNF-alpha in stroke patients versus controls, and a negative association of IL-1-alpha, IL-8 or TNF-alpha with the Barthel Index[114]. But the study only covers the first two weeks after stroke. More recently, Blicher and colleagues have studied the neurotransmitter GABA by magnetic resonance spectroscopy in relation to motor function after 2 weeks of constrained-induced movement therapy showing an association between motor improvement and the magnitude of the GABA/creatine ratio[115]. Finally a recent study has reported the use of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a sensitive marker for DNA damage that is detectable in urine samples, in a cohort of stroke patients undergoing rehabilitation therapy reporting a negative correlation between FMA scores and 8-OHdG level after rehabilitation treatment and lower levels of baseline 8-OHdG in patients that exhibited more improvement in motor function, suggesting the use of 8-OHdG as a biomarker of stroke recovery[116].

Given the complexity of the CNS responses to cerebral ischemia and the role of neural plasticity in tissue regeneration and functional recovery, it is likely that future rehabilitation treatments will be combined with pharmacological/biological therapies to enhance certain molecular pathways. In this context, to identify the key molecules responsible for these tissue regeneration mechanisms will be of great importance to influence the natural history of the disease. Part of this thesis focuses on the study of matrix metalloproteinases during intensive rehabilitation therapy related to the

motor/functional improvement achieved at different moments of the rehabilitation program.

### 1.3.3 Cell-based Therapy

Cell-based therapies have been demonstrated to be an effective approach for brain repair by enhancing the endogenous restorative mechanisms of recovery in preclinical models of stroke[117], and some of them are being tested in the clinical trials (/www.clinicaltrials.gov). Most of these treatments do not aim to replace the damaged cells, but to remodel the total system by modifying the cerebral microenvironments, thereby enhancing the neuroplasticity through angiogenesis, neurogenesis, oligodendrogenesis or synaptogenesis. Multiple stem/progenitor cells have been tested as potential sources for cell-based therapies including neural stem cells, mesenchymal stem cells or bone marrow-derived cells proving their efficacy in animal models of stroke improving the functional recovery or reducing brain lesions[118]. However, there are several types of stem cells, and to identify which subtype is the best one contributing to the robust therapeutic improvement is still a challenge. Moreover, there is still no consensus regarding to issues related to the therapy such as how long will these cells survive, what time point is the best one to administer those treatments or which group of patients are expected to benefit most from these therapies[119].

As the main goal of cell-based therapy is to create a favorable microenvironment for tissue repair in the desired peri-infarct area, and angiogenesis plays a pivotal role during this process, EPCs have attracted increasing attention in the last years. EPCs were firstly identified as bone marrow derived cells responsible for the postnatal vasculogenesis both in physiological and pathological neovascularization, circulating in adult humans[120,63]. After ischemic damage, EPCs mobilize from bone marrow to the peripheral blood; home to the sites of vascular remodeling in the brain where they differentiate into mature endothelial cells and/or regulate preexisting endothelial cells via paracrine or juxtacrine signals[121], contributing to neurovascular repair after stroke. Importantly, the number and function of EPCs could be modulated after stroke to improve cell-based tehrapies[122]. In this line our group has demonstrated that the genetic or pharmacological inhibition of MMP-9 diminished the pro-angiogenic actions of EPCs[95], and that the post-stroke neurovascular niche requires MMP-9 for an appropriate vascular remodeling and pro-angiogenic action of an EPC-based therapy in a mouse model of stroke[96]. These studies highlight the role

of MMPs in post-stroke neurorepair cell-based therapies.

# 2. Objectives

The main objectives of this PhD thesis are:

1) To conduct a systematic review of the literature studying Endothelial Progenitor Cells role in vascular remodeling and cell-cell interactions related to neurorepair after stroke.

2) To determine the role of MMP-13 both in brain injury after cerebral ischemia and in post-stroke neurorepair mechanisms such as angiogenesis and neurogenesis.

3) To identify the temporal profile of MMP-3, MMP-12 and MMP-13 serving as biomarkers of recovery during intensive rehabilitation therapy after ischemic stroke.

# 3. Methods and Results

**Article 1**

**Endothelial progenitor cells and revascularization following stroke**

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Review

# Endothelial progenitor cells and revascularization following stroke

*Feifei Ma, Anna Morancho, Joan Montaner, Anna Rosell**

*Neurovascular Research Laboratory and Neurology Department, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain*

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ABSTRACT

Brain injury after ischemia induces the mobilization of endothelial progenitor cells (EPCs), a population of bone marrow-derived cells with angio-vasculogenic capabilities. These cells have been also tested in pre-clinical models and proposed for neurorepair therapy aiming to treat patients in the delayed phases of stroke disease. Promising results in the pre-clinical field encourage the translation into a clinical therapeutic approach. In this review, we will describe EPCs actions for enhanced revascularization and neurorepair, which on one hand are by their direct incorporation into new vascular networks/structures or by direct cell–cell interactions with other brain cells, but also to indirect cell–cell communication thorough EPCs secreted growth factors. All these actions contribute to potentiate neurovascular remodeling and neurorepair. The data presented in this review encourages for a deep understanding of the mechanisms of the cross-talks between EPCs and other brain and progenitor cells, which deserves additional investigations and efforts that may lead to new EPCs-based therapies for stroke patients.

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## Contents



*Corresponding author.
E-mail address: anna.rosell@vhir.org (A. Rosell).

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## 1. Stroke disease and neurorepair

Ischemic stroke is a leading cause of mortality, long-time disability and morbidity in the world. Data from the World Health Organization confirms that stroke affects 15 million people worldwide each year; of these, 5 million people will die and another 5 million will survive with neurological deficits that limit their functional independence (http://www.who.org). Once a vascular occlusion in the brain happens, a complex chain of events develop at molecular and cellular level leading to irreversible tissue injury and cell death (Kalladka and Muir, 2014), if vascular occlusion is not resolved in a short period of time. As a consequence an ischemic core is formed as a result of irreversible cell necrosis not only affecting all the cellular elements like neurons, glial cells and blood vessels but also affecting the extracellular matrix components. The ischemic penumbra surrounding the ischemic core, transiently maintains a collateral blood supply sufficient for cell viability. However, tissue in the penumbral region could progresses to cellular death by expanding the core lesion without an appropriate restored perfusion and recanalization in time (Rha and Saver, 2007). In addition to early brain injury caused by the reduction of blood flow, post-ischemia reperfusion may worsen initial tissue damage by secondary damage by triggering an inflammatory response, blood–brain barrier breakdown and cell apoptosis (Lopez-Neblina et al., 2005). On the other hand, stroke also triggers a regenerative response such as the proliferation of endogenous neural progenitor cells, an increase in the number of immature neurons or an enhancement of peri-infarct angiogenesis, among other processes (Parent et al., 2002; Jin et al., 2001; Zhang et al., 2004), demonstrating the plastic nature of the brain in opposition to more classical views of passive dying brains.

The only proven effective drug treatment for acute ischemic stroke is thrombolysis with recombinant tissue plasminogen activator (rtPA), which was approved by the US in 1995 (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995), and now given to patients in a limited therapeutic time window of 4.5 h from symptoms onset (Hacke et al., 2008). Although thrombolysis with tPA is effective and lifesaving, only 2–5% of all stroke patients receive this treatment. Therefore, it is necessary to develop new stroke therapies that could be used to treat a large number of patients in the delayed phases to repair and rewire the injured tissue. The new concept for research in stroke therapy is that endogenous neurovascular plasticity and remodeling are also activated in the most-early phases of the ischemic event and participate in the functional recovery after stroke.

### 1.1. *Angiogenesis, neurogenesis and oligodendrogenesis after stroke*

The classical view of neurorepair as the replacement of death neurons has changed in the last decades for a more global understanding of the brain as a whole, including other cell types (glial cells, inflammatory cells and stem/progenitor cells) but also the extracellular matrix and their interactions. Therefore endogenous mechanisms of neurorepair include angio-vasculogenesis (formation of new blood vessels), gliogenesis (formation of new glial cells), neurogenesis (formation of new neurons), re-myelination (new myelin sheaths on demyelinated axons), among others. Immediately after stroke, some early functional recovery mechanisms can be attributed to the resolution of edema or inflammation, which are usually limited in time and extent, whereas other process including neurogenesis, angiogenesis and oligogenesis are involved in the longer-time recovery of neurological function and tightly regulated by many factors (Arvidsson et al., 2002). Thus, neurorepair of the damaged tissue to rewire neuronal networks and enhance neuronal regenerative mechanisms becomes one promising therapeutic approach that involves other remodeling steps. Several of those endogenous mechanisms are activated in minutes following the ischemic trigger in peri-infarct areas (Pepper, 1997; Carmichael, 2008) and several populations of newborn progenitor cells have been identified in remodeling areas (Carmichael, 2008; Ohab et al., 2006; Jin et al., 2006), such as neural progenitor cells (NPCs), endothelial progenitor cells (EPCs) or oligodendrogyte progenitor cells (OPCs).

Neurogenesis in the adult brain takes place in two areas: the hippo-campal sub-granular zone (SGZ) and sub-ventricular zone (SVZ) (Ohab et al., 2006; Ohab and Carmichael, 2008) which renew cells of the dentate gyrus and olfactory bulb, respectively. Neural stem cells (NSCs) reside in specific niches such as the SVZ, and display partial differentiation and enhanced proliferation with specific fates, for example to neuroblasts or oligodendrocyte progenitor cells (OPCs). After cerebral ischemia it has been observed that in those areas rich in neural progenitor niches these cells can proliferate, migrate and graft into the most peri-lesional brain areas where they can differentiate into new neurons or glial cells and renew the cell population (Ohab and Carmichael, 2008). For this to happen there must be signals that guide migrating neuroblasts to areas where cell renewal can take place. In this process, neurons facilitate each others migration in chains; astrocyte end-feets influence migration by surrounding and creating encased tube; blood vessels provide a path of neuronal migration by releasing some factors and acting as a physical scaffold (Gage, 2000; Arvidsson et al., 2002). It appears that peripheral areas of infarction, and no other healthy areas of the brain, present such signals. In these areas bordering the injured tissue, the induction of endogenous angiogenesis has been observed. Neuronal migration is influenced by cell-secreted factors and by cell-bound molecules including gamma-aminobutyric acid (GABA), vascular endothelial growth factors (VEGF), brain-derived neurotrophic factor (BDNF), polysialylated neuronal cell adhesion molecule (PSA-NCAM), matrix metalloproteinases (MMP), β1 integrins, angiopoietins (Ang) and extracellular matrix components (Kahle and Bix, 2012). Importantly, today we recognize the so-called neurovascular niche that promotes the coupling between angiogenesis and neurogenesis with the establishment of migratory neuroblasts in the peripheral infarction in co-localization with small blood vessels especially in areas with active vascular remodeling (Ohab et al., 2006) where an increase in growth factors such as stromal derived factor (SDF)-1 occurs days after stroke. Other authors have demonstrated that cerebral endothelial cells are activated by ischemia enhance NPC proliferation and differentiation mediated by VEGF receptor 2 (VEGFR2)

(Teng et al., 2008) and studies using animal models of cerebral ischemia show that as early as three days after the event a secretion of angiogenesis-stimulating factors (Hayashi et al., 2003). The balance between factors that affect neuronal migration and differentiation will likely influence how the process of post-stroke neurorepair occurs.

Classically, the formation of new blood vessels was thought to be mediated exclusively by embryogenic vasculogenesis, followed by the sprouting of endothelial cells from pre-existing vessels during angiogenesis (Pepper, 1997). However, this dogma was called into question upon the discovery of bone marrow-derived CD34+ cells with endothelial characteristics and circulating in adult human blood (Asahara et al., 1997). These cells, referred to as endothelial progenitor cells (EPCs), were capable of differentiating *ex vivo* into endothelial-phenotyped cells, and represent a new model for endothelial regeneration and vessel repair. In this context, EPCs have been shown to be highly migratory to ischemic areas and a powerful therapeutic potential by directly enhancing angiogenesis or by forming new vessels through vasculogenesis (Vasa et al., 2001; Griese et al., 2003). In addition to the therapeutic interest of endothelial progenitors for vascular regeneration, it is extremely important to know more on the role played by the growth factors balance secreted by these cells. Therefore the benefit of EPCs may also be caused by the paracrine effects of EPCs secreted factors such as VEGF (Urbich et al., 2005; Horie et al., 2011) and angiogenic cytokines such as interleukin (IL)-8 (He et al., 2005) or interleukin (IL)-1β (Rosell et al., 2009). This might be of special interest if the cell secretome (and not recombinant proteins) can be delivered into the injured brain to induce angio-vasculogenic responses (Rosell et al., 2013).

Oligodendrocytes (OLGs) are one of the major cell types in the white matter in the CNS which produce myelin enwrapping axons and facilitating axonal conduction. Evidence of OLG regeneration after ischemia has been demonstrated in previous studies (Tanaka et al., 2003; Miyamoto et al., 2010) and the existence of a specific and unique oligovascular niche was proposed as many trophic factors may affect non-neuronal cells (Arai and Lo, 2009). OLGs differentiate from oligodendrocyte precursor cells (OPCs) and their differentiation into OLGs occurs in a multiple steps including migration, proliferation, differentiation and final maturation (Arai and Lo, 2009) and the efficiency of this differentiation is known to be severely compromised after injury (Kako et al., 2012) because they are vulnerable to energy failure. These OPCs migrate into injury areas of white matter to differentiate into pre- and mature myelinating oligodendrocytes, and participate in myelin repair in the lesions of adult brains. Several nourishing factors have been reported to modulate OPCs differentiation and function. VEGF-C, as a regulator of the vascular system was identified to be a regulator of the proliferation of neural progenitors expressing VEGFR-3 since proliferation was severely reduced in mice lacking VEGF-C (Le Bras et al., 2006). The same study showed a selective loss of oligodendrocyte precursor cells (OPCs) in the embryonic optic nerve of mouse embryos. Moreover, VEGF-C stimulates the proliferation of both early and late oligodendrocyte progenitors in a hypoxia-ischemia model (Bain et al., 2013) whereas VEGF-A does not influence proliferation or differentiation in OPC but strongly promotes OPC migration in a concentration-dependent manner, *in vitro* (Hayakawa et al., 2011). At the same time OPCs signaling influences other neurorepair processes such as angiogenesis by the secretion of molecules responsible for the crosstalk between OPCs and endothelial cells such as matrix metalloproteinases (Pham et al., 2012).

## 2. Endothelial progenitor cells and stroke

Early regenerative strategies followed the discovery of EPCs in 1997 (Asahara et al., 1997). As explained in Section 1.1, EPCs

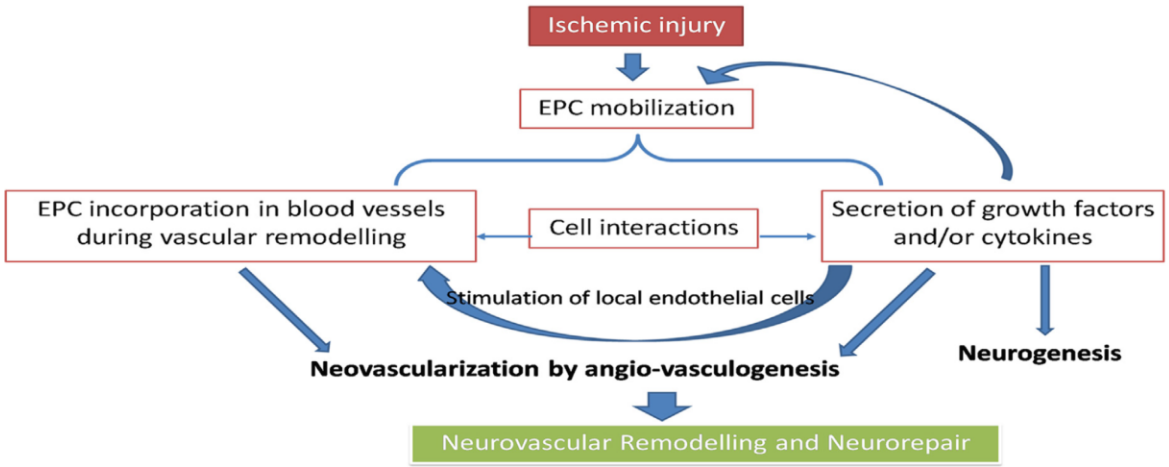


**Fig. 1 – Principal EPCs actions after ischemic stroke. Ischemic injury triggers EPC mobilization immediately after stroke. Mobilized EPCs migrate to the tissue where endothelial damage occurs, and incorporate into vessels during vascular remodeling by directly differentiating to endothelial cells. At the same time, EPCs guide migrating neural progenitor cells or localizing at the sites of injury by secreting several growth factors and/or cytokines. Some of these secreted factors are capable to communicate with brain resident cells such as endothelial cells, astrocytes and pericytes, which contribute to the accomplishment of neurorepair.**

have been viewed as adult stem cells that contribute to neurovascular repair after stroke or neurodegeneration (Fig. 1). Since no single marker has been identified for this type of cells and they are defined as cells expressing both stem cell markers and endothelial cell markers, it is still controversial and challenging to define EPCs. Cell culture and flow cytometry are the two most common approaches applied to isolate or count EPCs (Fadini et al., 2012) and the most widely accepted current definition is by the co-expression of cell-surface markers CD34, CD133 and VEGF receptor-2 (VEGFR-2) (Liman and Endres, 2012), although other progenitor or endothelial markers are being used by different authors.

More and more evidences show that EPCs are present after ischemic stroke since the first study of EPCs mobilization in response to tissue ischemia (Takahashi et al., 1999). The maintenance of endothelial integrity plays a critical role in vascular-related diseases and EPCs have been shown to be incorporated into neovessels (Asahara et al., 1997; Carmeliet, 2003). Taguchi firstly reported that CD34+/CD133+ cells, as a EPCs-enriched population, provided a marker of cerebrovascular function (Taguchi et al., 2004). Following injury, locally damaged tissue induces EPCs modulation in blood and produces a variety of growth factors as well as cytokines that recruit EPCs for neovascularization and tissue repair (Reinisch et al., 2009; Navarro-Sobrino et al., 2010; Massot et al., 2013).

Several studies have proven that endogenous EPCs participate in the neovascularization via C-X-C chemokine receptor type (CXCR) 4/SDF-1 axis after permanent middle cerebral artery occlusion (MCAO) in rats (Mao et al., 2014), and several studies have demonstrated the therapeutic potential of EPCs for the treatment of many ischemic diseases in experimental models. Regarding cerebral ischemia, Ohta and colleagues first demonstrated that the autologous intra-arterial transplantation of early bone marrow-derived EPCs after 90 min of ischemia reduced infarct volume and improved motor function (Ohta et al., 2006). Later on, in a mouse model of transient MCAO, Fan and others showed again that the acute intravenous acute administration of human early EPCs was associated with a reduction in infarct volume and brain atrophy, a reduction in long-term neurological deficits, and an increase in vessel density (Fan et al., 2010). More recently, it was reported for the first time that the administration of outgrowth populations of human EPCs improved neurological function in a rat model of ischemia-reperfusion. This improvement occurred along with an increase in angiogenesis, a decrease in apoptosis in peri-infarct areas and an increase in neurogenesis in the SVZ (Moubarik et al., 2011).

### 2.1. *Neurovascular niche and EPCs secretome*

The so-called neurovascular niche (Ohab et al., 2006; Shen et al., 2004) refers to the factors released by the tissue cells, which create an appropriate niche for neurorepair. This was demonstrated when ischemic mice receiving systemic endostatin to inhibit angiogenesis abolished the normal patterns of neuroblast migration thorough the peri-infarct areas from the subventricular zone (Ohab et al., 2006). In this study the authors elegantly demonstrate that vascular production of SDF-1 and angiopoietin1 (Ang1) promote neuroblast migration to peri-infarct cortex where newly generated neurons expressed CXCR4 or Tie-2 (the receptors for SDF-1 and Ang1, respectively). At the same time other authors have identified proliferating neuroblasts in peri-infarct areas of the human brain in close vicinity to blood vessels (Jin et al., 2006). Moreover, it has been described that the trophic support of endothelial cells to the neural stem cell niche also occurs in non-pathological conditions, where brain endothelial cells secrete soluble factors that maintain CNS stem cell self-renewal and neurogenic potential (Shen et al., 2004), *in vitro*. The crosstalk between endothelium and oligodendrocyte/oligodendrocyte progenitors by nourishing factors released by endothelial cells has also been described in the normal and ischemic brain where TGF -β, BDNF, VEGF or matrix metalloproteinases (MMPs) could be responsible to maintain the oligovascular niche (Miyamoto et al., 2014; Pham et al., 2012).

Could EPCs contribute to the homeostasis of this neurovascular niche after stroke? As mentioned, EPCs enhance post-stroke neurorepair by physical incorporation of the cell into a vascular network under active remodeling but also by the secretion of growth factors or cytokines (Zhang et al., 2002; Rouhl et al., 2008). Several studies demonstrate that beneficial effects of EPCs have been due to paracrine mechanisms that stimulate local endothelial cell mobility, growth and function (He et al., 2004; Gnecchi et al., 2008; Baraniak and McDevitt, 2010; Yang et al., 2010; Rosell et al., 2013). Other studies supporting the therapeutic effect of EPCs secreting factors have reported that cultured cortical neurons exposed to oxygen-glucose deprivation have less axon degeneration when treated with EPCs-conditioned media (Park et al., 2013). Rehaman demonstrated that acetylated LDL (+) ulex-lectin (+) cells secrete angiogenic growth factors such as VEGF, HGF, GSCF, and GM-CSF with pro-angiogenic effects (Rehman et al., 2003). It is also known that cultured EPCs secrete a number of cytokines that could stimulate proliferation, migration, and survival of endothelial cells, including VEGF and FGF, which could enhance the expression and enzymatic activity of endothelial NO synthase (eNOS) (He et al., 2004). In this study He and colleagues demonstrate that neurotrophic proteins like BDNF and leukemia inhibitory factor (LIF), secreted by EPCs, play important roles in the dynamic interaction between vascular endothelium and neuronal tissue. EPCs also release thrombo-inflammatory mediators and chemokines, such as IL-8, monocyte chemoattractant protein (MCP)-1 and regulated upon activation normal T cell expressed and secreted (RANTES) (Zhang et al., 2004). A total of 82 proteins were identified in the conditioned medium of human EPCs with proteomic characterization, including the alternative macrophage markers chemokine (C–C motif) (CCL)-18, platelet factor (CXCL4) and platelet basic chemokine (CXCL7) with "platelet $\alpha$ granule" (Urbich et al., 2011). Similarly, several cytokines secreted from cultured endothelial colony-forming cells ("late" populations of EPCs) have been identified such as BDNF, MCP-1, Ang-2, growth-regulated oncogene-α (GRO-α), neurotrophic growth factor (NGF), epidermal growth factor (EGF), insulin growth factor (IGF)-1, platelet-derived growth factor homodimer BB (PDGF-BB) and tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) as well as MMP-2 and MMP-9. Navarro-Sobrino found that EPCs from subacute stroke patients expressed more mRNA of HGF and VEGF (which are crucial proteins for angiogenesis)

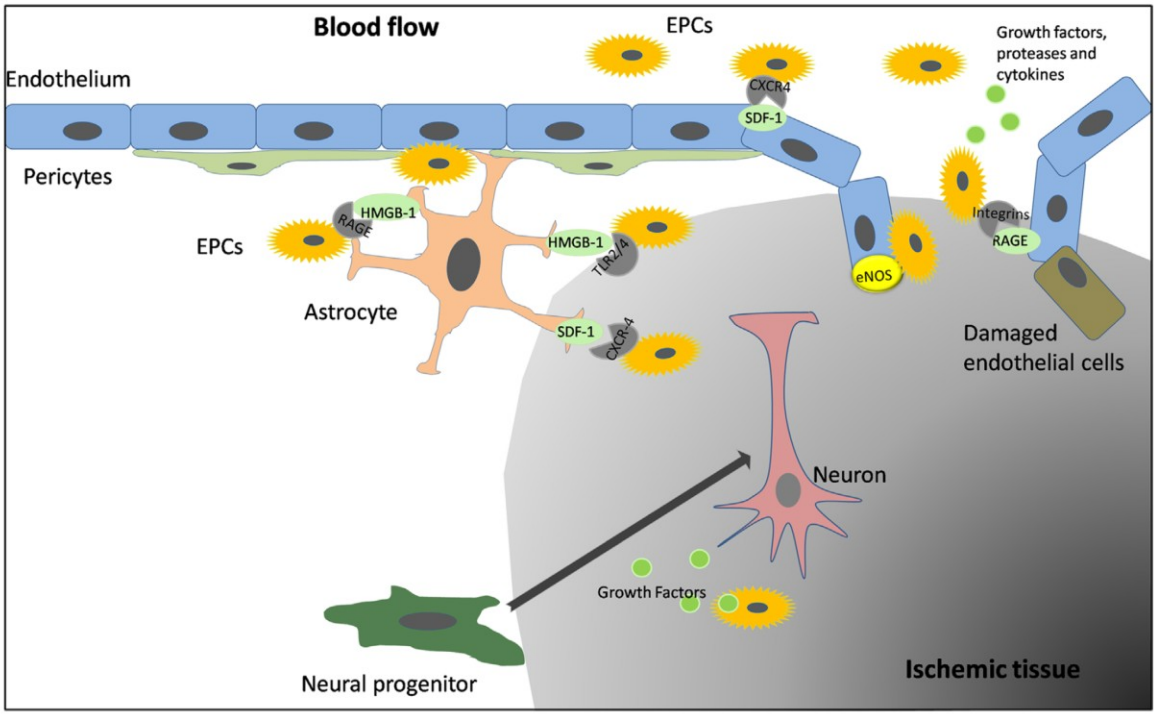


**Fig. 2 – Interactions between EPCs and brain tissue cells after ischemia. The figure represents the most relevant cell–cell interactions involving EPCs in the ischemic brain as detailed in the text. Some of these interactions are with endothelial cells thorough integrins or with astrocytes thorough HMGB1 release. At the same time, endothelial cells from peri-infarct vessels or astrocytes can guide EPCs transendothelial migration via CXCR4/SDF-1 signal pathway since EPCs highly express CXCR4. There is also a crosstalk between EPCs and astrocytes also by the actions of HGMB-1. EPCs secreted factors also serve as mediators for maturation of neuronal progenitors at the sites of injury.**

compared to acute stroke patients (Navarro-Sobrino et al., 2010). Other authors have reported that conditioned medium from EPCs support endothelial cells proliferation but does not sustain their adhesion *in vitro* (Burlacu et al., 2013), that magnetized (with iron oxides) human and mouse EPCs secrete VEGF, FGF and HGF (Carenza et al., 2014) or the secretion of soluble VEGFR-1 and VEGFR-2 from cultured EPCs (Buttler et al., 2014).

## 3. EPCs–cell interactions after stroke

Several types of brain resident cells may work together to maintain, remodel and repair the brain functions and dynamic interactions between these cells leading multiple processes after stroke. One example is the neurovascular unit which refers to a complex network of interactions and communication including neurons, astrocytes, microglia and endothelial cells but also the extracellular space (Lo and Rosenberg, 2009). Hirase and colleagues also proposed a similar concept of neuronal–glial–vascular interaction and communication working unit (Hirase, 2005; Zonta et al., 2002; Mulligan and MacVicar, 2004). Thus, interactions between EPCs and brain resident cells will probably be of great importance and should be included in a new working unit, Fig. 2.

Brain endothelial cells, central components of vascular system, are responsible for the selective transport of molecules, cells and releasing soluble factors to nourish neighboring cells of the brain parenchyma (Weiss et al., 2009; Dugas et al., 2008; Guo et al., 2008). The re-endothelization of blood vessels is regarded as an early event that results from the very onset of vascular disorders, including stroke. Early observations suggested that EPCs contributed to neovascularization by differentiating into cells forming structural components of the vasculature (Asahara et al., 1997; Takahashi et al., 1999; Orlic et al., 2001). However the paracrine hypothesis that EPCs contribute to vascular growth primarily by secreting proteins and recruiting additional progenitor cells to the injury site has become more acceptable (Zhang et al., 2002; Rehman et al., 2003; Yang et al., 2010). Interesting examples of the interactions of EPCs with endothelial cells have been reported in an *in vitro* study by blocking receptor for advanced glycation end products (RAGE) on endothelial cells significantly decreased EPCs-endothelial adherence while the presence of reactive astrocytes significantly promoted adherence between EPCs and ECs, involving the release of soluble high-mobility group box 1 (HMGB1) from astrocytes (Hayakawa et al., 2014). Other examples are the localization of EPCs residing at the edge of brain vessel wall and the predominant expression of SDF-1 in co-localization with CD-31 positive cells within the vessel wall in arteriovenous malformations (Gao et al., 2010). SDF-1 has been also proved to be expressed in endothelial cells after ischemia, while the majority of cultured EPCs could express CXCR4 inducing EPCs migrating to the injured site via SDF-1/CXCR4 signal pathway (Fan et al., 2010). More recently Di Santo (2014) demonstrated that conditioned

medium from EPCs enhance the viability, migration as well as tubular network formation of endothelial cells derived from rat brain which might be explained by the factors secreted from hypoxic EPCs. Regarding astrocytes, it has been described that in brain tissue, the interaction of brain microvascular endothelial cells with astrocytes (and pericytes) might be important to induce BBB characteristics and to suppress endothelial proliferation: astrocytes reduced the number of vessel-like structures but increased diameters and length, whereas pericytes presented the opposite effect (Al Ahmad et al., 2011). Fan demonstrated that SDF-1 is mainly expressed by activated astrocytes after transient MCAO, which induced EPCs migration to the ischemic regions (Fan et al., 2010). And as mentioned before, to investigate astrocyte–EPCs crosstalk, Hayakawa used an *in vitro* cell culture system to prove that astrocyte-conditioned medium significantly increased EPCs proliferation via HMGB-1-RAGE signaling and provided strong proofs of this crosstalk in a mouse model of stroke (Hayakawa et al., 2012, 2013), Fig. 2. Others previously reported that HMGB-1 is released by activated astrocytes (Passalacqua et al., 1998) and receptors for HMGB-1, such as Toll-like receptors (TLR2/4) and RAGE, are found to be expressed by EPCs (He et al., 2010).

Mesenchymal stem cells (MSCs), also known as bone marrow-derived stromal cells, are multipotent stromal cells that can differentiate into a variety of cell types. These cells also secrete a large amount of factors which contribute to collateral remodeling though paracrine mechanisms (Kinnaird et al., 2004; Shi et al., 2012). It is known that the cross-talk between EPCs and MSCs occurs through both paracrine and direct cell contact mechanisms leading to modulation of the angiogenic response. The therapeutic effect of MSCs in stroke models has already been documented (Honmou et al., 2012; Chen et al., 2014; Buttler et al., 2014). Both EPCs and MSCs are present at sites of injured tissue where vascular and nervous tissue repair are necessary, and they are likely to establish direct cell contact under these conditions. In this regard, some studies have shown that combined seeding of EPCs and MSCs resulted in a higher degree of host-derived neovascularization (Lemischka and Moore, 2003; Ball et al., 2004). One proof of the cell–cell interaction between these progenitor/stem cells is that differentiation of MSCs towards pericyte-like phenotype cells (as part of newly formed capillary-like structures) is enhanced when they are co-cultured with EPCs (Loibl et al., 2014). The interaction between MSCs and EPCs has been also proved in other research areas such as bone regeneration, demonstrating that blood-derived EPCs contribute to osteogenic differentiation of MSCs *in vitro* with direct MSCs support to proliferation and vascular network formation of EPCs and that, *in vivo*, more EPCs are found in blood vessel networks in the presence of MSCs (Fedorovich et al., 2010). Other studies have shown that, *in vitro*, tube formation was greatly enhanced when MSCs and EPCs were co-cultured whereas cell proliferation was significantly reduced in those conditions accompanied by an up-regulation of angiogenic markers (Aguirre et al., 2010). *In vivo*, MSCs provide an angiogenic environment by producing growth and differentiation factors such as VEGF-A, HGF, MMP-9 and MMP-3, and induce blood vessel formation when co-injected with EPCs in mice (Lin et al., 2012) or by secreting cytokines, such as IL-6 and IL-8 (Locksley et al., 2001). Another study has recently proved that the conditioned medium derived from tumor necrosis factor (TNF)-α-treated MSCs induced migration of human cord blood-derived EPCs through IL-6- and IL-8-dependent mechanisms and, *in vivo*, enhanced the homing of injected EPCs (Kwon et al., 2013). Regarding VEGF-A, it is known that MSCs release high amounts of VEGF-A, whereas EPCs produce soluble VEGF receptors in co-cultures of MSCs and EPCs (Buttler et al., 2014). Finally, the addition of either EPCs or SDF-1α to MSC-based constructs presented more luminal vessel-like structures as well as more complex and interconnected vessel networks (Eman et al., 2014).

## 4. Molecules for EPCs signaling

The key of EPCs-therapy could be that on one hand they directly participate into the development of vascular remodeling but on the other hand they enhance the mobilization and differentiation of local progenitors by some secreted and nourishing factors (Fig. 1). The underlying mechanisms of EPCs signaling and potential mediators continue to be investigated. Beneficial effects of EPCs have been described to occur due to paracrine mechanisms that promote the angiogenic response of brain microvascular endothelial cells (Gnecchi et al., 2008; Yang et al., 2010) at the same time that dozens of cytokines have been identified in EPCs secretome (Rosell et al., 2013; Urbich et al., 2005; Di Santo et al., 2014; Navarro-Sobrino et al., 2013). In fact, the role of some of these secreted factors has been clearly demonstrated by several authors in vascular remodeling, but also in other neurorepair processes. For example, VEGF is a crucial regulator of vascular survival and angiogenesis but can also stimulate the migration and proliferation of new neurons (Hansen et al., 2008). Factors secreted from EPCs into the medium (such as VEGF and EGF), can activate AKT and ERK pathways which are pivotal signaling steps for angiogenesis (Dimmeler and Zeiher, 2000; Wu et al., 2000). EPCs-derived conditioned medium induces activation of PI3K and ERK pathway in endothelial cells derived from rat brain and enhances cell migration and tubular structures formation on Matrigel matrix (Di Santo et al., 2014). Importantly, stroke treatment with intravenous EPCs conditioned medium increases the density of vessels in peri-infarct areas as much as treatment with cells, in a mouse model of cortical ischemia (Rosell et al., 2013). And we have learned from multiple studies that after the administration of thousands or millions of cells in rodents only a very small portion of these are found in the brain, despite the positive effects observed in terms of vascular remodeling and neurorepair (Rosell et al., 2013; Fan et al., 2010; Moubarik et al., 2011), pointing out that other actions must be orchestrating vascular remodeling.

Hypoxia-inducible factor (HIF) is the most common known transcription factor expressed in acute and chronic response to hypoxia and the activation of the HIF pathway is generally considered to be neuroprotective. HIF-1α is the major direct regulator of endothelial cell function and its over-expression in HIF-1α-transfected EPCs promoted EPCs differentiation, proliferation and migration in a model of hindlimb ischemia. In addition, HIF-1α-transfected EPCs presented higher revascularization potential as increased capillary density was

observed at the injury site (Jiang et al., 2008). Other authors have recently reported that EPCs mobilization and recruitment could be also mediated by hypoxic gradients via HIF-1-induced expression of VEGF (Paczkowska et al., 2013).

EPCs homing is an active process involving direct interaction between molecular targets expressed on homing tissues and adhesion molecules, namely integrins, expressed by EPCs (Vajkoczy et al., 2003). One of the major integrin subunits regulating EPCs homing to active angiogenic sites is the β2 integrin, a leukocyte specific receptor (Harris et al., 2000). First studies showed that β2-integrins mediate the adhesive interactions of EPCs to mature endothelial cells and to extracellular matrix proteins, being critical for chemokine-induced transendothelial migration of EPCs *in vitro*, thus regulating the *in vivo* homing and vascular integration of progenitor cells to sites of ischemia (Chavakis et al., 2005). Similar findings were also reported after blocking β2-integrin, *in vitro* (Hayakawa et al., 2014). α4β1, α5β1 and α6β1 integrins are also reported to increase EPCs mobilization and homing to vascular injury sites and thus promoting re-endothelization of these sites (Watson et al., 2010; Jin et al., 2006; Qian et al., 2006). CXCL12 (also known as SDF-1), CXCL1, VEGF and recently discovered macrophage migration inhibitory factor (MIF) promote EPCs adhesion *in vitro*. Essentially all factors promote EPCs chemotactic migration and transmigration through endothelial layers *in vitro* (Kanzler et al., 2013). There is also substantial literature demonstrating that SDF-1/CXCR4 contributes to the migration of different cells, including EPCs. CXCR4, a unique receptor of SDF-1, is highly expressed in EPCs (Fig. 2). SDF-1 is reported to mediate EPCs homing to brain ischemic regions and further inducing subsequent angiogenesis and vasculogenesis via a VEGF/eNOS-related pathway (Hill et al., 2004; Hiasa et al., 2004). In a transient MCAO model in mice, SDF-1-mediated signaling was determined to play a critical role in EPCs-mediated neuroprotection (Fan et al., 2010) and binding of CXCR4 with SDF-1α activates the PI3K/Akt/eNOS signaling pathway and decreased EPCs apoptosis. Other authors have shown that pre-treatment of EPCs with SDF-1 increased their pro-angiogenic potential by up-regulating the expression of integrins and enhancing EPCs adhesion to activated endothelium (Zemmani et al., 2008). Another central molecule, HMGB1, an important pro-inflammatory cytokine released by most eukaryotic cells during injuries including stroke, it is known to increase the expression of vascular cell-adhesion molecule 1 (VCAM-1) and endothelial-cell selectin (Lotze and DeMarco, 2003). Astrocytic-HMGB1 was found to promote EPCs adherence by up-regulating the receptor for RAGE via early growth response protein 1 (Egr1) signaling on endothelial cells and enhance EPCs transmigration across brain endothelial monolayers after adherence (Hayakawa et al., 2010, 2014), Fig. 2. HMGB1 up-regulation in post-ischemic brain could promote neurovascular remodeling during stroke recovery by modulating paracrine function of human peripheral blood derived-EPCs (Hayakawa et al., 2012). Additionally, beneficial effects of EPCs transplantation in ischemic mice, including enhanced neovascularization, were abolished when HMGB1 inhibitor glycyrrhizin was intraperitoneally injected (Chen et al., 2014).

Another family of molecules that regulate EPC function is the matrix metalloproteinases (MMPs) since MMPs are key players in the degradation of the basal membrane, one of the earliest steps that occur during new vessel formation (Fig. 2). For example it has been reported that angiogenic responses of EPCs were impaired in the absence of MMP-9 in different models of ischemia (Morancho et al., 2013; Huang et al., 2009; Johnson et al., 2004). Regarding stroke, Morancho and colleagues have reported that in MMP-9 deficient mice the number of spleen-derived EPCs cell-culture yields was decreased and the abilities of EPCs derived from those mice were severely compromised when shaping vascular-like networks intubulogenesis assays. These abilities could not be restored by adding exogenous recombinant MMP-9 protein (Morancho et al., 2013). Nonetheless, the role of other MMPs in EPCs function and signaling is still unknown.

## 5. Conclusions

In conclusion, mobilized or therapeutically-administered EPCs contribute to re-vascularization and neurorepair after cerebral ischemia. In this context EPCs secretome mediates multiple cell–cell interactions playing a pivotal role to achieve brain remodeling after stroke. These cell–cell interactions connect post-stroke angiogenesis, neurogenesis and oligodendrogenesis in a whole neurorepair niche, in which other types of cells such as neurons, astrocytes, endothelial cells, pericytes and neural progenitor cells are comprised. With these background we envision that EPCs-secreted paracrine factors will be crucial in future research in the field to further improve EPCs-based therapeutic approaches and its translation into the clinical practice by optimizing new therapies for stroke patients.

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**Article 2**

**Matrix Metalloproteinase-13 Controls Neuroprotection and Neurorepair after Cerebral Cortical Ischemia in Mice**

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# Matrix Metalloproteinase-13 Controls Neuroprotection and Neurorepair after Cerebral Ischemia in Mice

Feifei Ma, Pablo Martínez-San Segundo, Verónica Barceló, Anna Morancho, Marina Gabriel-Salazar, Dolors Giralt, Joan Montaner, Anna Rosell

Neurovascular Research Laboratory and Neuroscience Department, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Spain. Passeig Vall d'Hebron 119-129, 08035, Barcelona, Spain

**Corresponding Author:**

Anna Rosell, anna.rosell@vhir.org

Neurovascular Research Laboratory, Vall d'Hebron Research Institute, Passeig Vall d'Hebron 119-129, 08035, Barcelona, Spain. Tel. +34 934894029

**ABSTRACT**

New neuroreparative and neuroprotective therapies are being sought to treat stroke patients. One approach is the remodeling of extracellular matrix, which participates in both brain injury and neurovascular repair when matrix metalloproteinases (MMPs) are thought to be key players. Our aim was to investigate the role of MMP-13 (collagenase-3) in the acute (24 hours and 3 days) and delayed (2 weeks) phases of stroke. Permanent and transient cerebral ischemia models involving the cortex were induced in MMP-13 knock-out (KO) and wild-type (WT) mice. In the transient model, MMP-13 deficiency reduced the amount of TTC-stained infarct tissue, reduced hemorrhagic events and improved functional outcomes ($p$<0.01). At two weeks, normal neuroblast (DCX+) migration from the subventricular zone toward the peri-infarct area was observed. However, MMP-13 deficiency significantly reduced the number of newborn neuroblasts (DCX+/BrdU+) in the cortical peri-infarct area ($p$<0.01). This result occurred in parallel with aberrant cortical vascular remodeling: post-stroke peri-infarct vessel density increased in the WT mice ($p$<0.01) but this increase was blocked in the MMP-13 KO mice. Prior to these vascular alterations, the levels of pro-angiogenic factors, including G-CSF, VEGF-A and angiopoietin-2, were lower in the ischemic cortex of MMP-13 KO mice than in WT mice ($p$<0.05). In vitro, gene-silencing of MMP-13 in endothelial progenitor cells (EPCs) confirmed the reduced ability of these cells to build tubulogenic networks in Matrigel™ substrate. Together, our results indicate that MMP-13 is a central protease in infarct development and cortical remodeling during post-stroke neurorepair, which is critical for optimal angiogenic and neurogenic responses.

**KEY WORDS:** cerebral ischemia, MMP-13, angiogenesis, neurorepair, neurogenesis

## INTRODUCTION

Stroke is one of the most common causes of death and disability worldwide. Our investigation focused on ischemic stroke, which occurs when blood flow into the brain is interrupted by an artery occlusion. Recombinant tissue plasminogen activator (rtPA) and mechanical thrombectomy interventions are the only therapies that have shown efficacy, but they must be administered within a short therapeutic time window (Hacke et al., 2008; Lees et al., 2010, Jovin et al., 2015). New post-stroke therapies are therefore needed to diminish damaging molecular events and cellular death, which are responsible for further damage, at the same time that new opportunities to recover tissue are investigated. Of special interest are treatments that induce neurological repair with the aim of improving functional outcomes and rescuing the limited capability the damaged brain has to self-recover, with the potential to be administered in a wider therapeutic time window (Zhang and Chopp, 2009). One approach to potentiating neurological repair is to target angioneurogenesis, which is the formation of new vessels and neural cells. Adult neurogenesis occurs primarily in the subventricular zone (SVZ) and the subgranular zone (SGZ), from which newly born neural cells migrate to the olfactory bulb or to the dentate gyrus under physiological conditions (Ming and Song, 2011). However, ischemic stroke alters this pattern of neurogenesis and stimulates neuronal cells to migrate toward the damaged area (Ohab and Carmichael, 2008). In this environment, the neurovascular niche has been described as linking angiogenesis and neurogenesis, demonstrating that, after stroke, neurogenesis occurs following angiogenesis in the peri-infarct areas and showing strong support for the idea that trophic factors are secreted by endothelial cells (Ohab et al., 2006) and glial cells (Hayakawa et al., 2014).

In this context, extracellular matrix remodeling is a key step in angiogenesis and neurogenesis during which matrix degradation is required to allow cell migration and capillary reorganization (Bovetti et al., 2007; Senger and Davis, 2011). Matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes, are involved in the regulation of the composition of the cell-matrix. Interestingly, some MMPs have been described not only as the focus of many neuroprotective pre-clinical therapies aimed at protecting the brain during the acute phase of stroke but also as playing multiphasic roles, including roles in neurovascular remodeling during neurological recovery in rodent models of stroke (Zhao et al., 2006; Rosell and Lo, 2008). Among all the MMPs, the role of MMP-13 (also named collagenase-3) in neurorepair and vascular remodeling is unknown. As a major collagenase, MMP-13 mainly cleaves collagen II, shows the highest gelatinase activity among the collagenases (Knäuper et al., 1996; 1997) and has a central position in the activation of other MMPs, through which it plays a key role in angiogenesis and the re-epithelialization of the skin during wound healing (Hattori et al., 2009). In the context of cerebral ischemia, increased expression of MMP-13 has been described in rat neurons, where it peaks on day 7 after ischemia (Nagel et al., 2005). Previous results from our group showed that high blood levels of MMP-13 were associated with lesion expansion in the acute phase of stroke (Rosell et al., 2005), whereas nuclear MMP-13 was found to be expressed in neurons and glial supporting cells in human and rodent ischemic brain tissue (Cuadrado et al., 2009a). In the present study, we

aimed to determine whether MMP-13 could acutely modulate neuroprotective and neurological repair functions during the recovery phase of ischemia, and we focused our interest on post-stroke vascular remodeling. For this purpose, both permanent and transient ischemic stroke models were induced in MMP-13 deficient mice, demonstrating that MMP-13 is involved in acute infarct expansion at the same time that it is required for neurorepair during recovery. To further study the role of MMP-13 in vascular remodeling, MMP-13 mRNA expression was transiently silenced in outgrowing populations of endothelial progenitor cells (EPCs), confirming the role of this collagenase in tubulogenesis assays. Our data suggest that MMP-13 is a key protease that can be therapeutically modulated during both the acute and the recovery phases of stroke.

## MATERIALS AND METHODS

### Animals

Age-matched 8- to 15-week-old male MMP-13 knockout mice (MMP-13 KO in a C57BL/6 background) and wild-type (WT, C57BL/6) were bred in-house in a temperature and humidity controlled room and maintained on a 12 hour light/dark cycle. The offspring were used for these experiments. The founder mice for our WT and KO colonies were the kind gifts of Dr. Lopez-Otin and were generated as previously described (Inada et al., 2004) and crossed into congenic C57BL/6 mice for several generations. Genotyping analyses were performed to confirm that the mice were MMP-13 null, as described below. A total of 99 animals (49 WT and 50 MMP-13 KO) were used in the present study, and no mortality was observed during the study period. All procedures were approved by the Ethics Committee of Animal Experimentation (CCEA 67/11) of the Vall d'Hebron Research Institute and were conducted in compliance with Spanish legislation and in accordance with the Directives of the European Union.

### Genotyping analysis

Freshly obtained mouse tail tissue was used for DNA purification 116 to confirm the genotypes of randomly selected mice in our colonies. Genomic DNA was collected and purified using a Gentra Puregene Mouse Tail Kit (Qiagen, Germany) according to a protocol provided by the manufacturer. Polymerase chain reaction was performed using Platinum® Taq DNA polymerase (Life Technologies, USA). The following pair of Exon primers were used to genotype the wild-type mice: (Exon 5F) 5´TTTATTGTTGCTGCCCATGAG3´ and (Exon 6R) 5´AGTTTCTCCTCGGAGACTGGT3´. For the MMP-13 KO mice, a primer for the neomycin resistance gene, (neo) 5´GACCCACCCCTTCCCAGCCTCT3´, was used as the oligonucleotide primer. The approximate fragment sizes of each band were 1300 Kb for the wild-type and 1485 Kb for the MMP-13 KO on agarose gels.

### Focal cerebral ischemia models (MCAo)

The mice were given free access to food and water before surgery. Permanent electrocoagulation and transient compression of the distal part of the middle cerebral artery (MCA) were performed as previously described by our group (Morancho et al., 2012). Briefly, the animals were anesthetized using 5% isoflurane followed by 1.5 - 2% isoflurane for maintenance anesthesia via a facemask in medical air (79% $N_2$/21% $O_2$; Abbott Laboratories, Madrid, Spain).

A small craniotomy was performed in the left temporal bone (between the retro-orbital and ear area) to expose the MCA. This artery was compressed for 60 minutes with a 30-G needle using a micromanipulator to perform transient MCA occlusion (tMCAo) or electrocauterized for permanent MCA occlusion (pMCAo). A heating pad connected to a rectal probe was used to maintain body temperature between 36.5-37.5°C during all surgical procedures. Cerebral blood flow (CBF) was monitored using laser-Doppler flowmetry (Moor Instruments, UK) to ensure appropriate occlusion in both models and reperfusion in the tMCAo mice (Morancho et al., 2012). Only the mice that showed a reduction in CBF to below 80% and subsequent reperfusion to above 75% of the baseline CBF were included in this study. Buprenorphine (0.05-0.1 mg/kg), an analgesic, was administered subcutaneously immediately after the procedure. The animals in the sham group (n=6 for each genotype) were subjected to the same procedures (including 60 minutes of anesthesia for the tMCAo mice), except for electrocauterization or compression of the MCA.

A total of 17 mice were subjected to pMCAo (8 WT and 9 MMP-13 KO), 70 to tMCAo (35 WT and 35 MMP-13 KO) and 12 to sham surgery (6 WT and 6 MMP-13 KO), according to the design of the study, as presented in Figure 1.

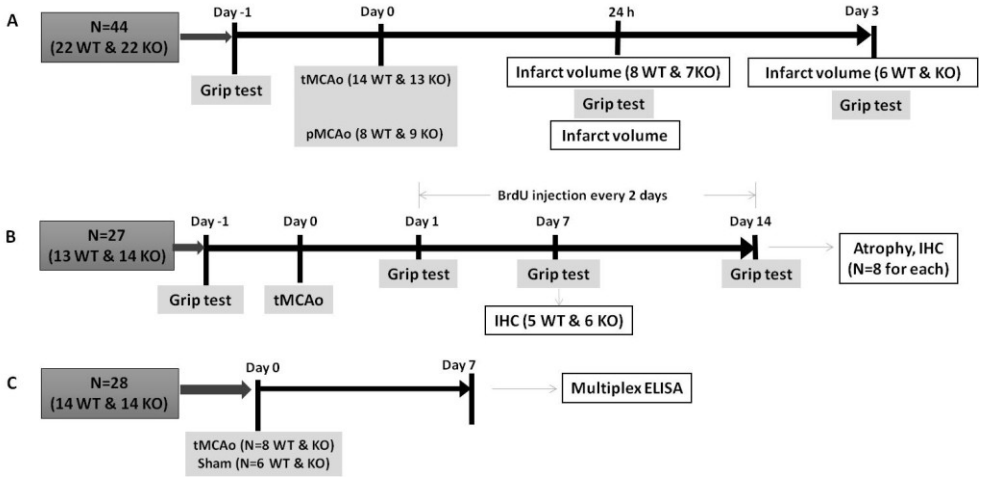


**Figure 1. The study design.** Three experimental protocols (***A***, ***B*** and ***C***) were followed to study the role of MMP-13 in neuroprotection and neurological repairs after cerebral ischemia.

**Grip strength neurological test**

The grip strength test is designed to assess the peak forelimb force executed by a mouse when the mouse releases its front paws from a grid. A computerized grip strength meter (Harvard Apparatus, USA) was used. The specific protocol is described elsewhere (Rosell et al., 2013b). A total of 6 trials were conducted for each test, and the mean value was calculated as the average force used at one time point. This test was conducted blindly by a trained researcher before the surgery (pre-surgery) and then repeated at 24 hours, 3days, 7 days and 14 days post-ischemia (according to the study groups, A and B, as detailed in Figure 1).

**Infarct volume and cortical atrophy**

The mice that were subjected to 60 minutes of

occlusion (tMCAo) and the mice in the pMCAo group that were included in the infarct volume protocol (study A in Figure 1) were transcardially perfused with ice-cold saline and euthanized at 24 hours or 3 days after ischemia. The dissected brains were cut into 1 mm-thick coronal sections and then stained with 2.5% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Sigma-Aldrich, USA) for 15 minutes at room temperature. The slices were post-fixed in paraformaldehyde (PFA, 4%) and digitalized to obtain the measurements. The extension of the pale colored ischemic lesion was calculated blindly, and the results are presented as the percentage of the infarct area (as a % of the ipsilateral hemisphere area) or as the corrected infarct volume ($mm^3$, corrected for edema), as described previously (Morancho et al., 2012). Cortical atrophy was evaluated using cresyl violet staining at 14 days after tMCAo (as shown in study B in Figure 1). Briefly, the mice were deeply anesthetized and then euthanized after the brain was perfused with ice-cold 4% PFA. The brains were harvested and post-fixed in PFA overnight at 4°C, followed by cryoprotection in 30% sucrose in PBS until the brains sank. Afterward, the brains were embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Fisher Scientific, USA) and stored at -80°C until used. Twelve-µm-thick coronal sections including the lateral ventricles and the hippocampus were collected. Two sections, from bregma +0.02 and bregma -1.70, were selected per animal from the WT and MMP-13 KO ischemic mice. The sections were stained with 0.1% freshly filtered cresyl violet containing 0.2% acetate and 0.15% acetic acid for 15 minutes at 37°C. The areas of the ischemic cortex and the area of the ipsilateral hemisphere were measured blindly and the results are presented as cortex percentage *vs.* ipsilateral hemisphere.

**Immunohistochemistry (IHC)**

The ischemic animals in protocol B received 5-Bromo-2'-deoxyuridine (BrdU, 50 mg/kg in saline, B9285, Sigma-Aldrich, USA) intraperitoneally every 2 days after ischemia. On day 7 or day 14, the animals were euthanized, and the brain sections were prepared for immunohistochemistry as described for the atrophy study. Later, the sections were fixed in ice-cold acetone and then blocked in 0.1% PBS-tween containing 1% BSA and 10% goat serum after acid denaturation with 2 M HCl PBS for 1 hour. Slides were incubated with the following primary antibodies overnight at 4°C (DCX) or for 3 hours at room temperature (BrdU or NeuN): rabbit anti-doublecortin antibodies (DCX, 1:1000, ab18723, Abcam, UK), rat anti-BrdU antibodies (BrdU, 1:100, ab6326, Abcam, UK) and anti-NeuN antibodies conjugated to Alexa Fluor® 488 (NeuN, 1:200, MAB377X, Merck Millipore, Germany). Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 647 goat anti-rat IgG (H+L) (1:500, A-1108 & A-21247, Invitrogen, USA) were the secondary antibodies, which were applied, where required, at room temperature for 1 hour. The samples were then mounted in VectashieldTM with DAPI (H-1200, Vector Laboratories, USA) to stain the cell nuclei.

To determine the number of neuroblasts migrating from the SVZ to the peri-infarct tissue, the DCX+/DAPI+ -labeled area in the SVZ and corpus callosum (CC) regions of one slide was quantified. In addition, triple-labeled cells (DCX+/BrdU+/DAPI+), which indicated the new proliferating neuroblasts, were also quantified in the SVZ and peri-infarct areas (three selected regions of interest (ROIs) in the infarct boundary area). The images were obtained at 400X

magnification using a confocal laser scanning biological microscope (FV1000, Olympus, Japan) and analyzed using FV10-ASW software. Finally, the mature (NeuN+/DAPI+) neurons in the peri-infarct cortical areas adjacent to the damaged tissue were imaged at 100X magnification and counted using ImageJ software (NIH, USA). The results are presented as the neurons per area. All analyses were conducted by a researcher who was blinded to the genotype and group. To study angiogenesis, the mice from study B (see Figure 1) were injected intravenously (retro-orbitally) with Dylight 594-labeled tomato lectin (80 µg/mouse, DL-1177, Vector Laboratories, USA) 10 minutes before euthanized. Two similar isotopically oriented slides per animal were selected from the brain sections. One image from each section that contained the infarct boundary area was randomly imaged at 100X magnification. Angiogenesis was evaluated as the lectin-positive blood vessel density in the selected ROIs and the corresponding contralateral areas. The total area of the lectin-positive perfused vessels was calculated using ImageJ software (NIH, USA) by an investigator blinded to the genotype group.

**Multiplex ELISA**

Both the WT and the MMP-13 KO mice (see study protocol C in Figure 1) were transcardially perfused with ice-cold saline and euthanized at 7 days after ischemia. The mouse brain tissue obtained from both the ipsilateral and contralateral cortex was homogenized with freshly prepared ice-cold lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, 0.05% BRIJ-35, 0.02% NaN3, 1% Triton X-100, 1% phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, Switzerland) and 0.5% aprotinin (Sigma-Aldrich, USA). Cortical lysates were collected from the supernatant after centrifugation at 12,000 rpm for 12 minutes at 4°C. A MILLIPLEX® map mouse angiogenesis/growth factor magnetic bead panel (MAGPMAG, EMD Millipore, Germany) was used to detect angiogenesis-related proteins at day 7 after ischemia. Briefly, 25 µl of the diluted lysates (1:2 in assay buffer) was loaded into the sample wells to quantify the levels of angiopoietin-2, granulocyte colony stimulating factor (G-CSF), amphiregulin, fibroblast growth factor (FGF)-2, vascular endothelial growth factor-A (VEGF-A) and stromal cell-derived factor- 1α (SDF-1), according to the manufacturer's instructions. The assay sensitivity for the different proteins was as follows: angiopoietin-2, 5.2 pg/ml; G-CSF, 0.8 pg/ml; amphiregulin, 2.2 pg/ml; FGF-2, 10.6 pg/ml; VEGF-A, 0.7 pg/ml and SDF-1, 24.6 pg/ml. Each sample was assayed in duplicate, and the coefficients of variation were <20%. The results are presented as the ratio of the amount of protein in the ipsilateral cortex to the amount of protein in the contralateral cortex. To evaluate the potential MMP protein imbalance in a MMP-13 knockdown, a MILLIPLEX® map mouse MMP magnetic bead panel 3 (MMMP-3 MAG, EMD Millipore, Germany) was run to detect the expression of MMP-3, MMP-8 and MMP-9 in brain homogenates obtained at 3 days after reperfusion (tissues were homogenized right after TTC staining), as described above. In brief, 25 µl of the brain homogenate (1:10 in lysis buffer) was loaded into the sample wells. The assay sensitivity of the different MMPs was MMP-3, 2.5 pg/ml; MMP-8, 3.4 pg/ml and MMP-9, 3.1 pg/ml. Each sample was assayed in duplicate, and the coefficients of variation were <20%.

**EPC cultures and MMP-13 silencing**

Outgrowth endothelial cells (OECs) with an endothelial-like phenotype and a highly proliferative nature were obtained from FVB wild-type male mice spleens for a previous study (Morancho et al., 2013) and stored in liquid nitrogen until use. These cells behave as competent endothelial cells both *in vitro* and *in vivo* (Fadini et al., 2012). MMP-13 gene silencing was achieved using delivery of passive siRNA via Accell SMART pool siRNA (Thermo Scientific, USA) according to the manufacturer's protocol. Briefly, $2\times10^4$ EPCs were seeded in 48-well plates and treated separately after 24 h with the respective siRNA solutions corresponding to each siRNA pool (2 µmol/L). Non-targeting/non-coding siRNA was used as a negative control to measure the minimal toxicity and side effects of the assay. Cyclophilin B (PPIB) siRNA was used as a positive control to test the efficiency and specificity of MMP-13 silencing. After 48 hours of treatment, the cells were thoroughly washed with PBS, trypsinized and collected in basal EBM media (Lonza, Spain) to perform Matrigel assays, which are described below. To confirm the mRNA expression in each condition, real-time PCR was conducted using the mRNA that was obtained from Matrigel-disaggregated cells, which were isolated using the Cell Recovery Solution (BD Biosciences, USA) for 60 minutes at 4°C. Briefly, an RNeasy® Minikit (Qiagen, Germany) was used, according to the manufacturer's instructions, and the quantity and quality of the RNA was measured using a Nanodrop Spectrophotometer and retrotranscribed as cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The incubation conditions for the retrotranscription were: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Finally, the samples were incubated at 4°C until use or frozen at -80°C (Thermal Cycler 2720, Applied Biosystems, USA). We included a negative control (RNase-free water) for the reverse transcription to cDNA to detect possible contamination. RT-PCR was performed by mixing 5 µl of Taq Man 279 Universal 2X PCR Master Mix (Applied Biosystems, USA), 0.5 µl of the Taq Man® Gene Expression Assay solution (PPIB: Mm 04208118_gl, GAPDH: Mm99999915_g1, MMP-13: Mm00439491_m1; Applied Biosystems, USA), 3.5 µl of RNase-free water and 1 µl of the sample (cDNA or RNase-free water). These experiments were performed in triplicate. A sample calibrator was used to compare the samples from different reading plates. Hybridizations and expression analyses were performed using a 7900 thermocycler HT Fast Real-Time PCR System (Applied Biosystems, USA), and the results were analyzed using SDS 2.3 software.

### *In vitro* tubulogenesis

To assess the angiogenic and/or vasculogenic ability of MMP-13 silenced cells, MatrigelTM matrix (BD Biosciences, USA) was used for *in vitro* tube formation assays (also named tubulogenesis). The experimental groups consisted of MMP-13-silenced OECs, non-targeting/non-coding siRNA OECs and Cyclophilin B (PPIB) siRNA OECs. Briefly, 9,000 cells were seeded in 96-well plates that were previously coated with 50 µl of Matrigel™ matrix and then maintained in a $CO_2$ incubator at 37°C. After 24 hours, two images per well were acquired using an Olympus IX71 microscope (100X magnification). Each experiment was run in duplicate in four independent experiments. In addition, to monitor the formation of vessel-like structures by mouse EPCs, a time lapse imaging assay was performed over hours, as described in a previous study (Morancho et al., 2013). A standard Matrigel™ assay was conducted as

described above; however, continuous image acquisition was performed starting 2 hours after seeding and then every 30 minutes (for up to 24 hours). An Olympus multi-dimensional-TIRFM cell-R microscope (Olympus, Japan) with temperature, $CO_2$ and humidity control was used. Two images per well were acquired at 100X magnification. For both assays, the number of complete rings, the number of branching points and the total tube lengths (the perimeter of complete rings) were automatically counted blindly using Wimasis® Image Analysis software. The results are expressed as a percentage of the control condition.

**Statistical Analysis**

SPSS version 15.0 was used for statistical analysis. Normality was assessed for continuous variables using the Shapiro-Wilk test. The values from the normally distributed variables are expressed as the mean ± SD and represented as bar graphs. The values from the non-normally distributed variables are expressed as the median (interquartile range) and represented as box plots. The differences between the different groups were analyzed using an independent t-test and ANOVA and, if significant, followed by the Dunnett *post hoc* test (all *vs.* the control condition) if normally distributed or the Mann-Whitney U-test and Kruskal-Wallis if not normally distributed. To assess differences between time-points, we used repeated ANOVA followed by Bonferroni *post hoc* tests. Finally differences between categorical variables were analyzed using Fisher's exact tests or Chi Square tests. A p-value less than 0.05 was considered statistically significant.

**RESULTS**

**MMP-13 deficiency protects the brain from ischemia-reperfusion injury and improves functional outcomes**

The WT and KO alleles for MMP-13 were confirmed using PCR (Figure 2A). The brain infarct was localized to the somatosensory and motor cortex in both the permanent and the transient model (Morancho et al., 2012). Unexpectedly, 4 WT mice presented some infarct expansion that affected the striatum at 3 days after MCAo. MMP-13 deficiency reduced infarct volume in the ischemia-reperfusion model at both 24 hours and 3 days (26.1±9.4 $mm^3$ for WT *vs.* 14.4±8.3 $mm^3$ for MMP-13 KO, $p$=0.025; and 24.8±4.7 $mm^3$ for WT *vs.* 9.3±3.1 $mm^3$ for MMP-13 KO, $p$<0.001; respectively), as shown in Figure 2B-C. However, this difference was not significant in the permanent occlusion model (19.1±8.7 $mm^3$ for WT *vs.* 14.5±6.5 $mm^3$ for MMP-13 KO, $p$=0.228). The difference between these two ischemia models was attributed to the increase in infarct extension observed in the WT mice subjected to tMCAo, although this difference did not reach statistical significance at 24 hours (19.1±8.7 $mm^3$ for pMCAo *vs.* 26.1±9.4 $mm^3$ for tMCAo, $p$=0.13) or at 3 days (19.1±8.7 $mm^3$ for pMCAo *vs.* 24.8±4.7 $mm^3$ for tMCAo, $p$=0.105). An analysis of functional outcomes also confirmed a significant decrease in forelimb force at 24 hours in the WT mice (decreased 83.8±15.8% from baseline, $p$<0.01); however, this was not clearly observed in the MMP-13 KO mice despite a statistical trend (90.2±23.9% from baseline, $p$=0.083); see Figure 2D. No differences were observed between mice with different genotypes at either 24 hours or 3 days (data not shown).

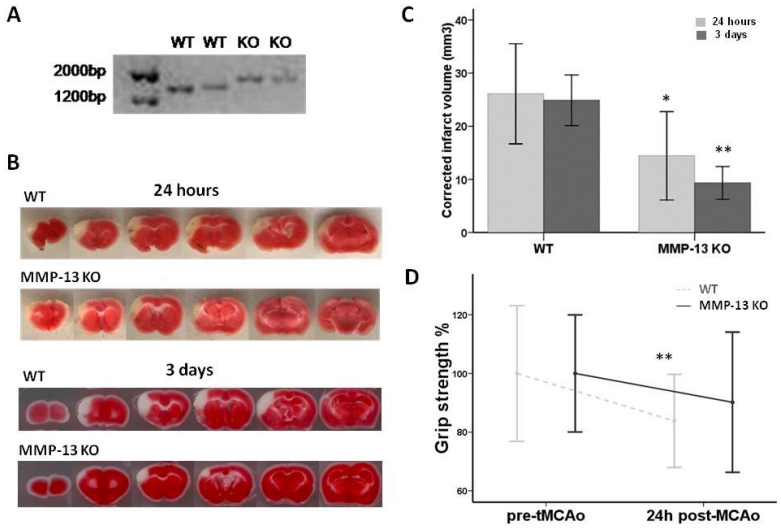


**Figure 2. MMP-13 deficiency protects brain tissue during cerebral ischemia reperfusion.** Genotype characterization of wild-type (WT) and MMP-13 knockout (KO) mice: a 1300Kb band indicates a WT allele, and a 1485Kb band indicates a MMP-13 KO allele (***A***). TTC staining showing infarct areas at 24 h and 3 days after 60 min of transient ischemia (***B***). Bar graph showing mean ± SD infarct volume at 24 h and 3 days; n=6-8/group, * *P*<0.05 and ** *P*<0.01 (***C***). Grip strength measurements confirming that the significant neurological impairment observed in WT mice was reduced in MMP-13-deficient animals; n=13-14/group, ** *P*<0.01 *vs.* pre-tMCAo (***D***).

Interestingly, we observed more spontaneous intracranial hemorrhagic transformations at 3 days than at 24 hours, and this was more common in the WT (6/6 and 4/8, respectively) than in the MMP-13 KO mice (3/6 and 1/7, respectively), as shown in representative images of the WT in Figure 2B. The differences between genotypes or reperfusion day were not statistically significant, however if the only presence of HT was considered, regardless the duration of MCAo, MMP-13 deficiency protected brains from spontaneous hemorrhagic conversions (10/14 in WT *vs.* 4/13 in MMP-13-KO, *p*=0.035, supporting Table 1 and Figure 1). The animals studied over a longer time (two weeks, study B) showed significant differences in forelimb force between WT and MMP-13 KO at 24 hours and 1 week after tMCAo (*p*=0.035 and *p*=0.015, respectively). When the neurological outcomes within the groups were analyzed, only the WT mice exhibited a significant decrease in forelimb force values *vs.* baseline at 1 week (66±4.6%, *p*<0.001) and 2 weeks (70.2±5.7%, *p*=0.017), as shown in Figure 3A. At two weeks, differences in forelimb force were no longer observed.

## MMP-13 and cortical damage

Cerebral ischemia leads to brain damage at the same time that it triggers neurorepair mechanisms. Cortical atrophy was evaluated at 14 days after tMCAo as a percentage of the remaining cortex in the ipsilateral hemisphere (35.7±1.9% for WT *vs.* 36.1±1.7% for MMP-13 KO, *p*=0.684) as shown in Figure 3B, regardless of large differences in baseline infarct volumes.

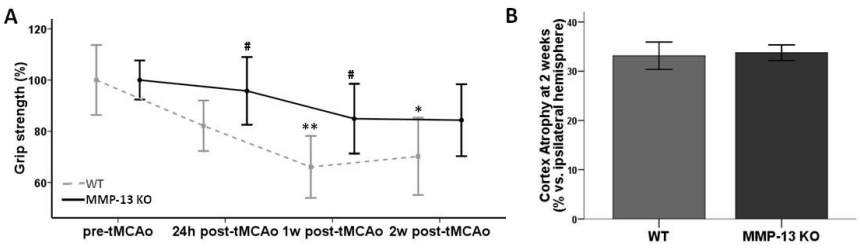


**Figure 3. Recovery from brain ischemia reperfusion.** Grip strength performance: MMP-13 KO mice were acutely protected from functional impairment following cerebral ischemia. After two weeks, differences between genotypes were no longer observed. n=8, **P* < 0.05, ***P* < 0.01 *vs.* pre-tMCAo in the same cohort; $^{\#}P$ < 0.05 *vs.* WT at the same time point (***A***). Cortical atrophy of the ipsilateral hemisphere at 2 weeks, n=8 (***B***).

**A reduced number of newly born neuroblasts was observed in the peri-infarct areas of the MMP-13 deficient mice during recovery**

DCX+ neuroblasts/immature neurons and proliferating cells were studied in the post stroke brain during recovery (at days 7 and 14), with a focus on the SVZ, the corpus callosum (CC) and peri-infarct areas. At the studied time-points, no enhanced neuroblast migration was detected at day 7 compared with the contralateral side in either the WT or the MMP-13 KO mice (Figure 4A), whereas at day 14, the number of neuroblasts was higher in the ipsilateral CC area than in the contralateral CC in both the WT and MMP- 13 KO mice (*p*=0.011 and *p*=0.011, respectively; see Figure 4B). Interestingly, when we analyzed the distribution of the BrdU+ replicating cells in the post-stroke brain (day 14), we found a reduced number of newly born neuroblasts (DCX+/BrdU+) in the peri-infarct areas of the MMP-13 deficient mice (18.0±2.8 cells in WT vs. 4.7±1.3 cells in MMP-13 KO, *p*=0.001), although a similar number of DCX+ cells was found in the same area (136±27 cells in WT and 130±20 cells in MMP-13 KO, *p*=0.873; see Figure 4C-D). An additional analysis of these cell counts also showed that the percentage of newly born neuroblasts (DCX+/BrdU+) out of the total number of neuroblasts was reduced in the peri-infarct areas of the MMP-13 deficient mice (16.9% in WT vs. 4.8% in MMP-13 KO, *p*=0.005). Immunostaining for mature neurons (NeuN+) did not reveal any difference between mice with different genotypes in numbers or size (data not shown).

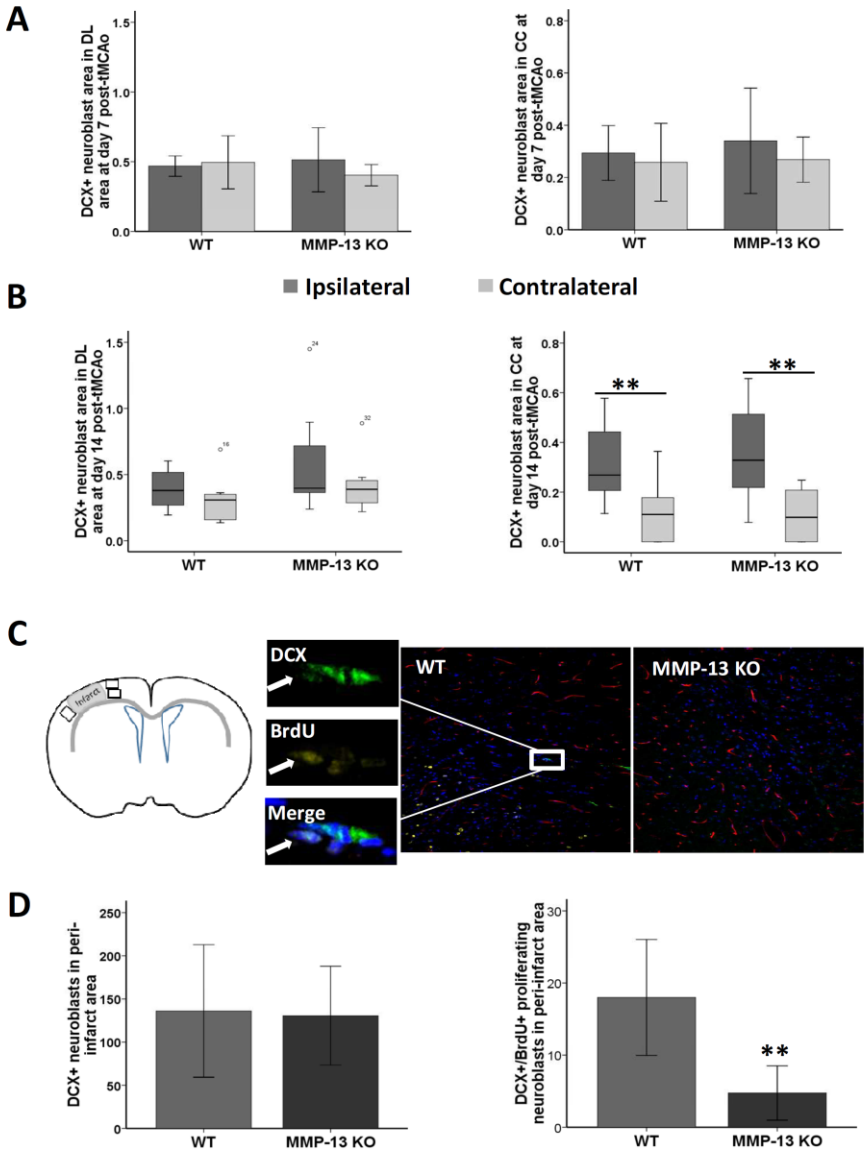


**Figure 4. Neuroblast migration from the SVZ through the CC to the peri-infarct cortex.** DCX+ (neuroblast) area quantification in the dorsolateral ventricle (DL) area and the corpus callosum (CC) area, as indicated, at day 7 (***A***, n=5-6) or day 14 after tMCAo (***B***, n=8); ***P* <0.01. The micrographs confirm peri-infarct neurogenesis (the areas studied in selected ROIs) as co-staining between DCX (green), BrdU (yellow), lectin (red) and DAPI (blue, nuclei) on day 14 after tMCAo (***C),*** the white arrows indicate a newborn neuroblast. The bar graphs show that MMP-13 deficiency decreased the number of DCX+/BrdU+-stained proliferating neuroblasts in the peri-infarct cortex; n=8, ***P*<0.01 (***D***).

## MMP-13 is a key protease during post-stroke vascular remodeling

Accelerated angiogenesis in the peri-infarct area is closely related to improved post stroke tissue repair (Hayashi et al., 2006). In the present study, we sought to study the functional brain microvasculature using *in vivo* endothelial labeling with lectin, as described. Our data show that 7 days after cerebral ischemia, no difference in peri-infarct vessel density (the percentage of

the lectin-stained area of the ROIs, as shown in Figure 5A) occurred as a consequence of ischemic insult in either the WT (3.11±0.35 in the ipsilateral vs. 3.15±0.47 in the contralateral side, *p*=0.861) or the MMP-13 KO mice (3.16±0.64 in the ipsilateral vs. 3.37±0.60 in the contralateral side, *p*=0.578). Nevertheless, on day 14, the WT mice presented higher vessel density in the peri-infarct cortex than in the corresponding contralateral area (3.03±0.41 vs. 2.45±0.19, *p*=0.003; Figure 5B-C), whereas the MMP-13 KO mice did not display ischemia-enhanced angiogenesis in the peri-infarct area (2.53±0.51 vs. 2.24±0.20, *p*=0.160; Figure 5B-C). Accordingly, the WT mice presented a more extensive vessel area than the MMP-13 KO mice in the peri-infarct cortex (3.03±0.41 vs. 2.53±0.51, *p*=0.049), and this was also observed, although less extensively, in the contralateral cortex, where it did not reach statistical significance (2.45±0.19% in WT vs. 2.24±0.20% in MMP-13 KO; *p*=0.05; Figure 5B-C). No differences were observed in the peri-infarct area in the striatum, as expected, in the distal cortical experimental model of stroke (data not shown). Interestingly, we studied whether the number of proliferating neuroblasts (DCX+/BrdU+ cells) was related to vessel density and observed a noticeable association between these studied parameters, as shown in the correlation plot in Figure 5D (p=0.056).

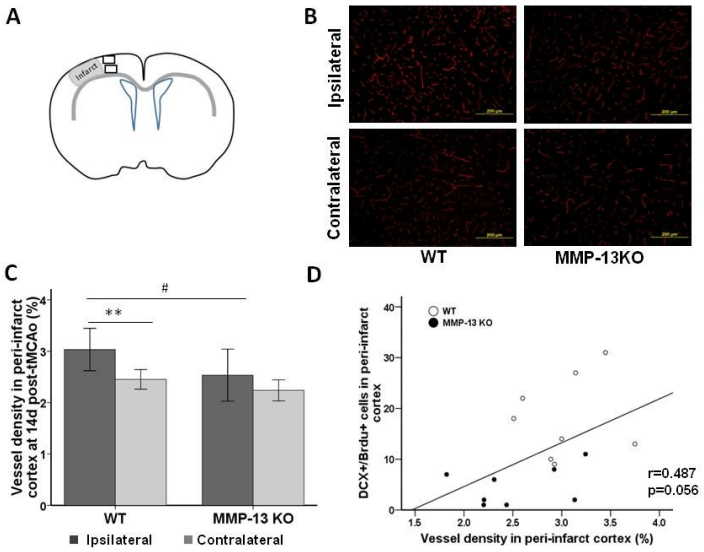


**Figure 5. Brain vessel density after ischemia reperfusion**. Representation of the studied areas (indicated by squares), (**A**). Micrographs of Lectin+ (red) vessels in representative brains; scale bar represents 200 µm (**B**). Bar graphs representing vessel density in the peri-infarct cortex and the corresponding contralateral area on days 7 and 14 after tMCAo; n=6-8, ***P*<0.01 and **P*<0.05 (***C***). Correlation plot between vessel density and the number of proliferating neuroblasts in peri-infarct areas on day 14 (**D**).

In agreement with this temporal profile, the levels of several angiogenesis-related growth factors were examined in the ischemic brain at day 7, which is just before the increase in vessel density observed in the peri-infarct tissue. Data obtained from immunodetection assays showed that G-CSF (shown as a ratio of ipsilateral/contralateral) was higher after ischemia in the WT mice than in the sham group (5.1 (1.9-10.5) vs. 1.1 (0.8-1.8), respectively;

*p*=0.003). At the same time-point, the MMP-13-deficient ischemic mice presented a lower level of cortical G-CSF than the corresponding WT mice (2.5±1.5 vs. 5.2±2.7, *p*=0.031; see Figure 6A). In agreement with these results, the expression of other growth factors associated with vascular remodeling was also significantly lower in the infarct cortex of MMP-13 KO mice than in the WT mice, for example, VEGF-A (0.9±0.3 vs.1.4±0.3, *p*=0.007) and amphiregulin (0.9±0.2 vs. 1.1±0.2, *p*=0.049; see Figure 6A). Other growth factors, such as angiopoietin-2, FGF-2 and SDF-1 were also down-regulated in the MMP-13 KO brains but these differences were not significant when compared to the WT (not shown). To explore effects on other members of the MMP family of proteins that might potential compensate for MMP-13 knockdown, the levels of three MMPs (including another collagenase, MMP-8) were quantified in the ischemic cortex and the corresponding contralateral tissue. Interestingly, our results show that the levels of MMP-3, MMP-8 and MMP-9 were significantly elevated in the damaged cortex in mice with both genotypes compared to the contralateral cortex, as shown in Figure 6B. Moreover, the amount of these MMPs was considerable higher in the ipsilateral cortex of WT mice than in the MMP-13 KO mice: 1.21 (0.55-1.78) ng/mg vs. 0.14 (0.09-0.20) ng/mg for MMP-3, p=0.002; 0.8±0.2 ng/mg vs. 0.4±0.1 ng/mg for MMP-8, p=0.001; and 2.4±0.9 ng/mg vs. 0.7±0.3 ng/mg for MMP-9, p=0.002. These results support the idea that MMP-13 plays a role as a central protease that regulates other MMPs after ischemia.

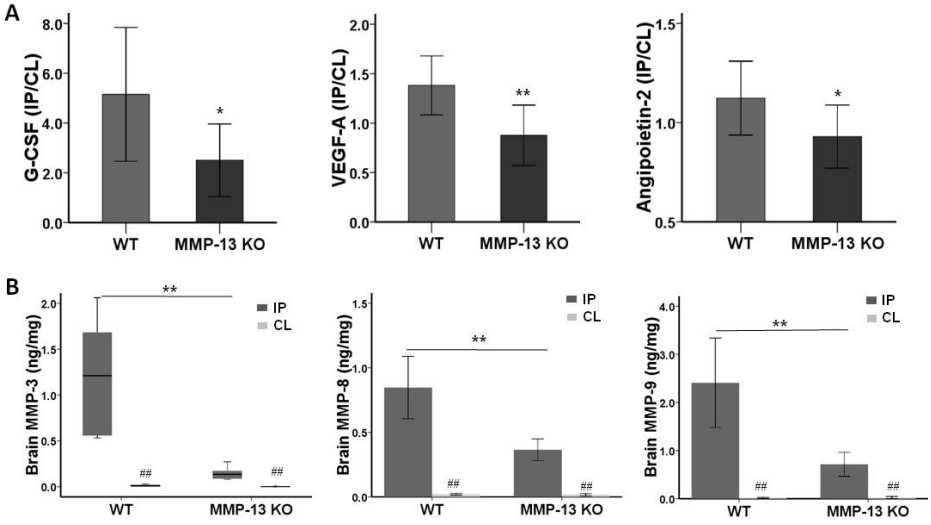


**Figure 6. Pro-angiogenic factors and MMPs in the cortex after ischemia.** Bar graphs representing the ratio of MMP proteins between the ipsilateral (IP) and contralateral (CL) hemispheres in WT and MMP-13 KO ischemic mice at 7 days after ischemia; n=7-8, **P*<0.05, ***P*<0.01 (**A**). Box plots and bar graphs demonstrating the overexpression of other MMPs in the ipsilateral hemisphere of WT brains and their dysregulation in MMP-13-deficient mice at day 3. **p<0.01 and $^{\#\#}$p<0.01 *vs.* ipsilateral (**B**). Data are expressed as ng of MMP per mg of total protein.

**Transient silencing of the MMP-13 gene impairs tubulogenesis *in vitro***

To determine whether the angiogenic/vasculogenic functions of OECs are controlled by MMP-13, an *in vitro* Matrigel$^{TM}$ assay was conducted after MMP-13 expression was silenced using siRNA. As shown in Figure 7A,

introducing MMP-13 siRNA into OEC cell cultures reduced the expression of MMP-13 mRNA by 68.62% compared with the control cells ($p$=0.001). This effect occurred without modifying the expression of housekeeping genes, such as PPIB. Additional experiments were conducted to demonstrate that the precise targeting of other genes, such as PPIB, successfully reduced their expression (up to 93.63, $p$<0.001) without modifying MMP-13 expression, as shown in Figure 7A. Nonspecific (non-targeting) siRNA did not modify any of the expression patterns of MMP-13 or PPIB.

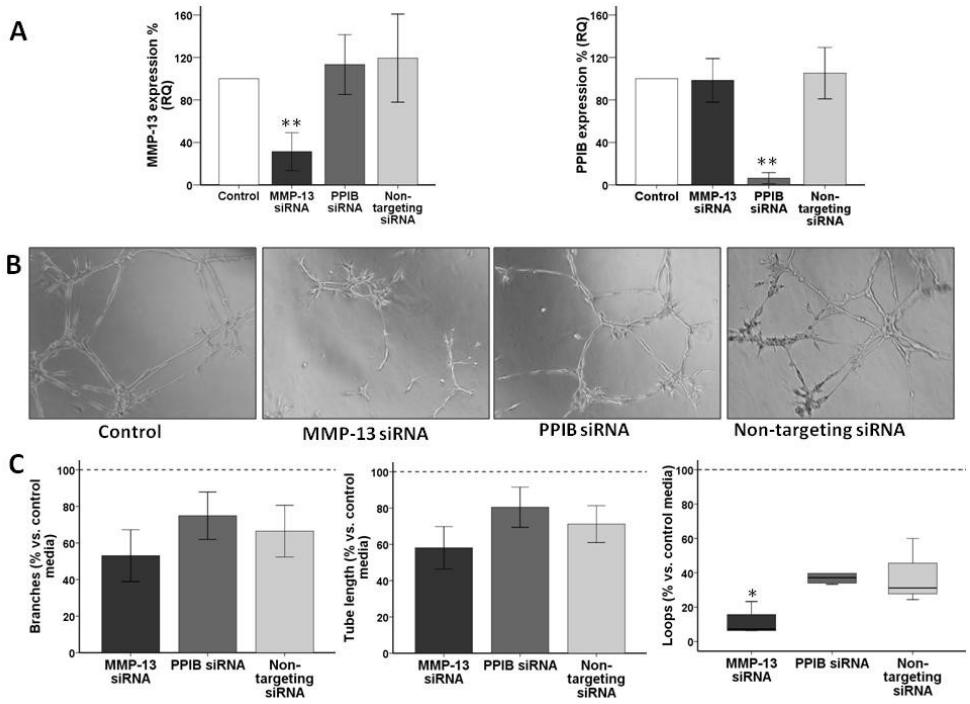


**Figure 7. Tubulogenesis network formation in Matrigel™ assays of mouse OECs after silencing MMP-13 expression.** Bar graphs represent the level of MMP-13 and PPIB RNA expressed in each culture condition; n=4, ***P*<0.01 *vs.* Control (***A***). Representative micrographs showing vessel-like structures shaped by OECs in different treatment groups (***B***). The bar graphs show the quantification of the number of branches, the total tube lengths and the loops of vessel-like structures that were induced in non-silenced, MMP-13-silenced and PPIB-silenced OECs; n=4, **P*<0.05 *vs*. non-targeting siRNA EPCs (***C***).

Matrigel™ functional assays showed that OECs that did not endogenously express MMP-13 formed aberrant tubulogenic networks compared with all the other tested conditions (see Figure 7B and supporting Videos 1-4). The total number of branching points and loops and the total tube lengths of the vessel-like structures were quantified. Compared with the non-targeting siRNA (control group), the MMP-13-silenced OECs showed decreased numbers of vessel-like structures, as assessed by the analysis of all parameters. The difference in the percentage of loops (circular structures) reached statistical significance ($p$=0.019), whereas the percentage of branches and the percentage of tube lengths are presented as statistical trends after the statistical analyses among the groups (p=0.132 and p=0.052, respectively), as shown in Figure 7C.

## DISCUSSION

The present study demonstrates for the first time that collagenase-3 (MMP-13) participates in tissue damage during cerebral ischemia and is simultaneously required for neurovascular remodeling during the recovery phases. MMP-13 deficiency reduced infarct size and acutely diminished the deterioration of functional outcomes observed after experimental transient cerebral ischemia. However, the absence of MMP-13 also reduced the amount of newly born neuroblasts in the peri-infarct areas during neurological recovery and altered stroke-induced angiogenesis by reducing peri-infarct angiogenesis and suppressing the secretion of G-CSF, angiopoietin-2 and VEGF-A. Finally, endothelial progenitor cell function (demonstrated in their high potential for vascular remodeling) was abolished when MMP-13 gene expression was silenced *in vitro*.

Stroke leads to cell and tissue death within minutes/hours because the brain spontaneously activates both injury and repair mechanisms that compete for the recruitment of tissue after an ischemic insult (Lo EH, 2008). This state opens a new battlefield wherein neuroprotection is needed to minimize the expansion of the injury and neurorepair strategies are needed to enhance endogenous repair. Among the known neurorepair mechanisms is the process by which cerebral ischemia triggers spontaneous neurogenesis (Ohab et al., 2006; Kreuzberg et al., 2010), angiogenesis (Ergul et al., 2012) and gliogenesis (Tanaka et al., 2003; Miyamoto et al., 2010) in peri-infarct areas. From a mechanistic point of view, our efforts have been focused on MMPs, which are zinc-dependent endopeptidases that degrade components of the extracellular matrix, including the basal lamina surrounding brain microvessels, which are modified during angio-vasculogenesis. Some MMPs have been found to contribute to brain injury (Asahi et al., 2001; Gidday et al., 2005) and also to orchestrate vascular remodeling (Morancho et al., 2013; Zhao et al., 2006). Knowledge regarding the role of MMP-13 is limited in the context of stroke.

In our study, both MMP-13 KO and WT mice were subjected to permanent or transient ischemia; however, the infarct size was only significantly reduced in the MMP-13- deficient mice in the transient model at both 24 hours and 3 days after MCAo, indicating that the role of MMP-13 in tissue damage is most likely more related to the reperfusion phase. The infarct volume did not change in the MMP-13-KO mice; however, more damage was observed in the WT mice in the transient model than in the permanent model. In addition, we unexpectedly observed spontaneous hemorrhagic transformations in our model (especially at day 3), but the knockdown of MMP-13 partially protected mice from suffering these complications. Previous results from our laboratory revealed a relationship between a high plasma level of MMP-13 and early brain lesion growth in stroke patients (Rosell et al., 2005). We identified an increase in MMP-13 protein levels in the infarct/peri-infarct areas in human and rat tissues, and the activation of neuronal nuclei has been related to cell death in oxygen- and glucose deprivation studies (Cuadrado et al., 2009a and 2009b). Recently, increased MMP-13 mRNA and protein levels were observed in brain tissue during the acute phase of ischemia reperfusion by Lenglet and colleagues (Lenglet et al., 2014). Other authors have reported that injured endothelial cells release MMPs, including MMP-13, which

attack the basal lamina and degrade the matrix components and tight junctions of endothelial cells (Hattori et al., 2003). Ueno and colleagues found that the expression of MMP-13 in hippocampal vessels was increased in rats with blood brain barrier (BBB)- damaged vessels compared with rats without BBB impairment, indicating the importance of MMP-13 recruitment to brain tissue during the healing process, where it participates in angiogenesis and neurogenesis (Ueno et al., 2009). Indeed, it has been demonstrated that a high level of MMP-13 activity lasts for up to 14 days after ischemia, and this appears to be closely associated with an increase in aggrecan, suggesting their role in neuronal reorganization (Nagel et al., 2005). All these data support a role for MMP-13 in the acute and subacute phases of stroke: MMP-13 participates in the development of acute ischemic damage, while at the same time contributing to favorable neurovascular remodeling during recovery. As we will discuss in detail, the results in this report support these hypotheses.

The distal MCAo model was chosen for this study due to its association with low mortality rates and for the precise infarct location it induces in cortical areas, which leads to a defined cortical peri-infarct area. However, cortical damage tends to produce milder neurological deficits over the long-term. In our experience, the grip strength test has been proven to be a feasible method for short- and long-term evaluations after distal MCAo (Rosell et al., 2013b). Our data show that the WT mice presented increased neurological deterioration in the first week. No difference was detected in the MMP-13 KO mice, supporting a protective role for MMP-13 in acutely induced neurological deficits. Moreover, significantly different effects on neurological functions were observed between the genotypes during the first week after ischemia (the WT mice performed worse); however, this difference disappeared at 2 weeks, at which time the WT mice showed improved results in the forelimb force test. These data support the idea that MMP-13 is implicated in the post-stroke spontaneous-recovery phase at a functional level. In this regard, our results also showed that similar cortical atrophy occurred in MMP-13 KO and WT mice at 2 weeks, despite the larger infarct volumes observed in WT mice, which could be a consequence of different capabilities related to neurorepair, the inhibition of cell apoptosis or the promotion of neuronal survival, among other mechanisms. It will be interesting to perform studies using longer time points to follow-up on these observations regarding the state of atrophy in the damaged tissue.

Neurogenesis and angiogenesis were studied to further explore the mechanisms affected by the lack of MMP-13 that could influence cerebral plasticity. Our results confirm that at 2 weeks, neuroblasts had migrated to the peri-infarct areas, as has been described to occur in post-stroke brains (Arvidsson et al., 2002; Carmichael, 2008). This effect occurred from the SVZ through the corpus callosum, as expected, and no difference was observed between the genotypes. Interestingly, the peri-infarct areas of the mice lacking MMP-13 contained a reduced number of proliferating neuroblasts (DCX+/BrdU+), which are responsible for the formation of new neural cells in the areas showing active neurorepair. Most neuroblasts die during migration and the generation of new neurons (Zhang et al., 2004). We hypothesized that either the microenvironment in peri-infarct areas and along the migration pathways in brains lacking MMP-13 did not support neuroblast

survival or that lack of MMP-13 may result in decelerated standard migration patterns. In this regard, neurogenesis has been shown to be coupled to angiogenesis in a post-stroke neurovascular niche (Ohab et al., 2006), and it has been demonstrated that blood vessels in the peri-infarct cortex secrete soluble factors that maintain neurogenic potential and guide neurogenesis toward the damaged cerebral cortex (Shen et al., 2004; Thored et al., 2007). In support of these hypotheses, we observed a positive association between the number of proliferating neuroblasts and lectin-positive vessels in tissues with more extensive vessel networks and the ones that presented more newborn neuroblasts in the peri-infarct area.

Importantly, our study shows that at day 7 after ischemia-reperfusion injury, lower amounts of some nourishing factors, such as G-CSF, angiopoietin-2 and VEGF-A, were observed in the infarct cortex of the MMP-13 KO mice than in the WT brains. This is an interesting finding because the administration of some factors in the early stages of stroke has been shown to stimulate angio-neurogenesis and to lead to improved functional recovery (Schäbitz et al., 2003; Rivera and Bergers, 2014; Beck et al., 2000). These data suggest that MMP-13 could be indirectly contributing to an appropriate angiogenic micro-environment. Therefore, we expected to observe changes in vessel density between the MMP-13 and WT mice. In agreement with this assumption, our observations support a role for MMP-13 in vascular remodeling after stroke: two weeks after cerebral ischemia, increased vessel density in the peri-infarct area was found only in the WT animals and not in the MMP-13-deficient mice. The present study does not identify the source of the trophic factors that are affected by MMP-13 deficiency. These could be secreted by brain resident cells, such as astrocytes or mature endothelial cells, as has been described by others (Ohab and Carmichael, 2008; Kahle and Bix, 2013). However, they could also be released by stem/progenitor cells, such as EPCs or NPCs, which secrete multiple growth factors (Rosell et al., 2013a; Drago et al., 2013; Urbich et al., 2011).

EPCs have been demonstrated to have therapeutic potential in that they 626 directly enhance angiogenesis and neurogenesis (Asahara et al., 1997; Carmeliet, 2003; Ma et al., 2015). These cells, which are also the source of multiple trophic factors, are able to differentiate into cells with an endothelial phenotype and have been proven to reduce the brain damage caused by ischemia and to enhance angio-vasculogenesis in stroke models (Ohta et al., 2006; Moubarik et al., 2011; Rosell et al., 2013a). In the present study, we further demonstrated a role for MMP-13 in tubulogenic remodeling in experiments in which we silenced the expression of MMP-13 in EPCs, which have been shown to be involved in angiognesis (Morancho et al., 2013; Rosell et al., 2013a). Our data demonstrate that EPCs develop abnormal tubulogenic remodeling patterns when MMP-13 expression is blocked, in which they failed to shape vessel-like networks in Matrigel™ substrate. This interesting observation should be considered for future therapeutic strategies, although additional research in an *in vivo* setting is needed to study the effects of MMP-13 deficiency in tissue EPCs or in putative EPC-based cell therapies.

In summary, our study highlights the role of MMP-13, a pivotal collagenase that remodels the ECM, in neuroprotection and neurorepair after cerebral ischemia. This central role is further supported by our findings regarding the

regulation of other MMPs in the ischemic brain. We demonstrate that in WT mice, other MMPs, including MMP-3, MMP-8 and MMP-9, are upregulated in the ischemic brain, as was reported by our group in human infarct tissue (Cuadrado et al., 2009b), but also that MMP-13 deficiency considerably diminished the overexpression of these other MMP family members.

Therefore, lacking MMP-13 protects the brain and improves functional outcomes over the short term, whereas over longer periods of time, this protease is required to support vascular remodeling and the production of growth factors that are linked to peri-infarct neurogenesis. In conclusion, the therapeutic modulation of MMP-13 during stroke is fully supported by the present data.

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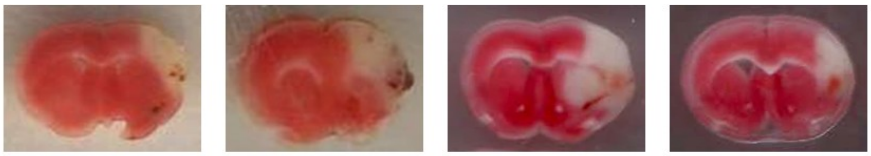
## SUPPORTING INFORMATION

### Supporting Table 1.

**Supporting Table 1. Summary of HT incidences in MMP-13 KO and WT mice after transient MCAo.**

| HT incidence | 24 hours post-ischemic reperfusion | 72 hours post-ischemic reperfusion | P value |
|---|---|---|---|
| **WT (10/14)** | 50% (4/8) | 100% (6/6) | 0.085 |
| **MMP-13 KO (4/13)** | 14.3% (1/7) | 50% (3/6) | 0.266 |
| 0.035 | 0.282 | 0.182 | |

### Supporting Figure 1.




**Supporting Figure 1. Representative images of HT at 24h or 72h after cerebral ischemia in WT or MMP-13 KO mice.**

**Supporting video 1**. 24 hours of time-lapse imaging Matrigel™ assay of mouse WT control OECs (100X)

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**Supporting video 2**. 24 hours of time-lapse imaging Matrigel™ assay of mouse WT MMP-13 silenced OECs (100X)

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**Supporting video 3**. 24 hours of time-lapse imaging Matrigel™ assay of mouse WT PPIB silenced OECs (100X)

http://ees.elsevier.com/ynbdi/download.aspx?id=341326&guid=12a5e1be-4f0d-4de9-9547-f115b74c6ad7&scheme=1

**Supporting video 4**. 24 hours of time-lapse imaging Matrigel™ assay of mouse WT No-targeting silenced OECs (100X)

http://ees.elsevier.com/ynbdi/download.aspx?id=341327&guid=0d57cc95-fd0d-4708-94de-058d8253d2da&scheme=1

**Article 3**

**Plasma matrix metalloproteinase levels in stroke patients during intensive rehabilitation therapy.**

Manuscript in preparation.

# Plasma matrix metalloproteinase levels in stroke patients during intensive rehabilitation therapy

Feifei Ma[1], BSc; Susana Rodriguez[2], MD; Xavi Buxo[2], MD; Anna Morancho[1], PhD; Iolanda Riba-Llena[1], MD, PhD; Ana Carrera[2]; Alejandro Bustamante[1], MD; Dolors Giralt[1], BSc; Joan Montaner[1], MD, PhD; Carmen Martinez[2], MD; Immaculada Bori[2], MD; Anna Rosell[1], PhD.

[1]Neurovascular Research Laboratory and Neuroscience Department, Vall d'Hebron Research Institute, Universitat Autonoma de Barcelona, Spain. [2]Unidad de Rehabilitacion Neurologica y Dano Cerebral, Hospital Vall d'Hebron, Universitat Autonoma de Barcelona, Spain.

**Corresponding Author:**

Anna Rosell, anna.rosell@vhir.org

Neurovascular Research Laboratory, Vall d'Hebron Research Institute, Passeig Vall d'Hebron 119-129, 08035, Barcelona, Spain. Tel. +34 934894029

**ABSTRACT**

**Background:** Rehabilitation therapies are still the only approved treatments used to improve neurological recovery in disabled stroke survivors. The use of biomarkers to monitor and predict patient's recovery could help to adjust individual rehabilitation programs and to identify mechanisms of repair. **Objective:** To study plasma levels of matrix metalloproteinases (MMPs) as potential markers of recovery during intensive rehabilitation therapy (IRT) after stroke. **Methods:** Patients with first-ever ischemic strokes enrolled to IRT (≥3 hours per day/5 days per week) were studied (n=15). The protocol consisted of a battery of motor/functional tests (Rankin, BI, FMA, FAC, MRC, CAHAI and the 10 meter walk) performed before therapy and at one-, three and six-month follow-ups, in addition to blood extractions to measure MMP-3, MMP-12 and MMP-13 levels. Healthy volunteers served as non-ischemic controls (n=15). **Results:** MMP levels remained stable during the study period and were similar to those in controls. However, baseline MMP-12 and MMP-13 levels were strongly associated with stroke severity as measured by NIHSS score ($p<0.001$ and $p=0.008$) and were elevated in the patients with the most extensive infarcts ($p=0.009$ and $p=0.058$). Interestingly, plasma MMP-3 was independent of baseline stroke characteristics but was found to be increased in patients with better motor/functional recovery and in patients with larger improvements during rehabilitation. **Conclusions:** MMPS might act as biological markers of recovery during rehabilitation therapy related to their roles in both injury and tissue remodeling. Future confirmatory investigations in multicenter studies are warranted by our data.

**Key Words**: Stroke, matrix metalloproteinase, intensive rehabilitation therapy, biomarker, recovery

## INTRODUCTION

Despite enormous advances in acute stroke management during the last two decades (1), approximately five million people survive a stroke each year worldwide with disabilities that limit independence for several types of daily activities. For these patients, the only approved treatment during the sub-acute and chronic phases of the disease is neurorehabilitation, with the objective of improving their independency status and to reach the best quality of life at long-term (2, 3).

During rehabilitation, improved outcomes in patients are the result of early mobilization, rehabilitation intensity and other measures used to prevent and treat medical complications (4, 5), and also to individual responses to therapy. From a biological perspective, we know that neurorehabilitation enhances different aspects of brain plasticity, such as axonal remodeling, cell genesis and angiogenesis (6), but this could be patient-dependent, and the biological mechanisms responsible for these individual responses to treatment are still being investigated. With this background, it is necessary to investigate the precise molecular mechanisms underlying tissue remodeling and plasticity to ameliorate or adjust current rehabilitation programs. Additionally, the accessibility of biomarkers that could support an accurate and individual prognosis of recovery during rehabilitation would help practitioners to decide on the intensity, type or duration of the program and to personalize treatments (7).

In this investigation, we have focused on matrix metalloproteinases (MMPs), enzymes that can degrade most components of the extracellular matrix that are involved in both injury and tissue repair after stroke (8, 9). We have investigated MMP-3, MMP-12 and MMP-13 as they were previously related to ischemic brain injury but not to recovery after stroke. Our hypothesis is that some of these MMPs can serve as biomarkers to monitor individual neurological status and/or functional improvement as a reflection of the brain plasticity phenomena and an individual response to rehabilitation therapy.

We designed a prospective study of ischemic stroke patients who were enrolled in intensive rehabilitation therapy (IRT) to measure plasma levels of MMPs and to determine the motor/functional status of the patients by using a battery of tests up to six months after stroke.

## METHODS

### *Study Subjects*

The study cohort comprised patients who were enrolled in IRT at the Vall d'Hebron University Hospital from February 2014 to February 2015. Inclusion criteria were: first-ever ischemic stroke, age < 70 years, somatosensory or ataxic hemiparesis, time until start of IRT < 3 weeks after stroke, stable medical condition, endurance to participate in a minimum of 3 hours/day in a therapy program and agreement to participate in the study. Exclusion criteria included sensory or global aphasias, cognitive deficits (Mini-Mental State Examination < 23), terminal illness, inflammatory diseases or previous deficits of the upper/lower limb. In parallel, fifteen non-ischemic healthy individuals with no known neurological, malignant or inflammatory disease volunteered to participate as part of the control cohort. Part of the control cohort were hypertensive subjects also studied in the YSSIS

study (10) free of ischemic events.

The Study Protocol was presented and approved by the local Clinical Research Ethics Committee (PRIR 317/2013). All patients and controls signed the corresponding informed consent.

***Rehabilitation Intervention***

Patients who met the inclusion criteria followed a comprehensive intensive rehabilitation program, including physiotherapy, occupational therapy, speech therapy and/or neuropsychology, if required, for a minimum of 3 hours per day and 5 days per week, as indicated by the Catalan clinical practice guidelines for the management of stroke (11). The protocol started with a physiatrist assessing patient's deficits within the first 24 hours after stroke and designing an initial rehabilitation program. Passive mobilizations performed in bed started within the first 24 hours after stroke if there are no medical conditions that contraindicate it, and at 48 hours patients started a moderate intensity rehabilitation program. Those patients who presented severe or moderate deficits in two or more functional areas and who met the inclusion criteria for an intensive rehabilitation program were transferred to the Neurorehabilitation Unit for inpatient IRT, whereas those patients who were able to walk with some support/assistance were released to their household and started the daily IRT at the Day hospital of the Neuro-Rehabilitation Unit. With the posterior release from the inpatient Neurorehabilitation Unit, the IRT then continued at the Day Hospital until completion of a minimum of 75% of the proposed objectives or when functional stability was achieved. If there were further objectives, the patient continued a high-moderate intensity outpatient rehabilitation program until the achievement of functional stability.

***Study Protocol***

A total of fifteen patients were finally included in the study. Of these, one patient was excluded following the baseline visit after having a second stroke, while another patient voluntarily discontinued his participation. Two other patients chose to withdraw from the study after the one-month follow-up visit (see Figure 1).

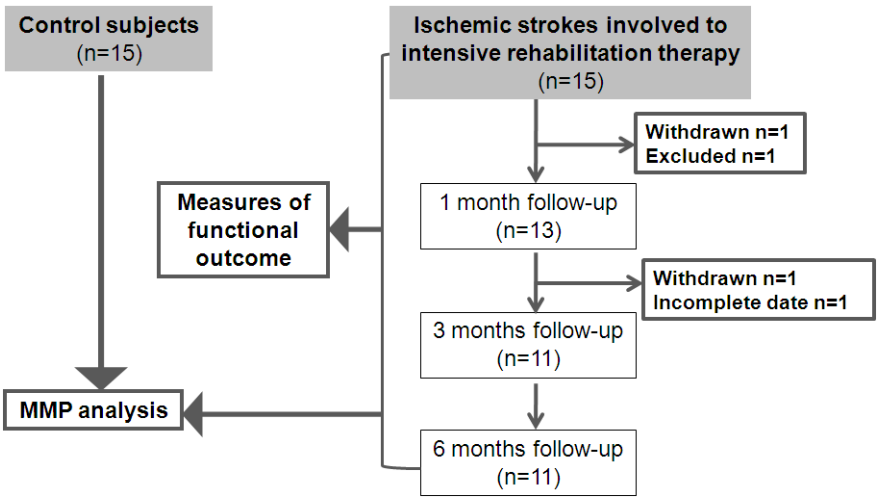


**Figure 1. Study flow diagram.**

Data related to subject demographics, risk factors, medication, comorbidities and exercise were obtained from patients and controls (see Table 1), along with clinical stroke characteristics (see Table 2).

Specific visits for the study were performed by an experienced physiatrist at baseline prior to starting the IRT and at one, three and six months after starting the therapy program (see Figure 1). During these visits, several tests and scales were performed to assess motor function and functional status (see details below), and blood was extracted in EDTA tubes and centrifuged at 1500 rpm for 15 minutes to obtain plasma, which was stored at −80 °C until use.

***Functional/Motor Measures Assessment and Definition of improvement***

The battery of tests consisted of the modified Rankin scale (mRS, 0-6), the Granger's modified BI (12) (BI, 0-100), the Fugl-Meyer Assessment score for the upper extremity (FMA, 0-66), the Functional Ambulation Categories (FAC, 0-5), the Chedoke Arm and Hand Activity Inventory (CAHAI, 13-91) (13), the 10-meter walk test and the Medical Research Council (MRC) (scale 0-5) of the disabled hemisphere (upper and lower extremities at the proximal/distal level).

Improvement classifications were obtained after comparing the scores during follow-up visits versus baseline scores: in FMA was defined as an increase ≥ 10 points, described previously as the minimal clinical important difference (14). For the CAHAI, an improvement was defined as an increase of ≥ 7 points (15). For the 10-meter Walk Test, the walking velocity was calculated and improvement was considered if walking velocity increased by more than 0.3 m/sec (16). The FAC was categorized into three categories: "cannot walk" (score 0), "dependent walk" (score 1-3) and "independent walk" (score 4-5), and improvement was defined as a shift to an upper category (17). For MRC, our analysis differentiated between normal (score 5) or impaired (score 0 to 4) muscle strength.

***Plasma MMP measure***

Plasma levels of soluble MMP-3, MMP-12 and MMP-13 were measured with the MILLIPLEXR map Human MMP Panel 1 (HMMP1MAG, EMD Millipore, Germany) for plasma samples obtained at baseline (n=15), 1 month (n=13), 3 months (n=11) and 6 months (n=8). Briefly, 25 µl of undiluted plasma were loaded per well and the assay was run following the manufacturer's instructions. The assay sensitivity for the different proteins was: MMP-3, 0.062 ng/ml; MMP-12, 0.038 ng/ml and MMP-13, 0.034 ng/ml. Each sample was assayed in duplicate and only values with a CV < 20% were accepted for the posterior analysis.

***Statistical analysis***

Descriptive statistics were used to define demographics, stroke characteristics, MMP levels and functional measures. Chi-square tests were run for categorical variables. Normality of continuous variables was assessed by the Kolmogorov-Smirnov test if N ≥ 30 or the Shapiro-Wilk test if N < 30. To assess differences between independent groups, independent t-tests and one-way ANOVA followed by Bonferroni post-hoc tests were used for normally distributed variables, and the values were expressed as the mean ± s.d., while the Mann-Whitney U-test was used to explore differences in non-normally distributed variables and was expressed as median and interquartile range (IQR). For repeated measures, Friedman

followed by Wilcoxon tests were used for non-normally distributed variables, whereas for variables with a normal distribution, repeated ANOVA followed by the Bonferroni post hoc test was run. Correlations between continuous variables were measured using the Pearson's or Spearman's coefficients, depending on their normality. Finally, a p value <0.05 was considered statistically significant. All analyses were completed using SPSS 15.0 software.

## RESULTS

### *Characteristics of the stroke and control cohorts*

Table 1 shows baseline characteristics of the stroke rehabilitation cohort compared to controls. Briefly, stroke patients were significantly younger and had smaller body mass indexes than controls (p=0.008 and p=0.048, respectively). Males were overrepresented in the stroke group (80%) but not significantly. All other registered variables were balanced between groups. The main clinical variables related to the ischemic event are described in Table 2, which shows common features of the cohort of ischemic stroke candidates for IRT. In summary, median NIHSS on admission was 8 (5-12), and after 3-4 days it was 5 (5-9), indicating an improvement (increase ≥ 4 points) in 33.3% of the patients. Most strokes occurred in the carotid territory (73.3%), and almost half of these were classified as total anterior cerebral infarction (TACI, (46.7%)). Time from stroke onset to IRT was 11.4 ± 4.4 days.

**Table 1. Baseline characteristics of the control and stroke cohorts.**

| | **Control** (n=15) | **Strokes** (n=15) | **P value** |
|---|---|---|---|
| Age (years) | 63.5±7.6 | 53.9±10.6 | **0.008** |
| Gender, male | 46.7 (7) | 80.0 (12) | 0.058 |
| **Risk factors** | | | |
| Alcohol | 53.3 (8) | 20.0 (3) | 0.058 |
| Tobacco | 13.3 (2) | 33.3 (5) | 0.195 |
| Hypertension | 80.0 (12) | 66.7 (10) | 0.409 |
| Dyslipidemia | 60.0 (9) | 53.3 (8) | 0.713 |
| Diabetes mellitus | 26.7 (4) | 20.0 (3) | 0.666 |
| Atrial fibrillation | 0 (0) | 13.3 (2) | 0.143 |
| Obesity | 60.0 (9) | 33.3 (5) | 0.143 |
| Body mass index (kg/m²) | 25.9±3.6 | 23.0±3.8 | **0.048** |
| **Comorbidities** | | | |
| Osteoarticular disorders | 26.7 (4) | 13.3 (2) | 0.361 |
| Ischemic cardiopathy | 0 (0) | 6.7 (1) | 0.309 |
| Psychiatric disorders | 26.7 (4) | 20.0 (3) | 0.666 |
| **Previous Exercise** | | | |

| | | | |
|---|---|---|---|
| Physical activity | 73.3 (11) | 50.0 (7) | 0.196 |
| Physical activity (hours) | 7 (0-10) | 7 (0-10) | 0.288 |
| **Previous Medication** | | | |
| Antiplatelets | 26.7 (4) | 20.0 (3) | 0.666 |
| Anticoagulants | 0 (0) | 6.7 (1) | 0.309 |
| Statins | 33.3 (5) | 33.3 (5) | 1 |
| Antihypertensives | 73.3 (11) | 46.7 (7) | 0.136 |
| Antidiabetic | 20 (3) | 13.3 (2) | 0.624 |

Data is displayed as mean ± SD, median (IQR) or percentage % and (number). Significant p values are highlighted in bold.

**Table 2: Clinical characteristics of stroke patients**

| Stroke Characteristics | | Strokes (n=15) |
|---|---|---|
| **NIHSS score at admission** | | 8 (5-12) |
| **NIHSS motor score at admission** | | 4 (2-7) |
| **NIHSS score after 3-4 days** | | 5 (5-9) |
| **NIHSS motor score after 3-4 days** | | 3 (1-5) |
| **Early neurological outcome** | | |
| | Improvement | 33.3 (5) |
| | Stability | 53.4 (8) |
| | Worsening | 13.3 (2) |
| **Etiology** | | |
| | Lacunar | 13.3 (2) |
| | Cardiopathy | 33.3 (5) |
| | Atherothrombosis | 13.3 (2) |
| | Others | 13.3 (2) |
| | Unknown | 26.8 (4) |
| **Location, Territory** | | |
| | Vertebrobasilar | 26.7 (4) |
| | Carotid | 73.3 (11) |
| **Hemisphere of stroke** | | |
| | Right | 53.3 (8) |
| | Left | 46.7 (7) |
| **OCSP classification** | | |
| | TACI | 46.7 (7) |
| | LACI | 20.0 (3) |

| | | |
|---|---|---|
| | PACI | 12.3 (2) |
| | POCI | 20.0 (3) |
| **Symptomatic stenosis** | | |
| | Extracranial | 26.7 (4) |
| | Intracranial | 20.0 (3) |
| | Both | 6.7 (1) |
| | No | 46.6 (7) |
| **Asymptomatic stenosis** | | |
| | Yes | 33.3 (5) |
| | No | 66.7 (10) |
| **Thrombolytic therapy** | | |
| | Yes | 26.7 (4) |
| | No | 73.3 (11) |
| **Hemorrhagic Transformation** | | |
| | Yes | 20 (3) |
| | No | 80 (12) |
| **Time Stroke-Intensive Rehabilitation (days)** | | 11.4±4.4 |

OCSP: Oxfordshire Community Stroke Project; TACI: total anterior cerebral infarct, LACI: lacunar cerebral infarct, PACI: parcial anterior cerebral infarct, POCI: posterior cerebral infarct; Early Neurological Outcome was defined as improvement (increase ≥4 points in the NIHSS), worsening (decrease ≥4 points in the NIHSS) or stability any other change in the NIHSS. Data is shown as mean ± SD, median (IQR) or percentage % (number).

### *Evolution of motor/functional outcomes and MMP levels over time*

The complete motor and functional progress of patients during IRT is detailed in Table 3. A significant improvement in the FMA, Rankin and BI tests had already been achieved at 1 month and was maintained during the complete follow-up period. At three months, the patients also showed some improvement in the FAC, CAHAI and 10-meter walk test. Patients showed minor improvements in MRC scores for the proximal segment of the extremities at three or six months.

**Table 3: Measures of functional/motor outcome and MMP profile**

| **Strokes** | **Time course** | | | |
|---|---|---|---|---|
| | **Baseline** | **1 month** | **3 months** | **6 months** |
| **Outcome assessment** | | | | |
| **Fugl Meyer Assessement (0-66)** | 30 (6-52) | 55 (14-61.5)* | 60 (16-63)* | 64 (26-66)* |
| **Modified RANKIN (0-6)** | 4 (3-4) | 2 (1-3)* | 1.5 (0-3)** | 1 (0-2)* |
| **Barthel Index (0-100)** | 57 (31-83) | 94 (64-100)** | 100 (88.6-100)** | 100 (100-100)** |
| **Functional Ambulation Categories (0-5)** | 1 (0-3) | 4 (1.5-5)† | 5 (5-5)* | 5 (5-5)* |

| | | | | | |
|---|---|---|---|---|---|
| **Chedoke Arm and Hand Activity Inventory (13-91)** | | 15 (13-71) | 72 (13-85)† | 82 (13-90) | 88 (13-91) |
| **10 meters walk test (velocity in m/sec)** | | 0.5 (0-1) | 0.9 (0.3-1.4)† | 1 (0.8-1.3) | 1.1 (0.8-1.4) |
| **Stroke Specific Quality Of Life Scale (49-245)** | | n.a. | 168 (152.5-183) | 193 (159-227) | 199 (171-229)$ |
| **MRC Superior-Proximal (0-5)** | | 4 (0-4) | 4 (2.5-5) | 5 (2-5)* | 5 (4-5)* |
| **MRC Superior-Distal (0-5)** | | 4 (0-4) | 4 (0-5) | 5 (0-5) | 5 (2-5) |
| **MRC Inferior-Proximal (0-5)** | | 4 (0-4) | 5 (4-5) | 5 (5-5)† | 5 (5-5)† |
| **MRC Inferior-Distal (0-5)** | | 4 (0-5) | 5 (2-5) | 5 (4-5) | 5 (1-5) |
| **MMP level (ng/mL)** | | | | | |
| | **MMP-3** | 28.2±14.8 | 23.6±8.8 | 32.5±13.4 | 27.7±12.3 |
| | **MMP-12** | 2.0±0.9 | 2.0±1.0 | 2.1±0.9 | 2.1±0.8 |
| | **MMP-13** | 4.5±1.7 | 4.5±2.6 | 4.3±1.8 | 4.6±2.1 |
| **Controls** | | | | | |
| **MMP level (ng/mL)** | | | | | |
| | **MMP-3** | 24.0±14.3 | | | |
| | **MMP-12** | 2.0±0.5 | | | |
| | **MMP-13** | 4.4±0.7 | | | |

Data is shown as mean ± s.d or median (IQR); n.a. (non available); *p<0.05, **p<0.01 and † p<0.1 vs. baseline. FMA (Fugl Meyer Assessement), FAC (Functional Ambulation Categories), CAHAI (Chedoke Arm and Hand Activity Inventory) and MRC (Medical Research Council).

Regarding MMPs measurements, a baseline sample was obtained 10.7 ± 5.0 days after stroke onset and no correlation was observed between the MMP levels and the number of days after stroke (MMP-3: r=-0.94, p=0.761; MMP-12: r=0.118, p=0.688; MMP-13: r=0.296, p=0.304). The patients exhibited stable MMP levels during the whole study period, and no differences with controls (see table 3). Interestingly, MMP-12 and MMP-13 were correlated both in controls (r=0.925, p<0.001) and in stroke patients (r=0.951, p<0.001 at baseline; r=0.984, p<0.001 at 1 month; r=0.993, p<0.001 at 3 months and r=0.979, p<0.001 at 6 months), but no relationship was found with MMP-3.

Regarding MMP-3 in stroke patients, age was positively associated with higher levels at three and six months (r=0.676, p<0.05 and r=0.873, p<0.01, respectively), hypertension with higher levels also at 3 and 6 months (36.2 ± 11.9 vs. 16.0 ± 17.9, p<0.05 and 32.6 ± 9.5 vs. 12.9 ± 4.6, p<0.05) and atrial fibrillation also with higher levels at 3 months (49 ± 6.5 vs. 28.8 ± 11.7; p<0.05). At 6 months higher levels of MMP-12 and MMP-13 were detected in dyslipidemic patients (2.7 ± 0.6 vs. 1.4 ± 0.4 and 6.2 ± 0.6 vs. 3.0 ± 1.2, p<0.01 respectively). In the control cohort males presented higher levels of MMP-3 (35.1 ± 13.6 vs. 14.1 ± 3.0, p<0.01) and higher levels of MMP-12 in subjects with diabetes (2.5 ± 0.4 vs. 1.9 ± 0.4, p<0.05). Regarding medication, higher levels of MMP-12 were found in subjects medicated with antiplatelets, statins and of MMP-13 in those subjects taking antiplatelets, statins or antihypertensive drugs (data not shown).

***High levels of MMP-12 and MMP-13 are associated with stroke severity***

We found a strong association between baseline MMP-12 and MMP-13 levels and stroke severity, as measured by the NIHSS score on admission (r=0.81 and p<0.001 and r=0.67 and p=0.008, respectively; see Figure 2A). MMP-12 was also increased in patients with higher motor scores at the NIHSS (r=0.625 and p=0.017). Interestingly, these two MMPs were elevated in patients who were diagnosed with a TACI and presenting the most extensive infarcts (p=0.009 for MMP-12 and p=0.058 for MMP-13 at baseline; see Figure 2B). No significant association were found for MMP-3 (see Figure.2A-B), except for a statistical trend correlating higher levels of baseline MMP-3 with patients with a lower motor NIHSS score on admission (r=-0.514 and p=0.072).

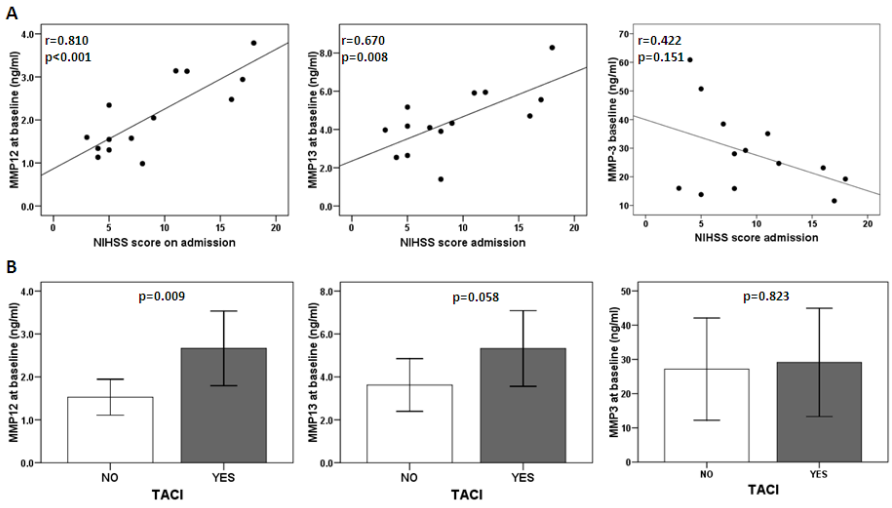


**Figure 2. Association of MMP levels and baseline stroke severity and extension. A)** Scatter plots showing significant associations between baseline MMP-12 and MMP-13 with NIHSS scores on admission. **B)** Bar graphs showing higher levels of MMP-12 and MMP-13 in patients classified as TACI vs. those with other classifications (LACI, PACI, POCI), according to the OSCP classification.

***Elevated MMP-3 and decreased MMP-12 or MMP-13 levels in patients with a better motor score during rehabilitation***

Despite the strong relationship between pre-rehabilitation and 6 months scores for several functional/motor scores (see supplementary Table 1), higher levels of MMP-3 were found in the patients presenting higher walking velocity at one month of IRT (r=0.579 and p=0.079). In addition, lower baseline levels of MMP-12 and MMP-13 were observed in patients who experienced a better outcome in the MRC upper distal extremity scale (p=0.006 for MMP-12 and p=0.039 for MMP-13) at one month after or in patients who had a better outcome at three and six months in the lower proximal extremity MRC scale (p=0.026, respectively for baseline MMP-12 levels). These data are also represented in supplementary Figure 1.

***The best motor and functional recovery is associated with higher plasma levels of MMP-3 and lower plasma levels of MMP-12 and MMP-13***

Our results reveal that patients with higher levels of MMP-3 at one month presented larger improvements in motor function, assessed by the FMA score, at the same time point (r=0.632 and p=0.05); this was also the case at 3 months (r=0.780 and p=0.013) but to a lesser extent at 6

months (r=0.596 and p=0.097), as shown in Figure 3A. Similarly, we found that higher levels of MMP-3 were associated with major improvements in the CAHAI score at three months (r=0.566 and p=0.07) and at the study end of the study (r=0.737 and p=0.037); see Figure 3A.

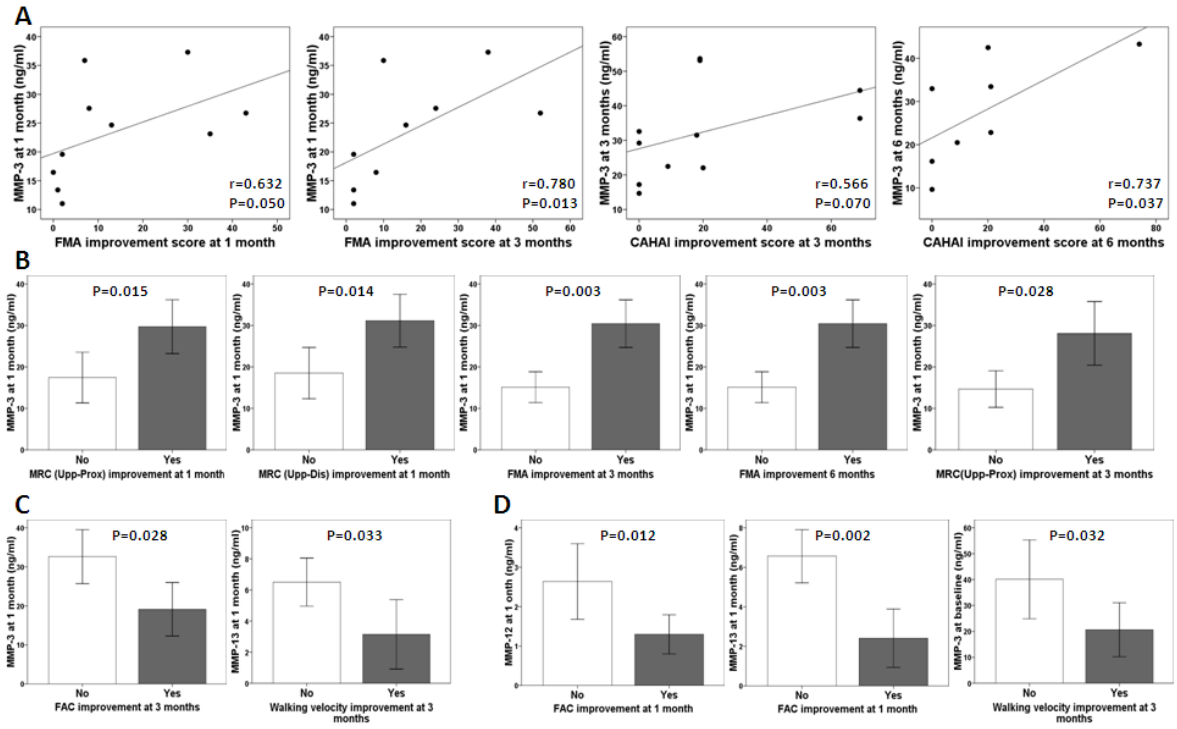


**Figure 3. Associations between MMP-3, MMP-12 or MMP-13 and motor recovery. A)** Scatter plots showing a positive correlation between MMP-3 and improvement in FMA or CAHAI scores at different time points during the study period. **B)** Bar graphs showing that high MMP-3 levels one month after starting rehabilitation therapy predicted motor improvement assessed by MRC or FMA scores. **C)** Bar graphs showing FAC score and the 10-meter walk tests improvement in patients with lower MMP-3. **D)** Bar graphs showing elevated plasma MMP-12 and MMP-13 in patients that did not improve in the walking and FAC tests.

During rehabilitation therapy, patients were grouped into those who improved and those who did not improve for each of the outcome measures in the terms defined in the Methods section. We confirmed our findings for MMP-3 because stroke patients who responded better to rehabilitation therapy presented higher plasma levels of this protein at one month than patients who did not show improvement in the MRC of the upper proximal extremity (p=0.015) or in the MRC of the upper distal extremity score (p=0.014). The same results were observed for FMA and MRC upper proximal scores (p=0.003 and p=0.028, respectively) at three months and for FMA scores at six months (p=0.003), as shown in Figure 3B. These results suggest the predictive value of MMP-3 for identifying patients who will respond better to rehabilitation.

Two contradictory results connected improvement on the FAC score and in the walking test at three months with lower levels of MMP-3 (p=0.028 and p=0.033, respectively), as shown in Figure 3C. In the same line of results, the increase/decrease in the walking velocity at three and six months was inversely correlated

with baseline MMP-3 levels (r=-0.647, p=0.031 and r=-0.683, p=0.02; respectively).

Finally, plasma MMP-12 and MMP-13 were associated with the poorest (Figure 3D). One month after starting the rehabilitation program, we observed the highest levels of MMP-12 and MMP-13 in the patients that did not show an improved FAC score (p=0.012 and p=0.002, respectively) and high MMP-12 in those that did not show an improvement in the walking test (p=0.051). Moreover, higher MMP-13 at one month was associated with the poorest level of performance in the walking test at the third month (p=0.032).

## DISCUSSION

The present study describes for the first time the temporal profile of plasma MMP-3, MMP-12 and MMP-13 up to 6 months after ischemic stroke in patients receiving IRT. Our results describe the association of MMP-12 and MMP-13 with stroke severity and the poorest neurological outcomes and identify MMP-3 as a potential molecule to monitor greatest improvements, suggesting that MMP-3 could serve as a biomarker of post-stroke recovery and could help in identifying those patients that will respond better to the rehabilitation therapy.

Over the last decades, acute stroke care has improved, with the development of stroke units, hyperacute treatment with recombinant tissue plasminogen activator, and new thrombectomy strategies (18-20). However, rehabilitation therapies remain the only approved treatments for the millions of patients who each year survive a stroke but experience functional deficits. Evidence-based stroke rehabilitation care includes several types of health interventions, including early admission to specialized stroke rehabilitation units, intensive rehabilitation therapies, and task specific oriented therapies (3, 5, 21) with the goal to help survivors become as independent as possible so that they can conduct daily living activities and achieve the best possible quality of life in the long-term. As we have reported for the stroke rehabilitation cohort in the present study, the most significant improvements are achieved within the first one to three months, but recovery continues for up to 6 months, as described (22).

Although recent meta-analyses have shown strong evidence for stroke rehabilitation intervention (23, 24), daily practice indicates that individual patients respond differently to therapy, which raises several questions regarding which patients will benefit the most and which patients will respond better to larger and more intensive programs. Questions relating to the underlying biological mechanisms of neural plasticity that drive improvements in neurological functions remain to be answered, to known whether they can be therapeutically modulated to improve rehabilitation therapies.

MMPs are proteolytic enzymes that can degrade most components of the extracellular matrix and remodel the pericellular space. They are normally expressed in most tissues and take part in multiple physiological processes (25). However, uncontrolled MMP activity is involved in the pathophysiology of many disorders with underlying inflammation processes. In cerebral nervous system-related diseases, some MMPs have been associated with neurodegeneration, demyelination, neurotoxicity and blood brain barrier dysfunction (26, 27), but emerging evidence has positioned these proteases as key

players in tissue regeneration processes, including post-stroke repair (8, 26). To our knowledge, our study shows the longest temporal profile of MMP levels in stroke patients: from the sub-acute to the chronic phase.

Plasma MMP-3, MMP-12 and MMP-13 remained stable over time without major fluctuation but with two opposite behaviors. In the current study, elevated baseline MMP-12 and MMP-13 levels were found in patients who had more severe infarcts, whereas MMP-3 remained independent of stroke characteristics. Previous results from our group have described the expression of nuclear MMP-13 in neuronal cells after ischemia, where it is acutely elevated in the infarct tissue (28, 29). In another study, we reported the role of MMP-13 as a plasma biomarker for lesion expansion, as measured in diffusion-weighted images in the first days following stroke (30). With regards to its function as a major collagenase, other authors have shown that MMP-13 expression is increased in the BBB-damaged vessels of spontaneous hypertensive rats (31). These data support our finding that higher baseline MMP-13 levels are related to infarct extension and stroke severity. However, other investigations have reported the activation of MMP-13 by two weeks after ischemiareperfusion, which is associated with perineuronal matrix remodeling (32) and suggests its role in neuronal remodeling. Less is known about MMP-12, which is also named macrophage elastase. In an experimental hemorrhagic stroke model, MMP-12 expression was associated with poor functional recovery of forelimb function and with the development of secondary injury (33). More recently, the specific knockdown of MMP-12 by the administration of a shRNA-expressing plasmid (silencing the gene) resulted in brain neuroprotection in a rat model of transient ischemia via MMP-9 downregulation (34). Again, published studies have related brain MMP-12 expression with larger infarct size and worse neurological outcome, which is in agreement with our results.

Finally, the most striking results of our study are related to plasma MMP-3, a member of the stromelysin sub-family that degrades multiple extracellular matrix components, activates growth factors and cleaves adhesion molecules, among other functions (26). We demonstrate, for the first time, an association for MMP-3 not only with better neurological status but also with improved motor recovery at later time points during rehabilitation therapy. Importantly, baseline MMP-3 levels were not related to stroke characteristics nor were they correlated with baseline motor/functional prerehabilitation scores. On the other hand, when assessing improvement during the follow-up using the 10 meter walk test we observed contradictory results. Here we could speculate that important achievements such as better stability could not translate into higher walking velocity. MMP-3 also, like other MMPs, plays a dual detrimental/remodeling role, and an extensive literature supports its central position in brain tissue embryonic and postnatal development, in addition to its involvement in axonal extension, synaptic plasticity and remyelination (26, 35). Experimental models of stroke have shown that MMP-3 expression is critical for intracranial bleeding after tPA administration (36) and for perineuronal agrin cleavage after ischemiareperfusion in rats (37). Interesting results how a single administration of a broad spectrum MMP inhibitor (GM6001) after ischemia, by protecting BBB integrity acutely, enhanced vascular remodeling at three weeks related to increased MMP-3 and tight junction

expression (38).

The use of biological samples to detect the presence of specific molecules with the aim of monitoring ongoing pathophysiological processes and predicting neurological outcomes for stroke survivors is the focus of current clinical and experimental research (39, 40). However, very few studies have investigated the use of biomarkers during rehabilitation therapy. Blicher and colleagues studied the neurotransmitter GABA by magnetic resonance spectroscopy in relation to motor function after 2 weeks of constrained-induced movement therapy and showed an association between the extend of motor improvement and the magnitude of the GABA/creatine ratio (41). A second study recently reported the use of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a sensitive biomarker for oxidative DNA damage that is detectable in urine samples, in a cohort of stroke patients undergoing rehabilitation therapy (42). The authors reported a negative correlation between FMA scores and 8-OHdG levels after rehabilitation treatment and lower levels of baseline in patients that exhibited more improvement in motor function, suggesting the use of 8-OHdG as a biomarker of stroke recovery.

Nonetheless, several caveats of this study should be discussed. First, our study does not provide a control cohort of stroke patients with similar characteristics that did not receive IRT to determine the exact effect of rehabilitation therapy on MMP levels. However, this is for the understandable reason that ethical principles demand that all patients admitted at our hospital who are candidates for receiving IRT must be enrolled in the program. In addition, the lack of power due to the number of patients did not allow us to perform multivariate analysis to explore possible interactions between the clinical variables and the biomarkers. The designing of future multicenter studies will overcome this limitation and allow a more detailed analysis.

## CONCLUSIONS/IMPLICATIONS

The ability to monitor stroke recovery using biomarkers would be of great help when making decisions related to a patient's management and rehabilitation programs. To our knowledge, this is the first report on ischemic stroke patients who were enrolled to IRT to describe the temporal profile of plasma MMPs over a 6-month follow-up period, and we show exciting results regarding the association of MMP-3 with the best motor recovery. This observation could be related to the role of MMPs in brain plasticity and tissue regeneration. Regardless of the preliminary nature of our study and the need to design larger studies in a multi-center setting, we can conclude that MMPs have emerged as potential biomarkers of recovery during rehabilitation therapy following stroke.

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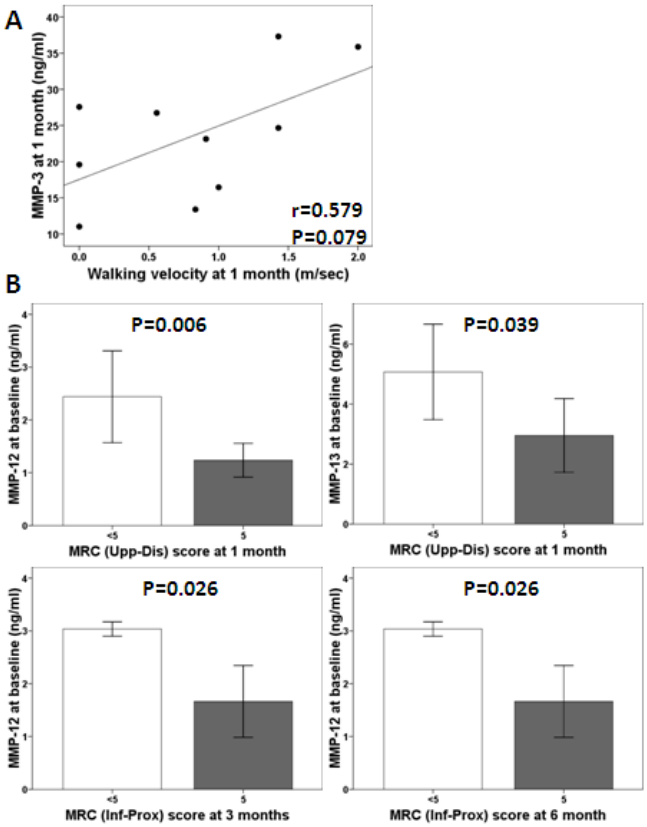
**Supplementary Table1. Association study between pre-rehabilitation and 6 months scores of functional and motor measures.**

| | RANKIN | FMA | CAHAI | WALKING | SS-QOL | MRC (Upp-Prox) | | MRC (Upp-Dis) | | MRC( Inf-Prox) | | MRC (Upp-Dis) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | r | r | r | r | 0-4 | 5 | 0-4 | 5 | 0-4 | 5 | 0-4 | 5 |
| **NIHSS (admission)** | | -0.55† | | | | | | | | 14.5 (12-17) | 7 (4.5-8.5)* | | |
| **NIHSS motor (admission)** | | | | | | | | | | 7.5±0.7 | 3.0±2.6* | | |
| **NIHSS (3-4days)** | 0.723** | -0.678* | -0.568† | | | 10.5 (8.3-13.5) | 5 (4-7)* | 10.5 (8.3-13.5) | 5 (4-7)* | 13 (12-14) | 5 (4.5-8.5)* | 12 (8-14) | 5 (4.3-8.5)* |
| **NIHSS motor (3-4days)** | 0.586* | -0.797** | -0.67* | -0.597† | -0.812** | 5.5±2.6 | 2.1±2.2* | 5.5±2.6 | 2.1±2.2* | 7.5±0.7 | 2.4±2.1* | 6.7±1.5 | 2.1±2.0** |
| **RANKIN** | 0.835** | -0.681* | -0.575† | -0.681* | -0.592† | 4 (4-4) | 3 (3-4)* | 4 (4-4) | 3 (3-4)* | | | 4 (4-4) | 3 (3-4)† |
| **BI** | -0.871** | 0.888** | 0.745** | 0.603* | 0.670* | 36 (18.3-41) | 79 (62-95)* | 36 (18.3-41) | 79 (62-95)* | 22.5 (14-31) | 70 (41-89)† | 31 (14-41) | 74.5 (46.3-92)* |
| **FMA** | -0.707* | 0.841** | 0.784** | 0.528† | 0.602† | 4 (2.5-6.3) | 50 (23-55)* | 4 (2.5-6.3) | 50 (23-55)* | 3 (2-40) | 47 (6.5-54)* | 4 (2-4) | 48.5 (11-54.5)* |
| **FAC** | -0.836** | 0.789** | 0.674* | 0.765** | 0.696* | 0 (0-0.8) | 3 (1-5)* | 0 (0-0.8) | 3 (1-5)* | 0 (0-0) | 2 (0.5-4.5)† | 0 (0-0) | 2.5 (1-4.8)* |
| **CAHAI** | -0.612* | 0.875** | 0.876** | | | 13 (13-13) | 70 (13-71)* | 13 (13-13) | 70 (13-71)* | | | 13 (13-13) | 68.5 (13-71)† |
| **WALKING** | -0.791** | 0.711* | 0.58† | 0.813** | 0.879** | 0 (0-0.4) | 1 (0.5-1)* | 0 (0-0.4) | 1 (0.5-1)* | 0 (0-0) | 0.5 (0.3-1)† | 0 (0-0) | 0.8 (0.5-1)* |
| **MRC (Upp-Prox)** | -0.536† | 0.860** | 0.879** | | | 0 (0-0) | 4 (4-4)** | 0 (0-0) | 4 (4-4)** | | | 0 (0-0) | 4 (4-4)* |
| **MRC (Upp-Dis)** | | 0.690** | 0.593† | | | | | | | | | | |
| **MRC (Inf-Prox)** | -0.566† | | 0.609* | 0.548† | 0.673* | 1 (0.3-3.3) | 4 (4-5)* | 1 (0.3-3.3) | 4 (4-5)* | | | 0 (0-1) | 4 (4-5)* |
| **MRC (Inf-Dis)** | | 0.571† | | 0.588† | 0.732* | | | | | 0 (0-0) | 4 (2-5)† | 0 (0-0) | 4.5 (4-5)* |

Note: ** p <0.01, *p <0.05 and † p<0.1




**Supplementary Figure 1. MMP levels and motor scores during rehabilitation. A)** Correlation plot showing the association between MMP-3 levels and the walking velocity assessed at 1 month. **B)** Bar graphs showing the difference in baseline MMP-12 and MMP-13 levels between patients that showed normal or weakened muscle strength at different time points assessed by the MRC score.

# 4. Discussion

## 4.1. MMP-13 deficiency protects mice from ischemia-reperfusion injury whereas high level of plasma MMP-12 or -13 is associated with stroke severity in ischemic stroke patients.

The present study firstly demonstrates that MMP-13 participates in acute damage which is enhanced during the reperfusion phase in a mouse model of stroke and related to the presence of spontaneous HT. In parallel we have described for the first time the association of MMP-12 and MMP-13 with stroke severity and with the poorest neurological/motor outcomes in patients under neurorehabilitation therapy.

It is known that ischemic damage induces the unregulated expression of several MMPs at multiple levels (brain and plasma), which has been documented previously both in humans and rodent models of cerebral ischemia[71,123,124,125]. Most of these papers focus on MMP-9, a gelatinase that has been extensively studied in the ischemia context, by proving that its gene or pharmacological inhibition protects from ischemia injury and reduces hemorrhagic transformations[123,126,127]. However we know that other MMPs are also dysregulated in the human infarct tissue suggesting its implication in tissue damage. In this regard a previous study from our group showed that several members of the MMP-family of proteases, including MMP-9 and -13 among others, were upregulated in the post-mortem infarct tissue of patients who had an ischemic stroke within the previous 4 days when compared to the contralateral hemisphere[81]. Furthermore, it is known that MMP-9 is able to be activated directly after stroke onset, with the plasma level correlated with the infarct size and functional outcome[87,128] , while only a few studies shows the MMP-13 activation in the brain/plasma related to lesion expansion in the acute phase[101,102]. More recently, increased MMP-13 mRNA and protein levels were observed in brain tissue during the acute phase in a rodent model of ischemia-reperfusion[129].

The two primary outcomes in preclinical studies are infarct lesion reduction and the improvement in neurological function which might translate to an improved neuroprotection or functional outcome clinically. The quantification of infarct size can be obtained by several methods such as TTC staining for experimental models or MRI for either animal or human subjects. However, more variability exists on the tests used to assess functional status or neurological outcomes in rodents[130,131,132,133,134], whereas in humans, the NIH stroke Scale score and the modified Rankin scale being the gold standard methods to evaluate stroke severity and functional outcome[135,136,137], respectively.

Our results show a significant reduction on infarct size in MMP-13 gene knock-out (KO) mice compared to wild-type (WT) at one and three days after cerebral ischemia-reperfusion, but not after permanent MCAo although infarct volumes were also smaller in MMP-13 deficient mice. In fact, differences between the two models were observed in the WT groups, being infarcts of the ischemia-reperfusion model larger than in the permanent occlusion but not significantly.

The observed neuroprotection in terms of infarct volume was correlated with our observation on the reduced incidence of spontaneous HT events in MMP-13 KO mice. Spontaneous HT occurs in patients with ischemic stroke mainly between the 3th and the 7th day of stroke onset[138], and the disruption of BBB is a central step that precedes HT development. Early within the first 2 hours, the opening of BBB has been observed in stroke patients and enhanced in later phases[139]. In the delayed HT, the opening of BBB is thought to be mediated by MMPs, other brain proteases, neuroinflammation, as well as vascular remodeling and neovascularization[30]. It has been described that MMPs derived from blood have a dominant role in the early BBB disruption and HT, while the brain derived MMPs such as MMP-9, MMP-2 and MMP-3 contribute to the delayed HT[140,141,142]. Related to endogenous neurorepair, we have explained that vascular remodeling and angiogenesis are important for the recovery after stroke[143], and pro-angiogenic factors may also promote post-stroke HT[144,145]. For example, VEGF is an important factor involved in angiogenesis and a decrease on VEGF reduces HT in rodent stroke model[146].

Our results show a tendency to an increased incidence of hemorrhage at day 3 which might indicate the involvement of MMP-13 in the delayed HT. Although HT is associated with the size of ischemic area[147], our study seems to be independent to this fact since similar infarct size were observed at 24 hours and 72 hours after ischemia-reperfusion in WT mice who increased the incidence of HT at 72 hours. At the same time, no such increase was found in MMP-13 KO mice, indicating that MMP-13 deficiency may protect the mice brain tissue not only from initial tissue injury but also from the upcoming hemorrhage. Regardless the day of euthanasis, WT mice presented increased incidence of HT events than MMP-13 KO mice.

We have also observed the decreased amounts of brain angiogenic factors including VEGF and Ang-2 in MMP-13 KO mice at day 7 after ischemia. The combination of VEGF and Ang-2 has been demonstrated to lead angiogenesis related to BBB disruption by increasing the activity of MMP-9 after 2 weeks treatment in mice[148]. The biphasic role of VEGF in stroke has been described in a previous study with early VEGF promoting HT and later VEGF promoting BBB integrity and vessel function[149].

With this background, MMP-13 may also play a similar dual role directly and indirectly in HT in the first 7 days after stroke.

One unexpected finding is the presence of striatal infarctions at day 3 after ischemia-reperfusion, related to the presence of hemorrhage within the infarct area. Since our model or MCA occlusion only affects the cortical territory, we hypothesize that the growing formation of a hematoma induced by the hemorrhage may constrict the vascular supporting blood flow to striatum. Unfortunately, most studies based on neuroprotection assess infarct size and extension at 24-48 hours making difficult to compare this finding with others.

However, our study also includes a longer time follow-up of the mice to study neurorepair mechanisms, and it seems that the final functional outcome at day 14 was not different between MMP-13 KO and WT mice which presented larger infarcts and more incidence of HT events acutely (although these observations are performed in separate study groups). This could suggest that WT mice have repaired/recovered better or faster than that in MMP-13 deficient mice and our next findings support this hypothesis.

The other primary outcome mentioned at the beginning of this section is the functional outcome. In this regard, the appropriate tests should be chosen accurately according to the stroke models, evaluation time and animal species even strains[130]. The grip strength test is highly specific, well-defined aspect of behavior and less stressful to the animals than other tests. KIlic *et al*. found that grip test is sensitive for detecting outcomes from different treatments in C57BL/6 mice after mild transient ischemia at day 7 as well as day 30[150]. In our hands, a significantly reduced forelimb force was also found from day 1 until day 14 in permanent distal MCA occluded Balb/c mice[130]. In the present study, the grip strength test has proven to detect reduced forelimb function after transient MCAo in C57BL/6 mice as long as 14 days after ischemia. At day 1, all mice decreased the forelimb force however this decrease was only significant in WT mice, in agreement with our findings on infarct volume and HT incidence. During the follow-up only the decreased forelimb force in WT versus MMP-13 KO was maintained at one week but not at two weeks. This fact could indicate different repair mechanisms between genotypes.

It is interesting to link the MMP-13 observation in the animal model of ischemia with the findings in patients under rehabilitation therapy after stroke: high levels of MMP-13 were observed in those patients with more extensive infarcts (TACI classification) and presenting a more severe neurological status at baseline

(measured by the NIHSS). Although blood samples were obtained in some cases days after the baseline neurological evaluation no association was found between plasma MMP levels and the day of baseline blood sampling. The expression of MMP-13 is increased in the BBB-damaged vessels of rats[151], and the role of plasma MMP-13 as a biomarker for the lesion expansion has been proposed in a previous study from our group[102]. Together with our finding in the experimental model, we can conclude that MMP-13 contributes to the acute cerebral damage further contributing in BBB damage. It would have been interesting to analyze the association of MMP-13 level with the presence of spontaneous HT, however the sample size was too small to speculate on this interesting point that certainly deserves to be further investigated.

Overall, we demonstrate the role of MMP-13 in brain injury after ischemia however our group and other have reported the active role of other MMPs related to brain infarcts, as detailed in the introduction section. Therefore, which MMP is more important? Are these MMP actions related?

The activation of MMP proteins by other members of the family has been widely described[152,153]. We have studied the protein levels of infarct MMP-3, -8 and MMP-9 compared to their contralateral hemisphere describing a significant increase at day 3 after ischemia in both genotypes, with a significant decrease of all studied MMPs in the ipsilateral hemisphere of the MMP-13 KO mice compared to WTs. In our study, the detection of MMPs was designed to measure the total amount of MMPs including pro and activated forms except for MMP-9 (only the pro-form) due to manufacturer setting of the kit. We included one collagenase (MMP-8), one relevant gelatinase as MMP-9 and one stromelysin (MMP-3) as representative proteins of the MMP family that could undergo compensatory, up- or down- regulations in the knockdown model of MMP-13.

Our conclusion is that the decreased level of MMP-3, -8 and pro-MMP-9 in MMP-13 KO mice in the context of cerebral ischemia indicates that the interaction of MMP-13 with other members of MMPs family might be controlling the MMP cascade. MMP-8 is known as the “neutrophil collagenase” targeting primary fibrillar collagen which is upregulated in the infarct tisue[81]. In our study, the knockdown of MMP-13 does not stimulate the overexpression of MMP-8, instead inhibits the expression of this collagenase after ischemia indicating the possible central and upstream role of MMP-13. In the present study, we may conclude that MMP-13 deficiency affects the expression of MMP-9, and this gelatinase has been directly involved in both injury and repair mechanisms after stroke[71,76]. In a previous study from our group both MMP-9 and MMP-13 showed an association with infarct lesion expansion after

stroke in a multiple screening protein array of the MMP family[102]. Other studies have demonstrated that the knockdown of MMP-12 inhibited the increased MMP-9 activity in ischemic brain[154] suggests that the interaction among MMPs in the ischemia context is possible.

### 4.2. MMP-13 deficiency inhibits spontaneous neurorepair by reducing the number of proliferating neuroblasts and angiogenesis in peri-infarct areas, while decreased level of plasma MMP-12 or -13 is associated with better motor recovery in patients.

This thesis shows how the deficiency in MMP-13 reduces the amount of newly born neuroblasts in the peri-infarct areas during neurological recovery and alters stroke-induced angiogenesis by reducing peri-infarct vessel density and suppressing the amounts of G-CSF, Ang-2 and VEGF-A after cerebral ischemia. In stroke patients, high MMP-12 and -13 levels during rehabilitation were found in patients with a worst recovery, however those levels were found associated to stroke severity at baseline.

We have learnt that the regulation of MMPs during post-stroke might promote a therapeutic effect[71] at both acute and stroke repair phases, and most therapeutic approaches under investigation now focus on the enhancement of endogenous repair mechanisms such as neurogenesis and angiogenesis[155]. In our experimental ischemia study, a similar cortical atrophy and functional outcome were observed among genotypes at day 14 after ischemia, which could be a balanced consequence of both the inhibition of cell apoptosis and/or promotion of post-stroke repair mechanism. We prove that as least angio-neurogensis progression is altered in mice lacking MMP-13.

Our results show a significant reduction in the number of proliferating neuroblasts (BrdU+/DCX+ cells) migrated from the SVZ towards to the peri-infarct cortex in MMP-13 KO mice at day 14 after stroke, despite the total amount of neuroblasts in those areas did not change among genotypes. We explored if new mature neurons were generated in those areas but numbers were similar among genotypes. In this regard, a study focused on toll-like receptor 4 in focal cerebral ischemia model described some BrdU labeled cells in the peri-infarct cortex co-stained with an interneuron marker at day 14, indicating that the new generated neuroblasts after stroke were able to maturate into neurons[156]. In the same line, Saha *et al.* found that, only a few number of proliferating cells present in areas surrounding the lesion expressed NeuN, while most of them co-express GFAP and Olig2[157]. Therefore, it seems possible that other neuronal cells or progenitors could be under proliferation in our peri-infarct tissue.

Importantly, a positive association between the number of proliferating neuroblast and vessel density was observed in the present study. Neurogenesis has been shown to be coupled to angiogenesis in a post-stroke neurovascular niche[158]. Other authors have also identified that the proliferating neuroblasts in peri-infarct areas are closely

associated to the blood vessels[159]. The mechanism underlying this crosstalk may involve the vascular network acting as migration scaffolds as well as being the source of pro-neurogenic and migratory factors such as VEGF and SDF-1[160]. Delayed inhibition of SDF-1 at day 7 suppresses neovascular remodeling and impairs functional recovery at day 14[93].

In line of these studies we have observed smaller vessel density in ipsilateral peri-infarct tissue of MMP-13 KO mice compared to WTs at 14 days, but not at day 7 post-ischemia. However, we found a suppressed amount of thropic factors in MMP-13 KO mice at day 7, such as VEGF-A, G-CSF and Ang-2, which occurs prior to the observation of decreased vascular density. Additionally, these factors may contribute to the recovery after ischemia damage: VEGF-A is one of the VEGF family, which plays multiple roles in the normal adult brain, including vascular homeostasis, neurogenesis, synaptic plasticity and neuronal activity[161] and similar functions have been found after pathological stimuli[162]. VEGF-A protein expression increases in neurons, astrocytes and macrophages after ischemia in rats[163]. At 24 hours after MCAo in rats, intraventricular administration of VEGF-A for 3 days increased the post-ischemic BrdU labeling in neuronal cells as well as immunoreactive endothelial cells [164]. What's more, the effect of a cell-based therapy aiming to enhance post-stroke functional recovery could be enhanced by the combination with VEGF-A in several MCAo models[162]. G-CSF is a hematopoietic growth factor produced by activated monocytes as well as endothelial cells, fibroblasts, mesothelial cells and platelets[165,33]. G-CSF and its receptors are expressed in response to ischemia in the penumbral region of ischemic stroke, and markedly improve long-term behavioral outcome[166]. The up-regulated expression in the subacute phase has been proven predominantly in vascular cells[167] and the G-CSF administration after tMCAo enhances angiogenesis as well as neurogenesis at day 7 after ischemic brain injury in rats[168]. Additionally, Ang-2 is typically expressed at sites of vascular remodeling in the adult tissue, which is dramatically increased in the infarct cortex and maintained for as long as 7 days after ischemia in mice[169,170]. The combination of VEGF and Ang-2 promotes more angiogenesis at 2 weeks after treatment in the adult mice brain compared to VEGF alone[148]. Besides, Ang-2-enhanced migration and neuronal differentiation of SVZ neural progenitor cells at day 7 after MCAo in mice; while Ang-2 enhanced migration could be blocked by matrix metalloproteinase inhibitor[171]. Finally, EPCs involved in vascular remodeling after ischemia have been proven to secrete a combination of these factors including fibroblast growth factor (FGF-b), platelet-derived growth factor (PDGF-bb) and VEGF, among others[122]. FGF has shown to reduce the infarct volume in ischemic rats in the subacute phase, which may

involves post-stroke angiogenesis and neurogenesis[172,173]. The expression of PDGF is upregulated in neurons, reactive astroglial cells and pericyte after ischemic stroke in humans and animals[174,175], and the platelet microparticles containing VEGF, FGF and PDGF significantly promote neural stem cell proliferation, survival and differentiation, which can be partly blocked by any specific inhibitor[176].

Whether the decreased neurogenesis in MMP-13 KO mice was finally caused by the direct actions of MMP-13 or by the relatively smaller infarct size was not fully addressed in the present study. A strong negative correlation between cell proliferation at the ipsilateral SVZ at day 14 and the infarct size at day 2 has been reported in a filament induced permanent MCAo model[156], while other studies have demonstrated that MMP-9 directly contributes to the increased neuroblast migration after stroke, with MMP-3 being involved in the neural progenitor cells' migration[94,89]. In this line we have shown results on suppressed expression of MMP-3 and -9 in the MMP-13 KO genotype which could explain a modulation on neuroblast migration.

However, in stroke patients, we have observed an association between decreased level of plasma of MMP-12 and -13 with better motor recovery during rehabilitation assessed with the MRC and FAC scores. This is possibly related to the relatively high baseline MMP-12/-13 levels in patients with more severe and extensive strokes. The statistical adjustment for baseline stroke severity and other factors such as age may solve this issue, but as explained larger studies increasing the sample size are needed.

### 4.3. MMP-13 gene silencing impairs the angio-vasculogenic function of Endothelial Progenitor Cells which shape simpler and less connected vascular networks *in vitro*.

As discussed we have demonstrated that MMP-13 deficiency impairs spontaneous vessel formation *in vivo* in the peri-infarct cortex at day 14 after cerebral ischemia in mice. In the context of tissue repair, this fact could depend on the capability of endogenous EPCs actions during vessel remodeling. In this regard, we also show that silencing the MMP-13 gene impairs the EPCs' function on tubulogenesis in Matrigel assays *in vitro*, indicating the key role of this protease in vessel formation.

Nowadays more evidences show that EPCs are present after ischemic stroke since the first study of EPCs mobilization in response to tissue ischemia[177]. The maintenance of endothelial integrity plays a critical role in vascular-related diseases and EPCs have been shown to be incorporated into neovessels[63,178]. Taguchi *et al.* firstly reported that CD34þ/CD133þ cells, as a EPCs-enriched population, provided a marker of cerebrovascular function[179]. And several studies have proven that endogenous EPCs participate in the neovascularization via CXCR-4/SDF-1 axis after permanent MCAo in rats[180].

After stroke injury, it is known that there is a modulation on circulating EPCs level which produce a variety of growth factors as well as cytokines or chemokines including FGF, VEGF, PDGF-bb, CXCL4, CXCL7 or several MMPs[122,181,182,183], these factors might stimulate local endothelial cell mobility, growth and fucntion[112] and other repair mechanisms such as neurogenesis or oligodendrogenesis as discussed in the EPCs' review publication[155]. In this regard the first observations after EPCs identification suggested that these cells contributed to neovascularization by differentiating into cells forming structural components of the vasculature[63,177,184]. However the paracrine hypothesis that EPCs contribute to vascular growth primarily by secreting proteins and recruiting additional progenitor/stem cells to the injury site has become more acceptable[185,186,187].

MMPs play a key role in the degradation of basal membrane, one of the earliest steps of new vessel formation during angiogenesis or vasculogenesis. MMP-13 acts as a collagenase by cleaving the interstitial collagens (I, II, and III) and has a key role in the MMP activation cascade[188]. The angio-vasculogenic response of EPCs has been reported to be impaired in MMP-9 deficient mice in an MCAo model[95,126] and in a hindlimb ischemia model[189]. In this context, we demonstrate for the first time the role of MMP-13 on EPCs function in an *in vitro* model of tube formation. The outgrowth endothelial cells (OECs) used in the present study are a homogenous and

highly proliferating cell population of endothelial-like cells which directly participate in tubulogenesis[190], while endothelial cells proliferation can be stimulated by the EPCs secretome[191]. Thus, the final *in vivo* influence of MMP-13 on endogenous or therapeutic EPCs function will influence not only the direct impairment of vessel formation, but also to the secretion of angiogenic/trophic factors. However, whether the *in vitro* tubulogenesis findings could translate into the *in vivo* setting of vessel formation stimulated by EPCs is still unknown.

## 4.4. Elevated plasma MMP-3 is associated to motor and functional improvement during rehabilitation therapy after stroke.

We have described for the first time the temporal profile of several MMPs over different time points in patients under an intensive neurorehabilitation program up to 6 months identifying MMP-3 as a potential monitoring molecule to detect those patients that will respond better to rehabilitation therapy.

The use of biological samples to detect the presence of specific molecules with the aim of monitoring ongoing pathophysiological processes and predicting neurological outcomes for stroke survivors is the focus of current clinical and experimental research[192,193]. However, very few studies have investigated the use of biomarkers during rehabilitation therapy as explained in the introduction section and none has explored the use of MMPs.

Why studying a biomarker for recovery in the context or neurorehabilitation?

The daily practice for physiatrists indicates that individual patients respond differently to therapy, which raises several questions regarding which patients will benefit the most from the proposed program or which patients will respond better to larger and more intensive programs. Moreover, it is accepted that rehabilitation enhances neural/brain plasticity but no pharmacological treatments are available to modulate or enhance this plasticity[111]. With this aim it is imperative to identify the underlying biological mechanisms of neural plasticity that drive improvements in neurological functions.

To know the temporal profile of a given molecule (such as MMPs) in circulating blood could provide us an amazing chance to develop its use as a biomarker to diagnose or predict the patient outcome. For example, plasma MMP-9 level within acute (<24 - 48 hours) phase of ischemic stroke has proved to correlate with severe outcome, large infarcts and has been considered as a biomarker for acute hemorrhage after ischemic stroke[194,195,196]. However, several promising markers have not finally shown sufficient evidence to support the use of any of them in the clinical practice[197] for several reasons (the source of plasma MMPs may differ from that of MMPs in brain, a single marker may not be able to reflect the complexity of a neurorepair process, etc.).

MMP-3, also known stromelysin-1 presents a broad substrate specificity for extracellular matrix proteins including fibronectin, collagen, lamina and proteoglycans, as well as for other proteases including pro-MMP-3, -8, -9 and -13[198,199]. Neuroscience literature supports its central position in brain tissue at

embryonic and postnatal development, in addition to its involvement in axonal extension, synaptic plasticity and remyelination[199,200]. Moreover, experimental models of stroke have shown that MMP-3 expression is critical for intracranial bleeding after tPA administration[201] and for peri-neuronal agrin cleavage after ischemia-reperfusion injury in rats[202] while the single administration of a broad spectrum MMP inhibitor (GM6001) after ischemia protected BBB integrity acutely and enhanced vascular remodeling related to increased tight junction expression[203].

Our study on MMPs as potential markers of functional response to rehabilitation therapy describes an association of MMP-3, for the first time, with better neurological status at one month but also with improved motor recovery at later time points during rehabilitation therapy. Importantly, MMP-3 baseline level was not associated to stroke severity measured by NIHSS, extensive infarcts, age or other factors that could compromise the final outcome of the patient. Although the reduced number of patients studied did not allow us to perform multivariate analysis to explore possible interactions between the clinical variables and the proposed biomarkers.

We have found similar levels between patients and controls and no change over time in MMP-3 levels, however those patients that showed better improvements in the FMA, MRC and CAHAI scores presented higher levels of MMP-3. Those tests are widely used and accepted to monitor motor/functional status and progression in rehabilitation programs[204]. In this line another publication has shown the potential use of 8-OHdG (a promising biomarker of oxidative stress) as a valid predictor of functional outcomes in patients under rehabilitation after stroke by using the FMA and MRC scores[205].

## 4.5. Future perspectives

Our results show the protective actions of MMP-13 deficiency in terms of infarct volume and hemorrhagic transformation which deserves further confirmatory studies such as a the specific pharmacologic in vivo inhibition of MMP-13 together with potential recovery studies in MMP-13 KO mice by administrating recombinant MMP-13. Also, confirmatory studies in other MCAo models will provide further evidences of our findings.

The *in vitro* tubulogenic remodeling study has demonstrated the promising role of MMP-13 in angiogenesis, but how will it modulated EPCs performance *in vivo* is still unknown. Since EPCs are being proposed as advanced therapies for stroke repair, new research is needed to relate the current *in vitro* observations to the *in vivo* context of cerebral ischemia in a putative EPC treatment.

Finally since MMP-13 is known to activate other MMPs such as MMP-2 and MMP-9 and we have demonstrated the important role of MMP-9 in EPCS function in other studies, it could be possible that the actions described for MMP-13 are up-stream in a proteolitic cascade. Additional studies investigating the consequences of MMP-13 activation/inhibition within the MMP family would provide valuable information to identify future therapeutic targets.

A multicentre study is needed to confirm our findings on MMP-3 as potential marker to monitor motor progression during rehabilitation therapy, which potentially could be used to adjust and adapt rehabilitation programs for stroke patients.

Finally to better translate our results into clinical studies, the use of aged MCAo models, studies in female rodents or in mice carrying stroke co-morbidities are needed since functional outcome, neuroblast migration, vascular remodeling and the brain plasticity response might be influenced by those factors.

# 5. Conclusions

The main conclusions of this PhD thesis are:

1) EPCs secretome contribute to post-stroke re-vasculatrization by mediating cell-cell communication actions. These interactions connect angiogenesis but also neurogenesis and oligodendrogenesis within a dynamic neurorepair niche.

2) MMP-13 is a crucial protease involved in both the development of acute infarct lesions after cerebral ischemia as well as vascular remodeling and neurogenesis in late phases by controlling the protein expression of other brain MMPs and trophic factors such as G-CSF, Ang-2 and VEGF-A. High levels of plasma MMP-13 as well as MMP-12 are associated with stroke severity in patients.

3) We firstly report the temporal profile of plasma MMP-3, MMP-12 and MMP-13 during rehabilitation therapy and demonstrate the potential use of MMP-3 as a biomarker of motor improvement.

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**Supplementary Information 1**

## Curriculum vitae of the author

# Curriculum Vitae

**Personal information:**

Name: Feifei

Surname: Ma

Date of birth: 11/08/1987

**Academic records:**

2012 November - 2015 November

Doctor degree candidate, major in Neuroscience, Universitat Autonoma de Barcelona, Vall d'Hebron Institut de Recerca, with the project of "Neuroprotection and neurorepair treatments for ischemic stroke"

2009 September - 2012 July

Master degree, major in Neuropharmacology, Zhengzhou University, with the project of "Neuroprotective potential and mechanism of 3-butyl-6-bromol (3H)-isobenzofuranone on cerebral ischemia in rats"

**Relevant skills and technical knowledge:**

Middle cerebral artery occlusion (MCAo) model in mice and rats

Animal experimental techniques

Immunology experimental skills (Immunohistochemistry, Immunofluorescence, Luminex assay, ELISA)

RT-PCR and Western blot techniques

Use of confocal microscope, Image J software, platelet aggregation instrument

Primary endothelial progenitor cells obtain

Statistical analyses with SPSS software

**Manuscripts under submission**

1. **F. Ma**, S. Rodriguez, X. Buxo, A. Morancho, I. Riba-Llena, A. Crrera, A. Bustamante, D. Giralt, J. Montaner, C. Martinez, I. Bori and A. Rosell
   Plasma matrix metalloproteinase levels in stroke patients during intensive rehabilitation therapy.
   *In preparation.*

2. **F. Ma**, P. Martínez-San Segundo, V. Barceló, A. Morancho, M. Gabriel-Salazar, D. Giralt, J. Montaner and A. Rosell
   Matrix Metalloproteinase-13 Controls Neuroprotection and Neurorepair after Cerebral Cortical Ischemia in Mice.
   *Neurobiology of Disease, under review.*

**Publications**

1. A. Morancho, **F. Ma**, V. Barceló, D. Giralt, J. Montaner and A. Rosell
   Impaired vascular remodeling after endothelial progenitor cell transplantation in MMP9-deficient mice suffering cortical cerebral ischemia.
   *Journal of Cerebral Blood Flow & Metabolism (2015), 1-5.*

2. **F. Ma**, A. Morancho, J. Montaner and A. Rosell
   Endothelial progenitor cells and revascularization following stroke.
   *Brain Research. 1623 (2015) 150-159.*

3. **F. Ma**, Y. Gao, H. Qiao, X. Hu, J. Chang
   Antiplatelet activity of 3-butyl-6-bromo -1(3H)-isobenzofuranone on rat platelet aggregation.
   *Journal of Thromb and Thrombolysis (2012) 33:64-73.*

**Presentation and poster**

1. **F. Ma**, A. Morancho, P. Martínez-San Segundo, M. Gabriel-Salazar, V. Barceló, D. Giralt, J. Montaner and A. Rosell
   MATIX METALLOPROTEINASE-13 (MMP-13) MODULATES NEUROPROTECTION AND NEUROREPAIR AFTER CEREBRAL CORTICAL ISCHEMIA.
   *1st European Stroke Organization conference (April, 2015)*

2. **F. Ma**, V. Barceló, A. Morancho, P. Martínez-San Segundo, M. Gabriel-Salazar, D. Giralt, J. Montaner and A. Rosell
   Matrix metalloproteinase (MMP)-13 modulates neuroprotection and neuro-repair after cerebral cortical ischemia.
   *8th VHIR Scientific Session (December 2014)*

## Supplementary Information 2

## Additional publication and poster

Journal of Cerebral Blood Flow & Metabolism (2015), 1–5

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BRIEF COMMUNICATION

# Impaired vascular remodeling after endothelial progenitor cell transplantation in MMP9-deficient mice suffering cortical cerebral ischemia

Anna Morancho, Feifei Ma, Verónica Barceló, Dolors Giralt, Joan Montaner and Anna Rosell

Endothelial progenitor cells (EPCs) are being investigated for advanced therapies, and matrix metalloproteinase 9 (MMP9) has an important role in stroke recovery. Our aim was to determine whether tissue MMP9 influences the EPC-induced angiogenesis after ischemia. Wild-type (WT) and MMP9-deficient mice (MMP9/KO) were subjected to cerebral ischemia and treated with vehicle or outgrowth EPCs. After 3 weeks, we observed an increase in the peri-infarct vessel density in WT animals but not in MMP9/KO mice; no differences were found in the vehicle-treated groups. Our data suggest that tissue MMP9 has a crucial role in EPC-induced vascular remodeling after stroke.

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**Keywords:** angiogenesis; endothelial progenitor cell; matrix metalloproteinase-9; neurorepair; stroke

## INTRODUCTION

Transplantation of endothelial progenitor cells (EPCs) has become a promising approach to enhance angio-vasculogenic responses after ischemia. EPCs can be mobilized in response to ischemia, can home to sites of neovascularization, and can differentiate into endothelial cells.[1] There are two subsets of EPCs: the so-called circulating angiogenic cells and the outgrowth endothelial cells (OECs).[2] Both types of EPCs have been demonstrated to increase brain angiogenesis in animal models of cerebral ischemia.[3–5]

The degradation and remodeling of the vascular basal membrane is required to allow endothelial cells to migrate, and matrix metalloproteinases (MMPs) have key roles in the initial steps of angio-vasculogenesis. Specifically, MMP9 has been shown to be essential for the invasion of endothelial cells and capillary branching, and *in vitro* studies have indicated that its absence impairs the angiogenic abilities of the EPCs.[6] Other authors have described that MMPs have dual roles after ischemia, where their upregulation is detrimental in the acute phase but is essential for effective neurorepair.[7] Experimental models of cerebral ischemia have proven that inhibiting MMPs can reduce the size of the ischemic lesion.[8] Published studies on MMP9-deficient mice have demonstrated the pivotal role of this protease in infarct expansion,[9,10] although the results remain controversial.[11]

Our core hypothesis is that tissue MMP9 is a key protease required for an effective and successful cell-based therapy to potentiate vascular remodeling. In this study, we investigated the effects of brain MMP9 deficiency on the cerebral ischemia after EPC-based treatment to potentiate angio-vasculogenesis. To examine this hypothesis, we evaluated the infarct size in the acute phase as well as the long-term peri-infarct angiogenesis in wild-type (WT) and MMP9 knock-out (MMP9/KO) mice after EPC transplantation in a model of cortical stroke.

## MATERIALS AND METHODS

### Animals

A total of 50 male WT and MMP9/KO mice (8-to-12-weeks old, FVB background) were obtained from Jackson Laboratories (Sacramento, CA, USA). Both genotypes were bred in-house and the offspring were used for experimentation. All procedures were approved by the Ethics Committee of Animal Experimentation of the Vall d'Hebron Research Institute (58/08/10) and were conducted in accordance with the Spanish legislation and the Directives of the European Union. The ARRIVE guidelines were considered in the design and report of the study.

### Permanent Focal Cerebral Ischemia

The left middle cerebral artery was permanently occluded (MCAO) by electrocauterization, affecting the cortex as described.[6]

### *In Vivo* Magnetic Resonance Imaging

Magnetic resonance studies were carried out 24 hours after MCAO using a 7-Tesla horizontal magnet (BioSpec 70/30, Bruker BioSpin, Rheinstetten, Germany). Sixteen coronal slices for T2-weighted fast spin-echo images (T2WI) and eight coronal slices for T2 map and

Neurovascular Research Laboratory, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain. Correspondence: Dr A Rosell, Neurovacular Research Laboratory, Vall d'Hebron Research Insitute, Passeig Vall d'Hebron 119-129, Barcelona 08035, Spain.
E-mail: anna.rosell@vhir.org
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diffusion tensor imaging were acquired. Maps of the trace of the diffusion tensor, also named the apparent diffusion coefficient (ADC), and the fractional anisotropy (FA) were derived using the Paravision software program, v5.0 (Bruker BioSpin). Regions of interest corresponding to the infarct area were traced on T2, ADC, and FA maps in all slices presenting infarcts and in matching contralateral hemispheres. The mean values are expressed as the ipsilateral/contralateral ratios.

### Treatments

Growing OECs were obtained and expanded as previously described[6] from WT FVB mice. The cells were trypsinized, and $5 \times 10^5$ cells were prepared in 200 $\mu$L of endothelial basal medium (EBM, Lonza, Allendale, NJ, USA). Treatments were randomly administered intravenously (200 $\mu$L via retro-orbital sinus) at 30 to 32 hours postischemia to the following groups: WT mice receiving vehicle (EBM, $n = 15$), MMP9/KO mice receiving vehicle ($n = 8$), WT mice receiving OECs ($n = 15$, $5 \times 10^5$ OECs in EBM), and MMP9/KO mice receiving OECs ($n = 8$), according to the power calculations described in the 'Statistical Analysis' section. Selected WT and MMP9/KO mice ($n = 4$) received the same cell treatment with green fluorescent OECs labeled *in vitro* with PKH26 (Sigma, St Louis, MO, USA), a general cell membrane green fluorescent dye.

### Measurement of the Infarct Lesion

The hyperintense area corresponding to the injured tissue and the total areas of both ipsilateral and contralateral hemispheres were traced manually on T2W images by investigators who were blinded to the genotype. The edema index and lesion volume were calculated as previously described.[4]

### Behavioral Testing

The corner test (used to assess sensorimotor asymmetries) and grip strength meter test (used to measure the maximum strength of the mouse forelimbs) were used to evaluate the functional outcomes. The tests were performed blindly before surgery and were repeated 1, 7, 14, and 21 days after MCAO as described elsewhere.[12]

### Evaluation of the Brain Vasculature

To assess the poststroke angio-vasculogenesis, mice were injected intravenously with 80 $\mu$g of Dylight 594-labeled tomato (*Lycopersicon esculentum*) lectin (Vector Laboratories, Burlingame, CA, USA) and were euthanized by cardiac perfusion of 4% paraformaldehyde under deep anesthesia 21 days after the induction of ischemia. Brains were collected, and 12-$\mu$m-thick coronal sections were selected from the anterior area (+1 to +0.2 bregma), including the lateral ventricles. Two images (×100 magnification) of the peri-infarct cortex were taken with a laser confocal spectral microscope (Olympus FV-1000, Olympus, Tokyo, Japan), and the total area of lectin-positive vessels was calculated (ImageJ software, NIH, Bethesda, MD, USA) by an investigator masked to the genotype/treatment. The results are expressed as the mean vessel density.

### Doublecortin (DCX) and NeuN Immunohistochemistry

EPC-induced neurogenesis was assessed in contiguous coronal sections, which were immunostained with antibodies for mature neurons (NeuN; Millipore, Billerica, MA, USA) and neuroblasts (DCX; Abcam, Cambridge, UK). The presence of DCX-positive cells was analyzed in the dorsolateral and subventricular zones of the ipsilateral hemisphere, and mature neurons were measured in the peri-infarct cortical area ($n = 7$ to 8/group).

### Statistical Analysis

SPSS 15.0 software (IBM, Armonk, NY, USA) was used for the statistical analyses. All variables were normally distributed, and the data are expressed as the mean ± s.d. Statistical significance was assessed by Student's *t*-test or an analysis of variance followed by the least significant difference *post-hoc* test. A *P*-value $< 0.05$ was considered to be statistically significant at a 95% confidence level.

After a pilot study including $n = 7/8$ mice/treatment, power calculations were evaluated using Ene 2.0 (http://sct.uab.cat) considering a *P*-value $< 0.05\%$ and 50% statistical power. The number of animals was raised up to 15 for WT vehicle and WT receiving OECs to assess vessel density.

## RESULTS

### MMP9 Deficiency Reduces the Cortical Ischemic Lesion

The ischemic lesions were evaluated by magnetic resonance imaging 24 hours after permanent MCAO in 32 animals. MMP9 deficiency entailed significant neuroprotection, reducing the infarct volume by 25% in MMP9/KO animals ($17 \pm 1.5$ mm$^3$ in WT versus $12.8 \pm 0.9$ mm$^3$ in MMP9/KO, $P = 0.025$; Figure 1A). The edema index was also significantly reduced in the MMP9/KO mice ($P = 0.026$), which indicated decreased swelling. In accordance with these findings, the T2 map values showed reduced lesions in the MMP9/KO mice ($P = 0.007$; Figure 1B). Measurements of the ADC and FA in the infarct area confirmed that the severity of the lesion (ADC) and axonal damage (FA) within the infarct core were similar between genotypes (Figure 1B). In addition, there were no significant differences between the treatment subgroups (vehicle vs. EPCs) before the treatment administration.

### Neurorepair with OECs Enhanced the Cortical Angiogenesis Only in WT Mice

The cortical vessel density was analyzed 21 days after the induction of ischemia. One animal in the MMP9/KO vehicle group died 2 weeks after treatment after suffering severe aggression from the other mice housed in the same cage. Interestingly, no correlation existed between the vessel density and the magnetic resonance imaging parameters (infarct volume and ADC and FA values) in any of the groups. Green fluorescent cells were observed to be associated with the brain vessels in the peri-infarct areas in both WT and MMP9/KO mice receiving cell therapy (see Figure 2A), confirming the arrival of administered cells to the brain and its incorporation to the brain vasculature. When we compared the amount of functionally perfused microvessels in the peri-infarct area, there were no significant differences between the WT and MMP9/KO mice that received the vehicle ($P = 0.220$). Regarding OEC treatment, there was a significant increase in the vessel density in WT mice that received OECs compared with the WT vehicle controls, as expected ($P = 0.028$, Figure 2B). However, there was no significant difference in the vessel density between the MMP9/KO vehicle vs. MMP9/KO OEC groups ($P = 0.672$), although there was an increased vessel density in the WT vs. MMP9/KO mice treated with OECs ($P = 0.001$), which suggested that endogenous MMP9 is required for the pro-angiogenic actions of OECs.

Immunohistochemistry studies showed a non-significant decrease in DCX+ and NeuN+ cells in the MMP9/KO mice that received OECs compared with WT mice with the same treatment ($0.51 \pm 0.26\%$ vs. $0.83 \pm 0.42\%$ for DCX+, $P = 0.087$ and $342.4 \pm 69.3$ vs. $437.9 \pm 131.4$ for NeuN+, $P = 0.09$). Moreover, when the differences were analyzed among all treatment groups, no statistically significant difference was observed ($P = 0.243$ for the DCX-positive area and $P = 0.359$ for the NeuN+ neuron counts).

The corner test and the grip strength meter test failed to show any impairment after ischemia (pre- vs. 24-hour scores; $P > 0.05$),

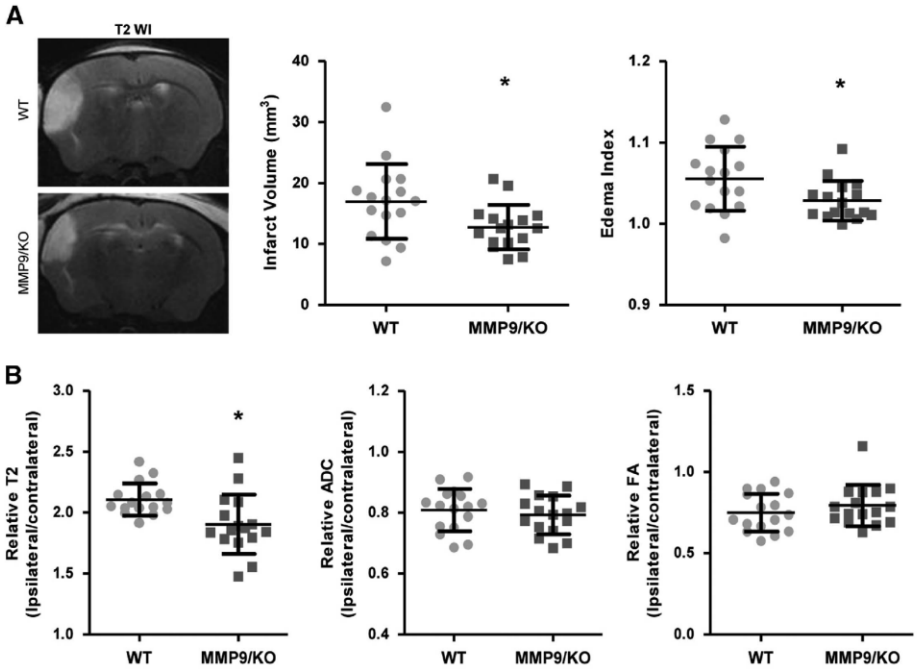


**Figure 1.** Magnetic resonance imaging 14 hours after cerebral ischemia. (**A**) A decrease in the infarct volume and edema index was observed in matrix metalloproteinase-9 knockout (MMP9/KO) versus wild-type (WT) mice by T2WI at 24 hours after ischemia. Representative images show severe cortical damage in mice of both genotypes. (**B**) The T2, apparent diffusion coefficient (ADC), and the fractional anisotropy (FA) were measured to assess the extent of brain damage, and relative values (to the contralateral cortex) are shown. The data are expressed as the mean ± s.d., and each individual value is represented. $N = 16$ per group; *t*-test, $^*P < 0.05$.

and no significant differences during the follow-up among the groups were observed ($P > 0.05$).

## DISCUSSION

The current results suggest that MMP9 has a key role in the tissue vessel remodeling mediated by cell therapy with EPCs after cerebral ischemia. The upregulation of MMPs, including MMP9, after cerebral ischemia and the contribution of the MMPs to brain injury have been widely documented.[7,8] Although pharmacological or immunological inhibition of MMP9 has proven to be neuroprotective, as evidenced by a decreased infarct size, discrepant results have been reported in MMP9-deficient mice.[8,9,11] These discrepancies could be attributed to the use of different experimental models of cerebral ischemia (proximal and distal MCA occlusion in the presence or absence of reperfusion) and/or to the use of different techniques to assess the lesion extension (tissue staining for 2,3,5-triphenyl-tetrazolium-chloride). The present study includes the largest sample size of WT and MMP9/KO mice used to evaluate the infarct volume ($n = 16$ per group), and our results support the previous findings showing that MMP9 inhibition reduces the inflammatory response, blood–brain barrier disruption, and the development of edema after cerebral ischemia.[9,10]

In addition to its detrimental effects in the acute phase, MMPs also participate in tissue remodeling after brain injury, and MMP9 has been suggested to participate in the unique tissue biobridge between neurogenic and non-neurogenic sites.[13] After a stroke, the pharmacological inhibition of MMPs with broad-spectrum drugs demonstrated that they have essential roles to ensure an effective neurorepair after cerebral ischemia and poststroke angiogenesis.[14] However, there have been no previous studies that have analyzed the specificity of MMP9 using genetically modified ischemic mice with poststroke EPC treatment to enhance angiogenesis.

We have recently described that there was an increase in the peri-infarct vessel density in WT ischemic mice compared with sham mice, which was not observed in ischemic MMP9/KO individuals.[6] In the present study, WT and MMP9/KO ischemic mice subjected to long-term brain ischemia showed a similar vessel density 21 days after cortical ischemia. The discrepancy between this finding and those of other studies analyzing the effects of MMP inhibitors in cortical remodeling after stroke[14] may be attributed to the small infarct volumes typical of the FVB strain in our model of cerebral ischemia,[12] the role of other MMPs, the longer time point for the tissue analysis in our study, or to differences in the neurorepair mechanisms in mice vs. rats. In this regard, a possible dual role of MMP9 in angiogenesis that depends on the endogenous tissue vascularization has been observed by other authors, as this protease was required for ischemia-induced angiogenesis after hindlimb ischemia,[15] whereas in cardiac tissue MMP9-deficiency facilitated angiogenesis after myocardial infarction.[16]

The most important finding of our study, which we believe will be of great importance to the development of future cell-based neurorepair strategies after stroke, is the MMP9 tissue dependency after EPC therapy. To date, several different subsets of EPC populations have been demonstrated to have therapeutic potential in experimental models of cerebral ischemia.[3–5] In the present study, we used OECs (a homogenous and highly proliferative cell population of endothelial-like cells) as a pro-angiogenic treatment for stroke, which was shown to be MMP9 dependent for tubulogenesis in an *in vitro* Matrigel substrate.[6] Here we showed that the intravenous administration of OECs enhanced the vessel density in the peri-infarct area in WT animals, but this augmentation did not occur in MMP9-deficient mice. These results suggest that tissue MMP9 has an important role in

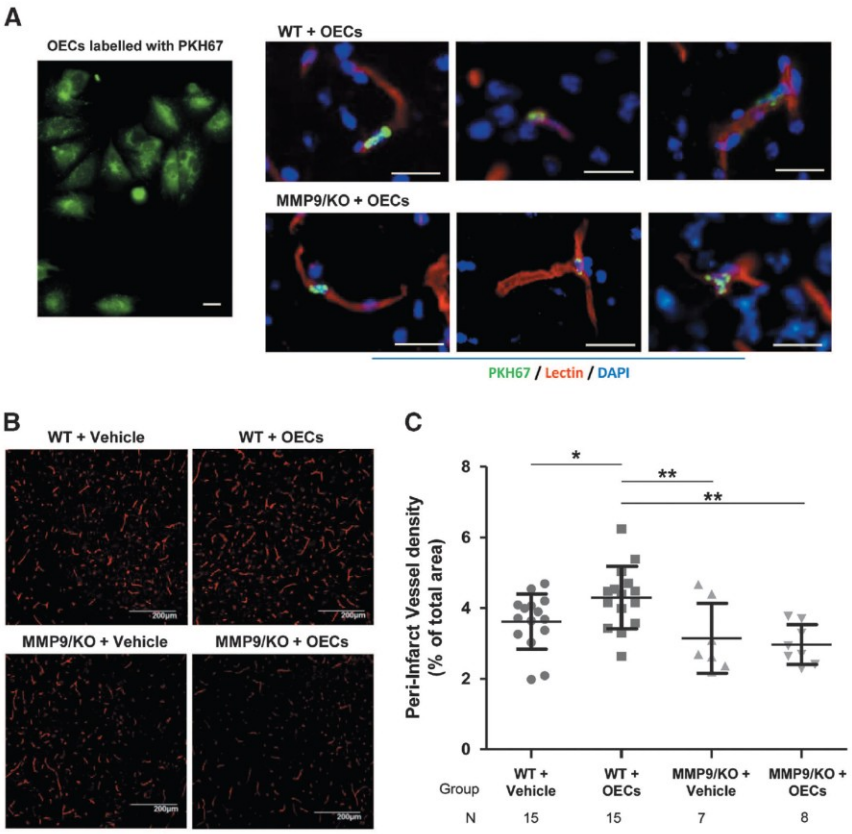


**Figure 2.** The brain peri-infarct vasculature in wild-type (WT) and matrix metalloproteinase-9 knockout (MMP9/KO) mice 21 days after cerebral ischemia. Functional blood vessels stained after lectin perfusion were quantified in the ipsilateral peri-infarct cortex 21 days after ischemia. (**A**) The right panel represents PKH67-positive outgrowth endothelial cells (OECs) *in vitro*. PKH67-stained OECs were detected in the brain vasculature (lectin-stained vessels) 24 and 72 hours after intravenous administration both in WT and MMP9/KO ischemic brains (bar = 50 $\mu$m). (**B**) Representative micrographs of lectin staining in cortical areas (bar = 200 $\mu$m). (**C**) A study of the vessel density showed a significant increase in the number of vessels in WT vs. MMP9/KO mice after OEC treatment (analysis of variance followed the least significant difference *post-hoc* test, $n = 7$ to 15/group). The data are expressed as the mean ± s.d., and each individual value is represented; $^{*}P < 0.05$; $^{**}P < 0.01$. DAPI, 4,6-diamidino-2-phenylindole.

the vascular remodeling as part of pro-angiogenic and vascular remodeling treatments. Because the administration route was systemic (intravenous), we hypothesize that the MMP9 in the tissue could inhibit the downstream pro-angiogenic effects of transplanted OECs at the ischemic site and/or the expected basal lamina remodeling during angiogenesis. Additionally, coupled EPC-induced neurogenesis was not altered in the MMP9 lacking brains, and this was not modified by EPC therapy, although further investigation will be necessary to confirm this data. A major limitation of our study is the lack of behavioral data supporting the findings for angiogenesis. In this regard, long-term behavioral assessments are difficult in distal MCAO models and might differ among strains,[12] thus suggesting the need for confirmation using other strains or ischemia models; multicentric studies would be an excellent platform for this purpose.

In summary, MMP9 deficiency provides protection against the adverse effects of cortical cerebral ischemia in mice, although the protease is unequivocally required for the success of neurorepair therapies based on vascular remodeling, such as EPC transplantation.

## AUTHOR CONTRIBUTIONS

AM, JM, and AR participated in the conception and design; AM, FM, and VB collected and assembled the data; AM, DG, JM, and AR analyzed and interpreted the data; and AM and AR wrote the paper. All authors corrected and approved the manuscript.

## DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Matrix metalloproteinase-13 (MMP-13) modulates neuroprotection and neurorepair after cortical ischemia

**Feifei Ma**, Anna Morancho, Pablo Martínez-San Segundo, Marina Gabriel-Salazar, Verónica Barceló, Dolors Giralt, Joan Montaner, Anna Rosell
Neurovascular Research Laboratory and Neuroscience Department, Vall d'Hebron Research Institute, Universitat Autònoma de Barcelona, Spain
feifei.ma@vhir.org

## Introduction

- New post-stroke therapies to antagonize brain damage and to increase the possibility of recovery are under urgent needed.
- Ischemic stroke stimulates neurogenesis in the damaged area which has been proven occurs followed by angiogenesis[1]. One approach to potentiate neurorepair is targeting angio-neurogenesis.
- Matrix metalloproteinases-13 (MMP-13 or collagenase-3) is involved in the regulation of cell-matrix composition, being increased in ischemic rat neurons[2] and associated to lesion expansion[3].

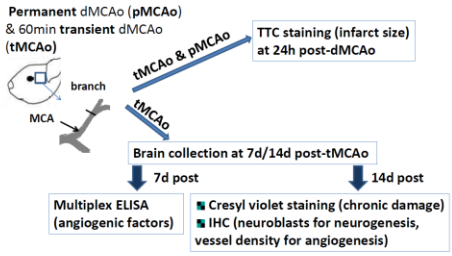
Less is known about the role of MMP-13 in neurorepair.

**Objective**: To determine the role of MMP-13 in neuroprotection and neurorepair after distal cortical ischemia by studying angiogenesis and neurogenesis.

## Methodology

➢**Focal cerebral ischemia models (MCAo)**

Distal middle cerebral artery (MCA) occlusion was induced in 8-12 weeks male MMP-13 gene knockout (KO) and wild-type (WT) mice (C57BL/6J).




➢**Grip strength neurological test**

Grip test was performed at pre-surgery, 24h post-surgery, 7d post-surgery, 14d post-surgery.

➢**Immunohistochemistry (IHC) in frozen brain sections (DCX & BrdU for neurogenesis and Lectin for angiogenesis)**

➢**Multiplex ELISA with cortex homogenate (angiogenic factors detection)**

➢**Endothelial progenitor cells (EPCs) culture and MMP-13 transient silencing study in Matrigel ™ (*in vitro* angiogenesis)**

➢**Statistical analysis**

Independent t-test or Mann-Whitney test for two groups, and repeated ANOVA for different time points.

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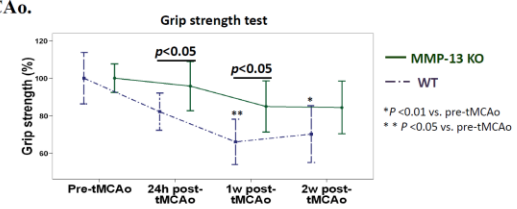
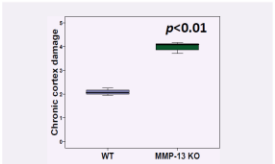
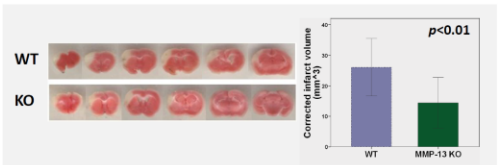
## Results

**1. MMP-13 deficiency protects the brain tissue at 24h post-tMCAo but impairs neurorecovery at 14d post-tMCAo.**



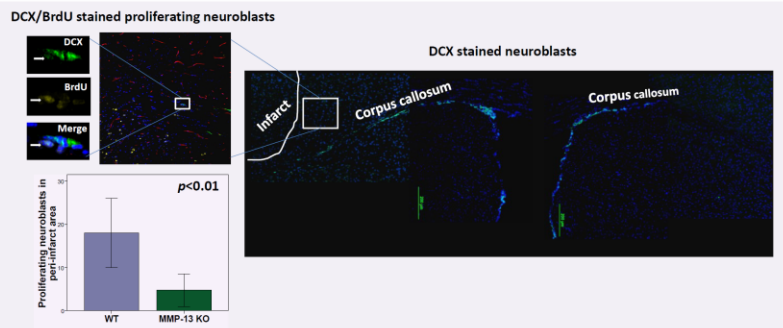




Cresyl violet stained brain tissue at 14d post-tMCAo corrected by infarct size at 24h post-tMCAo



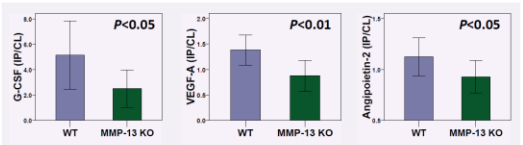

TTC stained infarct tissue at 24h post-tMCAo

**2. MMP-13 deficiency reduces proliferating neuroblasts migration from SVZ to peri-infarct cortex.**

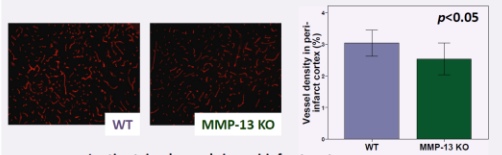



Proliferating neuroblasts at 14d post-tMCAo

**3. MMP-13 deficiency impairs cortical angiogenic factors secretion as well as vessel formation in peri-infarct cortex.**

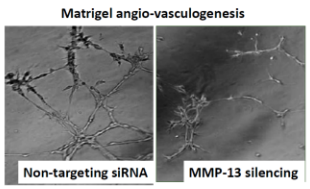


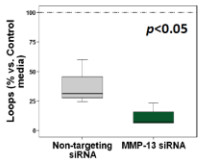

Angiogenic factors detection at 7d post-tMCAo




Lectin stained vessels in peri-infarct cortex at 14d post-tMCAo

**4. MMP-13 siRNA decreases the angio-vasculogenic capability of EPCs *in vitro***




## Conclusions

1. **MMP-13 plays an important role in both infarct formation and neurorepair after transient distal MCAo in C57/BL6J mice.**
2. **MMP-13 deficiency reduces established neurogenesis in mice in peri-infarct area at 14d post-tMCAo.**
3. **MMP-13 plays a key role in this vascular remodeling: when the expression of MMP-13 is blocked, *in vitro or in vivo*, the formation of vascular networks is impaired.**

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