Mariia Ermolaeva

COLLATERAL FLOW AS A PREDICTOR FOR ANGIOGENESIS IN MIDDLE CEREBRAL ARTERY ISCHEMIC STROKE

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular, com especialização em Neurobiologia, orientada pelo Doutor João Sargento-Freitas e coorientada pelo Professor Doutor Carlos Duarte e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Junho de 2023

UNIVERSIDADE D
COIMBRA

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ABSTRACT |

Ischemic stroke is a complex condition which causes deaths and comorbidities in adult population around the world. Different mechanisms of vascular remodelling, such as collateralization and angiogenesis, may impact the functional outcome of the patient after suffering stroke. Collateral flow is an important intrinsic mechanism of immediate blood supply restoration to the ischemic penumbra while a major vessel is occluded. Good collateral flow is associated with improved final outcome at 3 months. During the subacute phase of stroke, angiogenesis takes place. Usually, an increased permeability of blood-brain barrier at this stage represents changes of vascular system that are due to the sprouting angiogenesis. Similar to collateralization at hyperacute phase, increased BBB permeability at subacute phase is associated with better clinical outcome. Molecular mechanisms that trigger these two processes overlap to a certain degree, which might indicate a direct connection between them. Furthermore, some studies in animal models and in patients have proved association between collateralization and angiogenesis.

We performed a study of MCA stroke patients' data retrospectively collected with different techniques, namely CTA and TCCD for collateral flow and DCE-MRI for angiogenesis. Considering the suggested interactions, we aimed to understand if collateral flow at hyperacute phase of stroke is associated with angiogenesis at subacute phase, and if one can be reliably used to predict the other.

This work proved significant association between hyperacute collateralization and final outcome, as well as between subacute angiogenesis-related BBB permeability and final outcome. However, there was no direct association between the two vascular remodelling mechanisms. Considering these results, both mechanisms are important for stroke recovery. At the same time, there might be a more complex interaction that is not possible to detect in the current sample size.

Keywords: ischemic stroke, collateral flow, angiogenesis, BBB permeability

LIST OF ABBREVIATIONS |

ACA	Anterior Cerebral Artery
ADC	Apparent Diffusion Coefficient
AF	Atrial Fibrillation
AIF	AIF Arterial Input Function
AJ	Adherens Junction
ASVD	Arteriosclerotic Vascular Disease
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BECs	Blood-brain barrier Endothelial Cells
bFGF	Basic Fibroblast Growth Factor
CBF	Cerebral Blood Flow
CF	Collateral Flow
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CTA	Computed Tomography Angiography
DCE-MRI	Dynamic Contrast-Enhanced Magnetic Resonance Imaging
DWI	Diffusion-Weighted Imaging
EGF	Epidermal Growth Factor
EMCs	Extracellular Matrix Components
eNOS	endothelial Nitric Oxide Synthase
FLAIR	Fluid Attenuated Inversion Recovery
GDNF	Glial Cell Derived Neurotrophic Factor
GJ	Gap Junction
HT	Haemorrhagic Transformation
HTA	Arterial Hypertension
ICA	Internal Carotid Artery
ICAM-1	Intercellular Adhesion Molecule 1
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G

ISF	Interstitial Fluid
MCA	Middle Cerebral Artery
MMP	Matrix Metalloproteinase
MPRAGE	Magnetization Prepared Rapid Gradient Echo
MRI	Magnetic Resonance Imaging
mRS	Modified Rankin Scale
MTHFR	Methylenetetrahydrofolate Reductase
NECT	Non-contrast Enhanced Computed Tomography
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
NO	Nitrous Oxide
NSCs	Neural Stem Cells
NVU	Neurovascular Unit
PCA	Posterior Cerebral Artery
ROS	Reactive Oxygen Species
rtPA	Recombinant Tissue-Type Plasminogen Activator
SVZ	Subventricular Zone
SWI	Susceptibility Weighted Imaging
TCCD	Transcranial Colour-Coded Doppler
TJ	Tight Junction
TNF-α	Tumour Necrosis Factor
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
TOI	Stroke Onset Time
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
WHO	World Health Organization

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Chapter 1

| State-of-the-art

1.1. Stroke

1.1.1. Definition

The term *stroke* is commonly used nowadays in the medical and scientific community to refer to a condition in which the blood supply to the brain is compromised. However, the term is much more recent than the condition itself; beginning from the times of Hippocrates up until XVI century the term *apoplexy* was used, meaning "being struck with a deadly blow" (Schutta & Howe, 2006). The present view on stroke originates from the 1900s, when physicians set their eyes more on the characteristics of the condition that were mostly shared between all patients who suffered apoplexy (Pound et al., 1998). At this point the term *cerebrovascular accident* came into use, emphasising physiological changes rather than solely clinical representation. Still, the basis for stroke classification that is used today rests upon the work of mediaeval scholars who identified main causes of apoplexy as *sanguineous* for bleeding and *serous* for the intracranial fluid. This shift towards dichotomous classification contributed to latter reclassification of apoplexy causes as *sanguinea* (haemorrhagic) and *ischaemica* made by Rudolf Virchow (Engelhardt, 2017). Up until the 1960s the term *reversible ischaemic neurological deficit* was used due to misleading interpretation of neurological events between first post-stroke 24h and seven following days. It was eventually removed from the lexicon when it was proven that the events within this period are related to cerebral infarction.

According to the World Health Organization stroke is defined as "rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin" (Aho et al., 1980). At the same time, definition by the American Heart Association/American Stroke Association includes also silent infarctions in the brain, spinal cord and/or retina as well as silent haemorrhages, representing a shift of focus towards a radiological tissue-based definition (Sacco et al., 2013).

1.1.2. Epidemiology

Stroke is one of the most frequent causes of death and disability among the adult population worldwide. Studies of the global burden of neurological disorders show that the absolute number of people who died or remained disabled after a neurological event

has increased during the past years (Feigin et al., 2021). This clearly indicates that existing treatment strategies and management of these disorders is not adequate to the population growth and increased life expectancy that are in place. Even if a patient survives after a stroke there is a high risk of long-term disability, especially in elderly population. Well known is the fact that stroke risk increases with age, doubling after 55 years. The highest reported stroke incidence is in China with up to 378 affected per 100,000 individuals a year. The lowest stroke rate is reported in Latin America (up to 100 cases per 100,000 a year) (Collaborators G.S, 2019). Particularly, according to NINDS ischemic strokes account for 80-90% of total stroke cases with the remaining percentage of strokes having haemorrhagic origin (Saver et al., 2012). For ischemic strokes, the highest mortality rates were reported in Russia and Kazakhstan (Barker-Collo et al., 2015). Most of the causes have been identified as a consequence of scarce primary care to screen and address vascular risk factors, poor accessibility of basic diagnostic tools and treatment, post-stroke rehabilitation and secondary prevention. Another important problem is the economic burden of stroke. Despite similar diagnostic procedures, severe strokes (> 20 NIHSS) cost twice as much as mild strokes (Go et al., 2014).

Sex-related inequality in mortality due to stroke is observed. According to WHO reports, stroke-related deaths among women exceed those among men, and a number of studies carried out in Europe has shown that the risk of stroke in women increased by 10% compared to 9% in men (Redon et al., 2011). A possible explanation of these tendencies is the longer lifespan and higher predisposition of women to such risk factors as hypertension and atrial fibrillation, as well as other differences in vascular biology, hormonal profiles, lifestyle, societal status, etc. At the same time, stroke occurrence in men and women is also dependent on age. For example, it is higher in younger women than in younger men, while the incidence in men increases slightly with age. The higher risk for stroke in younger women can be attributed to pregnancy related factors, as well as to use of oral contraceptives and hormonal therapy.

The main cause of impairments, comorbidities and deaths after ischemic stroke is neuronal dysfunction and death caused by compromised blood supply to brain areas where ischemic core and penumbra were formed. In this case, ischemic core stands for an irreversibly damaged brain tissue, while penumbra denotes surrounding area at risk that is still salvageable given that reperfusion is established within adequate timespan. Extended periods of hypoxia cause irreversible changes in the brain structure, which is

why restoration of blood supply always remains the central therapeutic goal. Ischemic cell death involves a number of complex mechanisms that we will discuss further. This complexity makes it challenging to establish a treatment strategy that would fully prevent or even reverse functional impairments in stroke patients. Currently, the only available treatment for stroke is the use of thrombolytic drugs to restore brain perfusion. However, despite its proven efficacy, thrombolytic drugs are given to less than 5% of stroke patients due to its narrow therapeutic window and increased risk of intracranial haemorrhage (Bernardo-Castro et al., 2020). Therefore, there is a need for new stroke treatment strategies.

1.1.3. Classification and causes

As mentioned before, dichotomous classification that has been proposed by scholars in the 1600s with some changes still stands. Further classification is based on etiology. One of the simplest and commonly used classifications is the ASCO system, dividing strokes into four groups: atherosclerotic, small vessel disease, cardioembolic and others (Osborn et al., 2017). The stroke subtype classification system that we use in the current study is the one developed for the Trial of Org 10172 in Acute Stroke Treatment (TOAST) (Adams et al., 1993). This system suggests five ischemic stroke subtypes: large-artery atherosclerosis, cardioembolic stroke, small-vessel occlusion, stroke of other determined etiology and stroke of undetermined etiology. Atherosclerotic (ASVD) strokes are the most common type and represent up 50% of stroke cases. Large artery occlusion usually happens due to an embolization that originates from a thrombus that in turn originated from an ASVD plaque. Small vessel disease or lacunar infarcts are relatively small lesions (>15 mm) represent up to 30% of strokes. There are genetic variations identified that have been associated with lacunar strokes, such as *MTHFR* C677T polymorphism (Chita et al., 2020). Lacunar stroke can be of an embolic, atheromatous or thrombotic origin. Cardioembolic strokes represent up to 25% of total stroke cases. Myocardial infarction and atrial fibrillation are common risk factors.

Primary lesions in the cerebrovascular system. Further on we are going to address ischemic stroke causes in more detail based on the classification proposed in “Acute Ischemic Stroke” 2011 edition (González et al, 2011). The first group of strokes can be attributed to primary lesions in the cerebrovascular system. Suchwise, the presence of

severe carotid stenosis can predict the likelihood of stroke, as well as a carotid plaque. Since plaque is often calcified, it is dangerous not only due to stenotic effect but also because it traumatises vascular walls causing dissection and inflammation or even infection of the vessel. Besides, plaque debris can detach and lead to the downstream embolization. Intracranial atherosclerosis usually takes place in siphon portion of internal carotid artery (ICA), middle cerebral artery (MCA) stem and branches, anterior cerebral artery (ACA) branches and proximal segments of posterior cerebral artery (PCA), vertebral artery origin, vertebrobasilar junction and basilar artery. Atherosclerosis is a common cause of MCA territory stroke that is in focus of current research. Atherosclerosis in the aorta also increases the risk of ischemic stroke because the constant flapping of the aortic wall facilitates thrombus embolization. Free standing is the stroke caused by extra-cerebral artery dissection as it is the most common cause of stroke in young patients and children. When the wall of a vessel is dissected, blood enters the cavity and expands the wall outwards compressing the lumen. In this case symptoms often develop gradually, even in the course of a few days, starting with pain and possibility of tetraplegia in most severe cases.

Primary cardiac abnormalities. Primary cardiac abnormalities are the second group of stroke causes. Major risk factor here is atrial fibrillation (AF) because it significantly increases the chance of embolism if there is a mural thrombus present. Such heart conditions as myocardial infarction, valvular heart disease, patent foramen ovale and cardiac tumours are also associated with high stroke risk.

Embolic stroke. Embolic stroke includes local vascular lesions that result from a clot that travels along the vessel until the point when it reaches a segment narrow enough for the clot to be compacted into a plug and stuck. There is a possibility of microvascular changes in the ischemic brain such as maximal dilation regulated partially by nitric oxide. This vasodilation is required for attracting collateral flow via leptomeningeal vessels. However, as blood flow will decrease in microvessels the risk for microvascular thrombus formation increases. It is important to mention MCA embolism as it is a common stroke cause with often catastrophic consequences. Carotid stenosis and consequent artery-to-artery embolization often result in an occlusion in M1 or M2 segments of MCA. The complication of MCA embolization is often due to a reduced perfusion pressure resulting from carotid lesion. In case of hyperacute occlusion it is possible to dissolve the clot in the extracerebral segment of carotid followed by angioplasty. With time passing, a

solidified lot makes it harder for the catheter to pass. This serves as a good example demonstrating why time is crucial in stroke management.

Lacunar strokes and other causes. Lacunar strokes are especially common in patients with diabetes and/or hypertension. This type of stroke is often followed by immediate motor and sensory deficits, although the recovery rate is higher compared to, for instance, MCA territory strokes. Other stroke causes could be: inflammatory conditions such as arteritis, amyloid angiopathy, moya-moya; venous sinus thrombosis; vasospasm due to subarachnoid haemorrhage; migraine.

1.1.4. Pathophysiology

Pathophysiological processes of stroke evolve in a series of stages usually referred to as hyperacute, acute, subacute and chronic stages of stroke. We will discuss main pathophysiological events according to these stages.

Hyperacute stage of ischemic stroke. The ischemic events initiate with focal cerebral hypoperfusion. Within the first hours cellular bioenergetic failure develops, starting from excitotoxicity followed by oxidative stress. Thus, it is the matter of time if the area surrounding the ischemic core will become necrotic or saved. In the hyperacute phase of stroke blood-brain barrier (BBB) is mainly intact, but as the cerebral blood flow (CBF) is impaired the level of oxygen and glucose drop, triggering a cascade of reactions. First, the concentration of adenosine triphosphate (ATP) in the ischemic brain tissue reduces, which results in a lack of the substrate for the functioning of ionic transporters such as Na+/K+ ATPase and Ca2+ ATPase (Zhu et al., 2022). Consequently, Na+ accumulates within the cell, driving the movement of fluid inwards. This results in oncotic cell swelling termed as cytotoxic edema (Stokum et al., 2016). Cytotoxic edema is one type of a broader category of astrocyte swelling. The two types of ions that are involved in cytotoxic edema are primary drivers and secondary participants. Primary drivers, for example Na^+, are concentrated outside of the cell due to primary active transport. Secondary participants like Cl^- and water do not exhibit pre-existing electrochemical gradient but their flux is stimulated by the rearrangement of primary drivers in order to maintain electrical and osmotic balance. Cytotoxic edema always involves Na+ overload which by itself is sufficient to impair astrocyte volume regulation.

As it is clear from its name, cytotoxic edema is triggered by endogenous toxins (K^+, glutamate, H^+) and/or exogenous toxins.

Under normal conditions intracranial water is dynamic depending on neuronal activity but always maintained at homeostatic volumes inside impermeable compartments. Cytotoxic edema is a pathological increase of water mass in the brain that results in intracellular fluid accumulation. Extracellular ionic edema and vasogenic edema manifest later as brain tissue volumetric expansion or swelling. This volume marriage expansion of the tissue increases intracranial pressure up to the point when tissue pressure exceeds capillary pressure. If this happens, capillary lumens collapse and further edema and swelling are triggered. At the same time, for these physiological phenomena to develop, perfusion by an external fluid source is required. There are hypotheses on the new water sources. The first hypothesis is that fluid moves into parenchyma across endothelial cells of the capillaries under osmotic forces. BECs also exhibit high water permeability in the healthy brain, mediating water transfer. According to the second hypothesis, CSF serves as an immediate source of water, and swelling occurs when CSF influx into parenchyma exceeds the efflux of parenchymal interstitial fluid (ISF) (Chen et al., 2021). Nevertheless, these hypotheses do not have to be mutually exclusive as they might explain ionic edema formation at different levels.

Cytotoxic edema can be detected immediately after an arterial occlusion with diffusion-weighted MRI (DWI) since the decrease of the diffusion coefficient of water protons causes an increase of signal intensity in this sequence (von Kummer & Dzialowski, 2017). A decrease in apparent diffusion coefficient (ADC) 90 min after MCA occlusion was associated with extracellular fluid loss, swelling of cellular compartments and neuronal shrinkage. Although high-signal lesions that represent cytotoxic edema may disappear with reperfusion, these are closely associated with infarction when persistent.

Acute ischemic injury triggers endothelial dysfunction that results in loss of BBB integrity. There are three stages of endothelial dysfunction that starts with ionic edema, followed by vasogenic edema and, in the worst case, haemorrhagic transformation. There is an important difference between ionic edema and vasogenic edema. The influx of ions during ionic edema is mediated by endothelial ion channels and transporters, while physical disruption of BBB occurs only with extravasation of plasma proteins such as albumin and IgG. Vasogenic edema is extracellular and is characterised by the breakdown of BBB. Brain capillaries are a major contributor of vasogenic edema formation. The

dynamics of vasogenic edema depends on hydrostatic pressure gradient that is controlled by such factors as intracranial pressure, systemic blood pressure, capillary occlusion and vasospasm. Thus, hydrostatic pressure has direct clinical implications, such as that systemic blood pressure must be high enough to provide necessary brain perfusion and at the same time it promotes edema. It has been shown that vasogenic edema forms via paracellular transport through endothelial permeability pores. Ischemic injury and inflammation trigger BECs retraction which means increased permeability. At the same time, there must be other mechanisms involved to disturb BBB. Possible contributors are the leukocyte transmigration, increased VEGF expression, protease degradation through matrix metalloproteinase (MMP) activity (Barakat & Redzic, 2016). Haemorrhagic transformation (HT) as a secondary injury following ischemia represents the final phase of endothelial dysfunction, and like vasogenic, the driving force for HT is the hydrostatic pressure. Mechanisms of HT are complex and not completely understood. In stroke settings HT seems to be a sum of all above mentioned mechanisms coupled with unsuccessful acute treatment.

From the neuroradiological perspective, ionic edema may be present with brain tissue swelling, but brain tissue swelling is not necessarily associated with ionic edema. Brain tissue swelling without edema may develop with low perfusion pressure, meaning that perfusion is provided to some extent by compensatory arterial dilatation. In this case no tissue hypoattenuation would be present on CT scans, or no visible lesion on DWI-MRI. Such isolated focal brain swelling indicates tissue at risk but is reversible if perfusion pressure is enhanced. With the development of vasogenic edema, extravasation of protein-rich fluids into ischemic parenchyma takes place and causes mass effects such as midline shift, herniation and tissue compression that are visually detectable with conventional T_2-weighted MRI or CT. In general, vasogenic edema affects white matter more due to its compliance. Brain edema is a life-threatening complication that is often associated with MCA stroke. Up until today decompressive craniectomy remains the most effective therapy to lower intracranial pressure and reduce mortality, although this is an invasive method associated with additional risks.

Acute stage of ischemic stroke. Following the first six hyperacute hours of stroke and during the next 72-96 hours, the acute stage of stroke takes place. This stage is critical from a therapeutic point of view for saving the surrounding area of the ischemic core, known as peri-infarct tissue or penumbra. Over the course of days to weeks, the

neuroinflammatory response develops in the brain, resulting in BBB permeability alterations. Ischemia-induced cell death, residual cell debris and increased reactive oxygen species (ROS) production during the hyperacute stage leads to the neuroinflammation by triggering resident microglia and astrocytes. At this acute phase, higher permeability is associated not only with the severity of hemorrhagic transformation (HT) but also the likelihood to develop it. HT occurs when cerebral blood flow is restored to damaged blood vessels that are still weakened by an ischemic stroke. Early BBB disruption and early HT are driven by several abovementioned molecules. Reperfusion-induced ROS can disrupt the neurovascular units (NVU) – essential BBB building blocks – which will lead to the infiltration of neutrophils from the bloodstream. Neutrophils in turn have the capacity to secrete matrix metallopeptidase 9 (MMP-9) which is a pivotal mediator of early HT and BBB disruption. In contrast, delayed BBB disruption and HT (>36h) are believed to be related to the activation of brain-derived proteases, neuroinflammation, and factors that promote vascular remodelling such as VEGF (Rong et al., 2020).

Inflammation is especially important in the context of reperfusion. As it has been mentioned, establishing reperfusion is essential to save penumbra but it can also initiate the cascade of secondary injury via ROS generation, BBB disruption and different programmed cell death pathways as well as peripheral immune responses. In case of ischemic stroke, microglia are the central player of the inflammatory cascade. Resident microglia are activated within minutes after ischemic lesion and accumulate in the core and penumbra. Postischemic microglial proliferation peaks at 48–72 h after focal cerebral ischemia and may last for weeks. Upon activation, microglia can proliferate in two phenotypes: the “classically activated” M1 phenotype, which contributes to neuroinflammation and increased BBB permeability by releasing proinflammatory cytokines such as TNF-α and IL-1β, ROS and nitrous oxide (NO); the “alternatively activated” M2 phenotype, which is less inflammatory, produces less NO and more of anti-inflammatory cytokines and neurotrophic factors such as GDNF, BDNF, bFGF, insulin-like growth factor (IGF)-1 and VEGF (Xing et al., 2012). This microglial phenotype plays a crucial role in neuronal recovery at later stages that is associated with increased permeability. The biggest contradiction of microglia activity in stroke settings are the timing and number of cytokines expressed. TNF-α overexpression has been most associated with bad outcomes. It is probable that modulating duration and magnitude of

cytokine expression as a potential therapeutic approach could benefit stroke patients in the future. However, targeting inflammation especially in the brain is a non-trivial task.

Speaking of BBB disruption at acute phase, the function of matrix metallopeptidase 9 attracts particular attention. Neutrophile-derived MMP-9 concentration in patients' plasma has been proven to be a strong marker for BBB permeability in stroke and also for HT probability (Bernardo-Castro et al., 2020). This metalloproteinase provides proteolytic degradation of the ECM components and NVU proteins such as TJs' occludin and claudin. MMP-9 activity has been reported to increase secondary bleeding risk and worse clinical outcome in general (Rosell et al., 2008).

Subacute stage of ischemic stroke. At subacute stage neuroinflammatory cascade favours recovery through anti-inflammatory microglia phenotype that we have described above. Probably the most crucial for the brain recovery process that develops at subacute stage is angiogenesis. Clinical outcome can be predicted depending on how well the blood flow is restored to previously ischemic areas. Studies of patients' derived EPCs populations show that EPCs functional properties are associated with subacute BBB permeability and good clinical outcome, suggesting EPCs' contribution in poststroke angiogenesis. It is the capacity of EPCs to migrate and promote in vitro angiogenesis at day 7 that affects clinical outcome and angiogenesis in vivo (Sargento-Freitas et al., 2017). We will discuss this process in more detail in the section dedicated to angiogenesis.

Chronic stage of ischemic stroke. Eventually, successful brain recovery depends on the restoration of the NVU complex. It has been shown that posttranslational modifications such as phosphorylation of TJs' proteins result in an increased BBB permeability (Yamamoto et al., 2008). Animal studies support the idea that damping TJ protein modification could attenuate vascular hyperpermeability (Willis et al., 2010). Another important aspect of NVU restoration is junctional protein distribution that is altered in stroke condition. It is claudin-5 dissociation from actin cytoskeleton of BECs and actin polymerization that induce redistribution of TJ proteins and consequent BBB breakdown. Removal of factors that cause internalisation and degradation of NVUs is the prior step in permeability normalisation after stroke.

During the chronic phase, neovascularization and neurogenesis evolve. Unlike angiogenesis, neovascularization means de novo formation of blood vessels, so not only sprouting of the pre-existing vasculature. EPCs contribution to neovascularization has been observed in various vascular conditions, including ischemic stroke. EPCs migrate

from bone marrow to the sites of arterial injury and differentiate into mature endothelial cells that repair injured endothelium and form new blood vessels. Speaking of neurogenesis, for an extended period of time, it was believed that this process was not possible in the adult brain. However, it has been shown that there are two brain regions where neurogenesis takes place, namely the subventricular zone (SVZ) of the lateral ventricle walls and the subgranular zone of the dentate gyrus. Neural stem cells (NSCs) activated by a cascade of reactions in response to ischemic injury differentiate and migrate into the affected area to replace dying neurons. At the same time, NSCs' capacity to compensate for the damage is limited and decreases with age. Moreover, NSCs themselves can be damaged by stroke, although this mechanism is not very clear (Koh & Park, 2017). It has been reported that hypoxia triggers gliosis, as NSCs differentiate into astrocytes rather than neuroblasts and form glial scar tissue. Some clinical studies show that strokes that involve the subventricular zone result in a worse functional recovery (Lee et al., 2010). There is a therapeutic possibility to enhance neurogenesis after stroke by protecting NSCs from stroke-induced damage. Particularly, it has been shown that some growth factors have protective function, for example nerve growth factor neurotrophin (NT)-3, brain-derived neurotrophic factor (BDNF), some fibroblast growth factors (FGF), transforming growth factor (TGF)-α, epidermal growth factor (EGF) and many more (Tao et al., 1996; Seroogy et al., 2005). Currently the main research line regarding neurogenesis is understanding intracellular pathways that promote proliferation and enhancement of NSCs neurogenesis.

The leakage of BBB will persist to some degree despite several repair mechanisms in place. It has been proposed that TJ disorganisation is the reason for incomplete sealing of the barrier (Sladojevic et al., 2019). Underexpression as well as altered protein-protein interactions are among proposed mechanisms. Although it is debatable how favourable is the remaining incomplete closure of the barrier, it may benefit neurogenesis. Thus, incomplete sealing of BBB at chronic stage is a potential window to enhance neural recovery after an ischemic stroke.

1.1.5. Clinical management

The primary goal of acute stroke management is to reduce the time from patient's arrival to medical intervention to 60 minutes or less, the so-called "golden hour".

According to the consensus guidelines to manage a patient suspected of having a stroke, the sequence of events starts with retrieving information about patient's medical history, carrying out physical examination and taking blood samples, performing brain non-contrast enhanced computed tomography (NECT) within 30 minutes, interpret imaging results within 20 minutes after acquiring the sequence and decide on the treatment strategy. It is essential to know if the intracranial haemorrhage or a stroke "mimic" is present. Once intracranial haemorrhage is excluded it removes important contraindication for recanalization therapy. However, another critical issue to address is whether a major cerebral vessel is occluded. CT angiography (CTA) can be obtained immediately following the NECT scan in order to noninvasively visualise the blood flow inside intracranial vessels. The main advantage of NECT in emergency settings is that it is usually widely available and relatively fast to obtain. In the hyperacute phase of stroke NECT is used as a standard technique. In some cases, additional MRI scans (often DWI-MRI) are requested because its improved resolution and sensitivity can help identify lesions and differentiate stroke subgroups better. However, this technique cannot yet substitute CT both timewise and cost wise (Mizuma & Yenari, 2021).

Recanalization therapy aims to preserve balance between blood flow restoration to save penumbra and avoid haemorrhagic transformation. Conventional treatment is intravenous administration of recombinant tissue plasminogen activator (rTPA) within the therapeutic window of less than 3 hours. Mechanical thrombectomy is typically performed within less than 6 hours because this has been associated with good 90-day functional outcomes. Nevertheless, some patients may still benefit from mechanical thrombectomy performed up to 24 h after symptom detection, particularly in case of basilar artery thrombosis and in presence of a persistent significant perfusion-diffusion mismatch that represents the non-core hypoperfused area. While hyperacute and acute stages are crucial to save a patient's life and brain tissue at risk, in the late subacute to chronic stages, the main focus is on the functional recovery. Personalised rehabilitation and continued surveillance are pivotal for good recovery.

1.2. Collateral blood flow

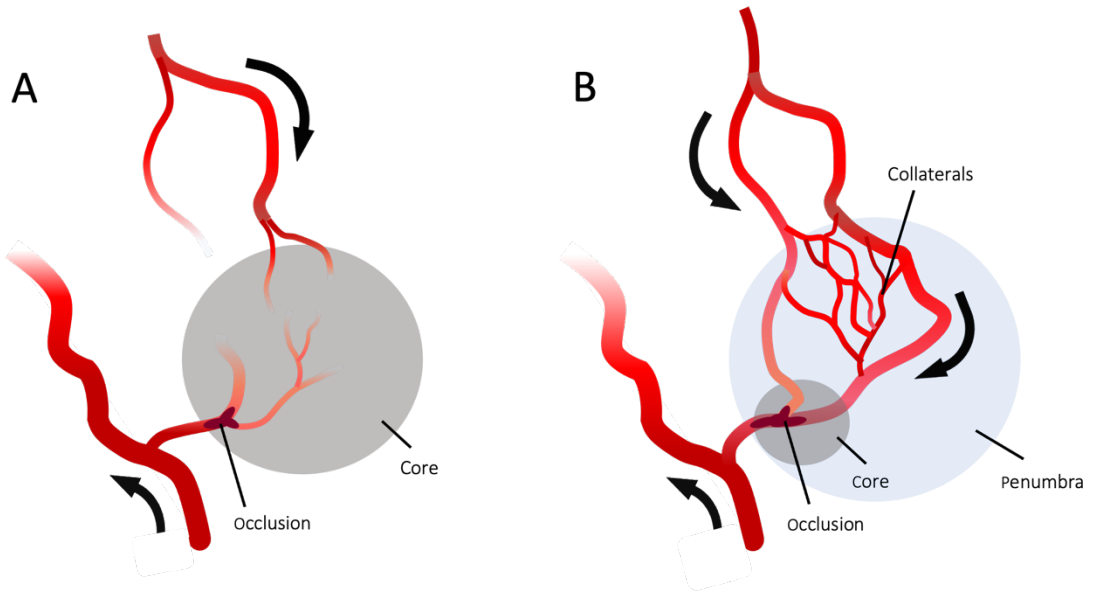

Figure 1. Schematic representation of an ischemic stroke with poor (A) and good (B) collateralization.

There are two main biological mechanisms for restoring neuronal function after a period of hypoxia caused by an ischaemic event, namely collateral blood flow (CBF) and angiogenesis. Usually, collateral circulation begins to develop immediately after an ischemic event to supply the area around the acute occlusion (Fig.1). It has been reported that better collateral flow is associated with smaller infarct volume and better clinical outcome (Iwasawa et al., 2016). Thus, collateral flow facilitation could help to avoid haemorrhagic transformation which is one of the most severe complications caused by conventional recanalization therapies. Collateral perfusion relies on three main anatomical features. First, the large-artery communications that exist between extracranial and intracranial circulations. For instance, external carotid arteries give rise to numerous branches in the neck that can serve as potential sources of collateral flow, particularly in cases of chronic internal carotid artery (ICA) stenosis or occlusion. In addition, collateral blood supply includes retrograde flow through the ophthalmic and superficial temporal arteries to the intracranial vessels that are normally supplied by the internal carotid artery. Similar anastomoses are also present in the posterior circulation, where many branches of the vertebral arteries communicate with muscular branches. Furthermore, the anterior and posterior spinal arteries connect with branches of the proximal intracranial arteries that supply the medulla and pons.

The second important opportunity for blood redistribution is possible because of the connection of major cerebral arteries through the circle of Willis. The third source of

collateral flow is the leptomeningeal anastomoses that connect major arteries and small arterioles allowing retrograde perfusion. These are especially important in case of acute MCA occlusion because they can supply anterior divisions of the MCA. Posterior collateral flow circulation for MCA arises from the posterior cerebral artery.

Under normal conditions, activity of CBF is determined by local metabolic load and regulated by the vascular innervations. In ischemic stroke the extent of infarction can be limited to some extent by collateral flow, but to which extent varies significantly among patients depending on various systemic factors, such as congenital lack of collateral anatomy, dehydration, hyperthermia, hyperglycaemia and many others. Another factor that affects robustness of collateralization is the pace of occlusion. In such conditions as moyamoya syndrome, occlusion happens gradually, usually beginning with carotid stenosis, which means that there are more chances for compensatory collateral flow to develop before complete occlusion happens. On the contrary, in case of a sudden thrombotic occlusion collateral circulation faces the demands that are often impossible to satisfy. Similarly, the location of the occlusion matters. For example, an occlusion of a proximal segment of a major artery would most likely result in a better collateralization because of a numerous network of vessels surrounding the area. More distal occlusive lesions have less chance for potential collateralization.

Good collateralization can mask neurologic symptoms which makes it hard to define clinical correlates for collateral flow. A good illustration for these is the possible preservation of sensitivity in presence of complete MCA occlusion. Given that some symptoms are masked it becomes harder to make decisions about reperfusion therapy for which time is a crucial factor. Moreover, the initial development of collateralization matters for collateral endurance and secondary collateralization. Altogether, the extent of tissue infarction is determined to a great extent by the balance between supply demand of the ischemic area and the actual collateral support. Some infarction patterns indicate poor history of collateralization, such as deep subcortical infarcts (Liebeskind, 2005).

Since clinical manifestation of collateral flow is inconsistent, imaging is the only reliable tool for collateral flow assessment. There are several techniques that provide accurate measurement of collateral blood flow. Cerebral angiography, CT angiography (CTA), MRI angiography and transcranial doppler are the possible imaging modalities, among which conventional digital subtraction cerebral angiography is the gold standard. This method allows for high level resolution, which makes it easy to define location of

the occlusion, its size and the anatomy of the occluded vessel. However, this is an invasive technique that requires contrast, X-ray and a qualified interventional neurologist, which is why CTA is often used as the first choice due to its accessibility and non-invasive nature. There are multiple scores to grade collateral flow views with CTA. In this study we used the score proposed by Tan et al., which is a four-point scale (Table 1). MRI angiography is often used to visualise intralaminar thrombi that appear hyperintense due to calcification. Transcranial colour coded doppler (TCCD) is a non-invasive ultrasonography test to evaluate circulatory velocity of the blood in basal cerebral arteries (Circle of Willis). This method is highly useful in acute stroke management since it does not require patients' transportation, it is non-invasive, fast and has no absolute contraindications.

1.3. Angiogenesis

In healthy condition, the genesis of blood vessels takes place during tissue's development and growth. The particular mode of new blood vessels formation from existing vessels is referred to as sprouting angiogenesis or just angiogenesis for simplicity. This is a complex process that occurs under both healthy and pathophysiological conditions, and yet a single growth factor, VEGF, is known to be its key regulator. This factor stimulates angiogenesis in health and disease through VEGF receptor-2 (VEGFR-2) (Miller & Sewell-Loftin, 2022). Eventually, this interaction seems to be a promising therapeutic opportunity in subacute stroke patients. Angiogenesis is tightly related to blood-brain barriers permeability and NVU in particular. For sprouting to happen partial reorganisation of NVU is required, which means alterations in BBB permeability. Angiogenesis after an ischemic injury and pathologic BBB disruption is caused by the same stimuli. At first, mobilisation of endothelial progenitor cells is required to the ischemic area. An inflammatory pathway results in a local degradation of the basal vascular membrane, meaning that tight junctions of NVU detach. Thus, the rupture of BBB allows for cellular migration and new vessel formation via sprouting.

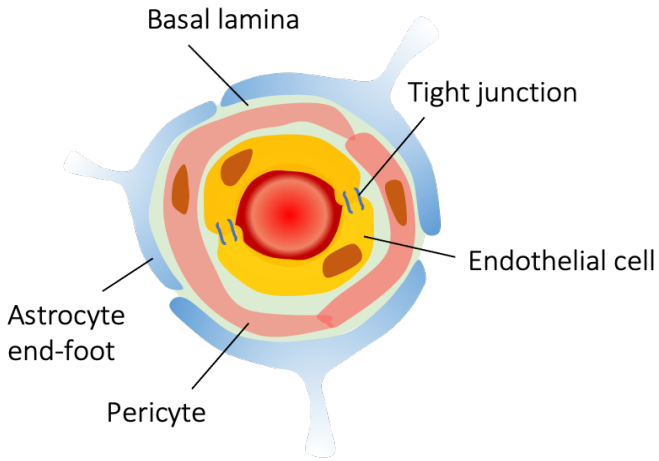

Figure 2. Schematic representation of NVU cellular components (frontal plane).

Neurovascular Unit and Junctional Complex. In order to understand the permeability of the blood-brain barrier it is important to know its structure. The dynamic functional units that form BBB are called the neurovascular units (NVU). NVUs consist of BBB endothelial cells (BECs), pericytes, astrocytes (mainly their end-foots), and extracellular matrix components (EMCs) that compose the basal membrane or lamina (Serlin et al., 2015). The junctional complex is what keeps BECs together. It is compromised by different types of junctions, such as tight junctions (TJs), adherens junctions (AJs), and gap junctions (GJs). These junctions in turn are composed of transmembrane proteins, scaffolding proteins and cytoskeleton. Basal membrane (BM) provides an anchor and stability for other cellular components of the NVU. Therefore, the disruption of BM damages junctions between BECs and compromises BBB integrity. Pericytes play an important role in preventing infiltration into CNS, synthesising some EMCs important for BM and modulating capillary dilation apart from their phagocytic function of degrading cellular debris. Astrocytes cover blood vessels with their end-foots that express a number of regulatory particles and thus provide connection between the vascular and nervous system, known as neurovascular coupling. Apart from that, astrocytes significantly contribute to the brain energy metabolism regulation. Microglia is also a part of NVU being in contact with microvessels and mediating primary inflammatory reactions.

Blood-brain barrier permeability. BBB permeability at each stage of stroke has a meaning. At first, increased BBB permeability occurs due to hypoxia and early BBB disruption in the hyperacute stage. During the acute phase of stroke neuroinflammation further contributes to BBB rupture, and this is when the second peak of increased permeability is observed. A number of studies suggest that BBB remains opened during the subacute and the chronic stages of stroke due to regenerative processes rather than pathology (Sargento-Freitas et al., 2018; Villringer et al., 2017). Speaking of

angiogenesis, it is at the subacute phase of stroke (>7 days) when increased permeability is a positive process that speaks for the new vessels' formation, even though there is always a risk of secondary bleeding (Bernardo-Castro et al., 2020).

By means of advanced imaging techniques, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), it is possible to non-invasively estimate BBB permeability by contrast agent extravasation. DCE-MRI measures the contrast-induced changes of the T_1 relaxation time that are due to contrast extravasation into brain tissue. The tissue T_1 relaxation time is directly related to the concentration of the contrast agent inside the vessels and brain parenchyma. This makes it possible to estimate the blood-to-brain transfer constant (K_i) by assessing local dynamics of the signal intensity of T_1-weighted MR images (Seevinck et al., 2010). The increase of K_i is observed in later stroke stages, and is associated with angiogenesis.

Important insights on the angiogenesis have been inferred from animal studies. A way to evaluate angiogenesis is to measure the density of microvessels. It has been reported that a higher density of new vessels in affected areas of mouse and rat brain is followed by functional recovery upon administration of VEGF (Zhang et al., 2000). Similarly, consequent studies in stroke patients have shown the same positive correlation between microvessels abundance and clinical outcome after stroke (Krupinski et al., 1994). Another approach to study interaction between angiogenesis and neuronal survival and genesis is co-culture systems. Such studies show that VEGF prompts axonal outgrowth (Namiki et al., 1995).

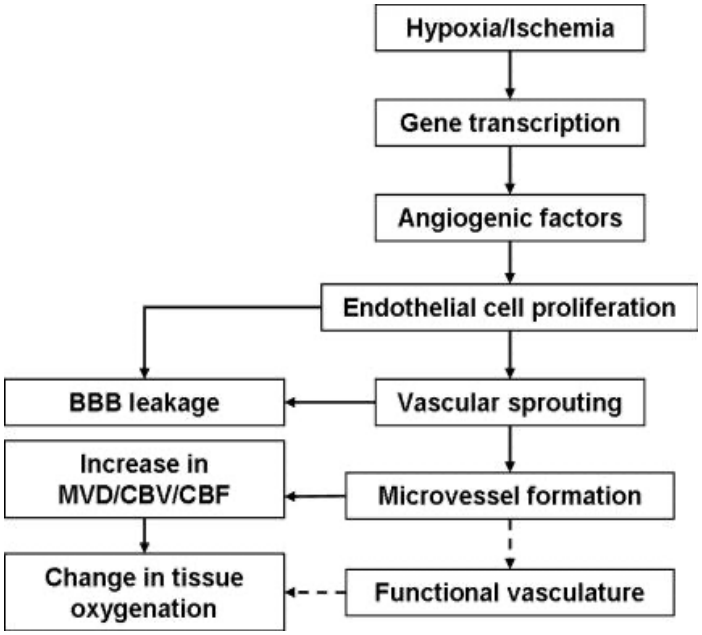

Figure 3. Schematic representation of the cascade of events associated with angiogenesis after ischemic stroke. The physiological phenomena such as BBB leakage, increase in microvessel density, cerebral blood volume and cerebral blood flow may be assessed with MRI (adapted from Seevinck et al., 2010).

BECs are the main targets in angiogenesis. Increased production of endothelial nitric oxide synthase (eNOS), increased VEGF expression and a number of other signalling pathways involving kinases benefits angiogenesis (Chen et al., 2005). At the same time, increased eNOS activity causes release of NO, which is known to mediate neurovascular coupling by fostering vessel dilation and remodelling (Lapi et al., 2013). Activation of BECs integrin matrix receptors, such as αvβ3, also plays a significant role in angiogenesis after stroke (Guell & Bix, 2014).

Chapter 2

| Hypothesis and Aims

The abundance and severity of MCA stroke justifies numerous studies that aim to find better treatment strategies. It has been previously shown that both collateral circulation and angiogenesis significantly contribute to neuronal survival and recovery after ischemic stroke. However, the relation between these vascular processes is not clear. A hypothesis that we tested was that post-stroke angiogenesis could be predicted depending on the collateral circulation around the acute occlusion.

Thus, in this study we aimed to:

• To assess the association between collateral flow and angiogenesis.

• To evaluate the association of collateral flow in the ischemic region with functional outcome at three months.

Chapter 3

| Materials and Methods

3.1. Study design and population

We performed a retrospective analysis of prospectively included patients in two independent cohorts. The data from the first cohort was collected between 2014 and 2017. Data from the second cohort was collected within the period from July 2020 to July 2021. All patients were admitted to the Neurology department of Coimbra University Hospital. We screened all patients included in both cohorts and excluded those that did not have information on collateral flow. The two cohorts had identical inclusion and exclusion criteria, as expressed in each published protocol (Sargento-Freitas et al., 2018b). In short, inclusion criteria were as follows: patients with ages within 18-80 years, and stroke classified as a non-lacunar cerebral infarction within the territory supplied by middle cerebral artery (MCA). Exclusion criteria of the cohorts were: intra-arterial therapy, premorbid mRS higher than 2, ongoing infection, contra-indications to MRI, premorbid neurocognitive pathology, symptom onset more than 24 hours, recent surgery, coma. For this study we excluded patients that did not have information on collateral flow based on CTA or TCCD (see Table 1).

Table 1. Inclusion and exclusion criteria for the study participation.

Inclusion criteria	n (%)
Patients with acute ischemic stroke within the MCA, age between 18-80 years, included in the study cohorts.	75 (100%)
Exclusion criteria	n (%)
No TCCD or CTA data available to evaluate collateral flow.	17 (22.6%)

Upon admission, patients' demographics and vascular risk factors data were collected and neurological condition was assessed using the National Institutes of Health Stroke Scale (NIHSS) and premorbid modified Rankin scale (mRS) by a trained vascular neurologist. Clinical outcome was assessed at 3 months after stroke using NIHSS and mRS.

3.2. Neuroimaging

All neuroimaging procedures in both cohorts were performed following the same protocol.

3.2.1. NECT and CTA

3.2.1.1. Scanning Protocol and Generation of Parametric Maps

Upon admission non-enhanced computed tomography (NECT) was performed in all patients. When indicated, computed tomography angiography (CTA) images were also acquired. Scans were performed following standardised multicentre protocol in a multi-slice scanner. Standard coverage (from the skull base to the vertex) axial NECT was performed with sequential sections in the traditional axial plane (parallel to the orbitomeatal line). CTA acquisitions were obtained immediately after NECT upon administration of single-bolus intravenous injection of 70–90 mL of a non-ionic contrast medium into an antecubital vein at 3-5 mL/s. Imaging was manually triggered by the appearance of the contrast medium in the ICA. Source images were reconstructed in axial planes with 1 mm thickness slices and 0.625 mm thickness intervals. Protocol adapted from Sousa et al., 2022.

3.2.2. TCCD

Transcranial colour coded doppler (TCCD) was used to investigate collateral vasculature in the first cohort of patients. The hemodynamic evolution was evaluated with serial cervical and transcranial neurosonological exams at admission (day zero), six hours, seven days and three months after stroke with 11MHz sector probe and 3MHz linear-probe (General Electric Logiq7). Data on flow diversion, recanalization and CBF was collected from patients in a supine position after 10 minutes of resting. Flow velocities were assessed bilaterally using TCCD with angle correction in ACA, MCA and PCA. Flow diversion (collateral flow) was defined as a high-velocity, low-resistance flow signal in the ACA-A1 segment or P1–P2 segments of the PCA ipsilateral to the occluded MCA; the ACA or PCA flow velocity had to be equal to or higher than the unaffected MCA. Protocol adapted from Sargento-Freitas et al., 2018b.

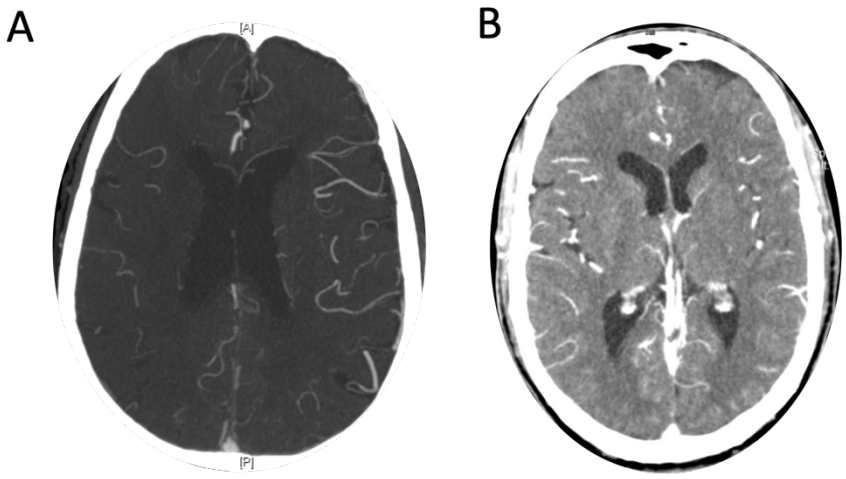

Figure 4. | Example of the poor (A) and good (B) collateral flow imaged with CTA (graded according to Tan et al., 2009). In each sequence, ischemic lesion is present in the right hemisphere; (A) Poor collateral supply ipsilateral to the lesion, <50% of occluded MCA territory is supplied (score = 1); (B) Good collateral supply of >50% of the occluded MCA territory (score = 2).

3.2.3. MRI

MRI imaging acquisition was performed on a 3.0 Tesla scanner with a standard phased array head/neck 20-channel coil (3T MAGNETOM PRISMA fit, Siemens Medical Solutions, Erlangen, Germany). First, structural MRI sequences including 3D T_1-weighted magnetization prepared rapid gradient echo (MPRAGE) (flip angle = 7º, echo time = 3.5ms, repetition time = 2530 ms, field of view = 256mm, reconstructed matrix size = 256x256, slice thickness = 1mm), axial T_2 fluid attenuated inversion recovery (FLAIR) (echo time = 440 ms, repetition time = 4800 ms, field of view = 230mm, reconstructed matrix size = 256x256, slice thickness = 0.90mm), axial susceptibility weighted imaging (SWI) (flip angle = 15º, echo time = 20ms, repetition time = 27ms, field of view = 235mm, reconstructed matrix size = 256x256, slice thickness = 1.50mm) and axial diffusion weighted imaging (DWI) (flip angle = 180º, echo time 1 = 61ms, echo time 2 = 105ms, repetition time = 3880 ms, field of view = 235mm, reconstructed matrix size = 200x200, slice thickness = 4.0mm) were performed to evaluate the ischemic lesion.

In order to retrieve BBB permeability, DCE-MRI axial T_1-gradient echo (GRE) was performed right after structural sequences. T_1 map was estimated using three variable flip-angle GRE sequences was acquired with following settings: flip angle 2º,5º or 10º,

repetition time = 5.9 ms, echo time = 2.11 ms, 128 × 128 matrix, field of view = 245 mm, slice thickness = 5 mm. 20 continuous measurements of the perfusion sequence were acquired with a flip-angle of 20º. A paramagnetic contrast agent – Gadobutrol (Gadovist 1,0 mmol/ml, Bayer Pharma AG, Berlin, Germany) – was injected after the fourth sequence with a programmed flow rate of maximum 4 ml/s and a contrast dose of 0.1mmol/kg body-weight. The rate and volume of saline-flush injection was 3.5 mL/s and 20 mL, respectively (analysis according to Sargento-Freitas et al., 2018b).

3.3. Image analysis

In both cohorts, all image analyses were performed by neurologists blinded to clinical data. Collateral flow was evaluated using CTA and/or TCCD. BBB permeability was assessed using DCE-MRI data.

3.3.1. Collateral flow evaluation

Collateral flow was assessed using two distinct methods, namely CTA and TCCD. Since collateral flow evaluation was made in retrospective analysis, the selection of the evaluation method was done merely on the basis of data availability. When both scans were available for one patient, CTA was preferred. In order to be able to integrate and compare data as a whole regardless of the used method, collateral flow was computed as a dichotomous variable as "poor" collateral flow or "good" collateral flow (see Table 2).

Table 2. Collateral vessel grading systems used in this study

Modality	Grading system	Comments
CTA	0: absent collateral supply to the occluded MCA territory	Recoded as poor collateral supply for the main analysis (binary variable)
	1: collateral supply filling ≤50% but >0% of the occluded MCA territory	
	2: collateral supply filling >50% but <100% of the occluded MCA territory	

	3: 100% collateral supply of the occluded MCA territory	Recoded as good collateral supply for the main analysis (binary variable)
TCCD	Good collateral supply: ACA or PCA flow velocity equal/ higher than ipsilateral MCA	
	Poor collateral supply: ACA or PCA flow velocity lower than ipsilateral MCA	

3.3.2. CTA evaluation of collaterals

Non-contrast single-phase CTA scans and maximum intensity projection (MIP) images were reviewed by a staff neuroradiologist (R.I.A., S.P.S. with 5 years' stroke imaging experience). The collateral grading system was scored on a scale of 0–3 proposed by Tan et al for patients with unilateral infarct with occlusion involving MCA with or without ACA occlusion (Table 1) (Tan et al., 2009). A score of zero indicated absence of collateral supply to the occluded MCA territory. A score of 1 indicated collateral supply filling ≤50% but >0% of the occluded MCA territory. A score of 2 was given for collateral supply filling >50% but <100% of the occluded MCA territory. A score of 3 was given for 100% collateral supply of the occluded MCA territory. A soccer of 0-1 was classified as "poor" collateral flow and a soccer of 2-3 was classified as "good" collateral flow.

3.3.3. TCCD evaluation of collaterals

The hemodynamic evolution was evaluated with serial cervical and transcranial neurosonological exams at admission (day zero) with 11MHz sector probe and 3MHz linear-probe (General Electric Logiq7). Data on flow diversion, recanalization and CBF were collected from patients in a supine position after 10 minutes of resting. Flow velocities were assessed bilaterally using TCCD with angle correction in ACA, MCA and PCA. Flow diversion (collateral flow) was defined as a high-velocity, low-resistance flow signal in the ACA-A1 segment or P1–P2 segments of the PCA ipsilateral to the occluded MCA; the ACA or PCA flow velocity had to be equal to or higher than the unaffected MCA (analyses according to Sargento-Freitas et al., 2018b).

3.3.4. MRI BBB permeability evaluation

3.3.4.1. ROI selection for BBB permeability calculation

For both cohorts, MRI image processing and post-processing analyses were performed using a Syngo workstation MRB19 (Siemens Medical Solutions, Erlangen, Germany).

To calculate BBB permeability, a region of interest (ROI) was defined including the entire ischemic area. Ischemic areas were recognised as hyperintense signal regions on T_2-FLAIR MRI sequences. In case haemorrhagic transformation (HT) was visible within the lesion on SWI image, it was excluded from the ROI. ROIs were manually drawn on the lesion site. Contralateral to the ischemic lesion, an additional ROI of similar size, shape and anatomical position was always identified and used as a control (Fig. 5). In order to assess the volume of the infarcted area, a manual planimetric calculation was performed on FLAIR sequences, tracing the perimeter of the entire ROI.

3.3.4.2. BBB permeability estimation

Post-processing of DCE-MRI data was performed using Syngo Tissue 4D workstation software. Dynamic series were corrected for any patient motion and then registered. After this, ROIs previously identified were allocated accordingly on the corresponding DCE sequence (Fig. 5C).

BBB permeability was assessed using the Tofts pharmacokinetic model. This model was applied to each of the selected ROIs to evaluate the rate at which contrast agent leaked into the brain parenchyma over time. The volume transfer coefficient (K^{trans}) was computed as the surrogate measure for BBB permeability. The arterial input function (AIF) for the model was set as the best fit for the contrast agent concentration-time curve provided by Tissue 4D. For each patient, overall BBB permeability in the corresponding ROI was taken as the median value of the K^{trans} maps and reported as min^{-1}.

The ratio between BBB permeability values in both hemispheres was calculated with the aim of obtaining a normalised value of BBB permeability. K^{trans} values of the ipsilateral ischemic tissue were normalised against K^{trans} in the contralateral hemisphere

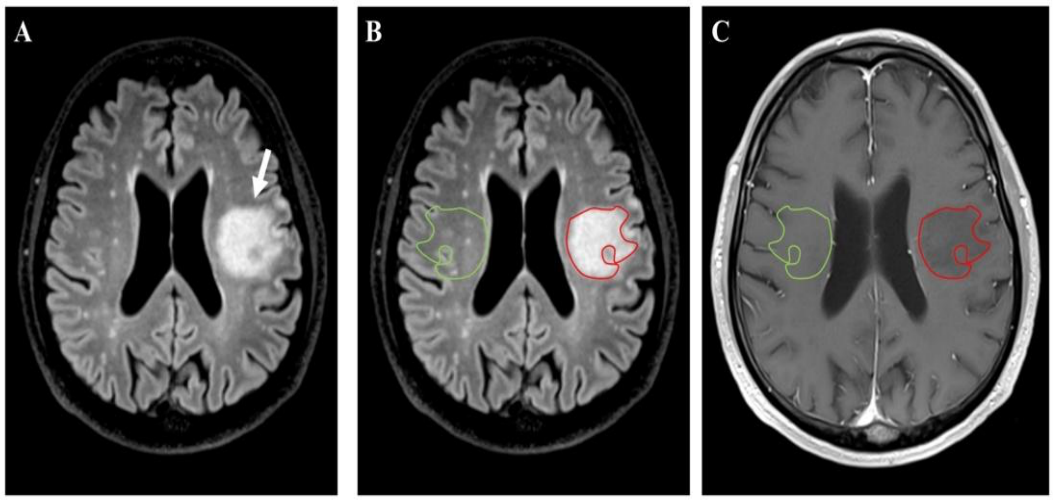

Figure 5. **ROI selection for BBB permeability calculation.** (A) FLAIR image. Lesion visible on the left hemisphere (white arrow). (B) ROI placement on FLAIR images. Ipsilateral ROI placement (red) and contralateral ROI placement (green). (C) ROI placement on the DCE sequence for BBB permeability assessment.

ROI. This measure allows for a measure with reduced variability attributed to individual clinical and permeability characteristics. A higher K^{trans} ratio will indicate greater differences between ischemic and contralateral permeability due to ipsilateral BBB status (analyses according to Sargento-Freitas et al., 2018b).

3.3.5. Statistical analysis

Quantitative variables were tested for normality using the Shapiro-Wilk test. Separate univariate association analyses for collateral flow and angiogenesis at day 7 after stroke were performed using binary logistic regression and linear regression, respectively. The threshold for significance was $p < 0.1$ for univariate, and $p < 0.05$ for subsequent multivariate analyses. The baseline clinical features of the studied population and their association with collateral flow score and angiogenesis are present in Table 2.

We used Pearson correlation analysis to study the main association between collateral flow and angiogenesis. We further analysed associations between collateral flow, angiogenesis and neurological outcome (NIHSS) and functional outcome (mRS) using ordinal and linear regressions adjusted for the baseline variables that were associated with good outcome.

Chapter 4

| Results

4.1. Clinical features

A total of 58 patients were included in the study (54.2% male, mean age 69.2 years) (Table 1). Three months after the stroke, 31 patients (53.4%) had good clinical outcomes (mRS between 0 and 2) and 5 patients (8.6%) were dead. No arterial reocclusions were documented in the study participants. Baseline NIHSS, NIHSS at 3 months after stroke and mRS at 3 months after stroke showed a normal distribution; all other variables did not have a normal distribution.

4.2. Collateral flow and angiogenesis

The univariate analysis of collateral flow with baseline clinical variables showed significant association of collateral flow with atrial fibrillation risk factor, stroke area and stroke volume (m^3) (see Table 3). However, no clinical variables were significantly associated with collateral flow on subsequent multivariate analyses.

The median BBB permeability at day 7 was 0.110 (K^{trans} ipsilateral). The univariate analysis with clinical variables revealed significant association of BBB permeability with age, intravenous thrombectomy and K^{trans} ratio.

We evaluated direct association between collateral flow and ipsilateral K^{trans} using non-parametric correlation analysis. There was no significant association between these variables: $r_s(43) = -.148$, $p = .331$ (Fig.6).

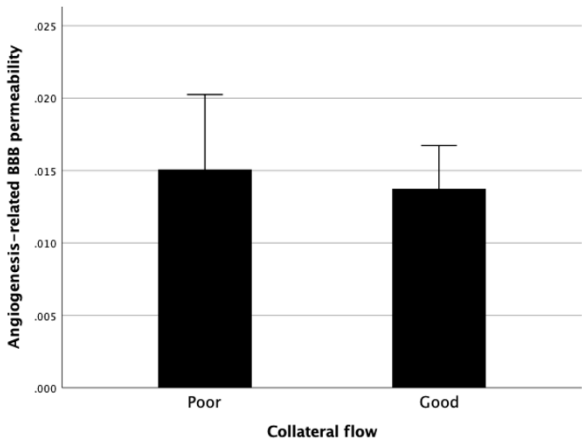

Figure 6. No significant association between the collateral flow and BBB permeability

Table 3. Coefficients of univariate analyses between clinical variables, collateral flow and angiogenesis-related BBB permeability

Factor	Collateral flow (day 0)			K^{trans} ipsilateral (day 7)		
	OR	95% CI	P-value	OR	95% CI	P-value
Age	0.962	0.898 to 0.031	0.271	-0.344	-0.001 to 0.000	0.021*
Sex	1.011	0.340 to 3.002	0.985	-0.106	-0.007 to 0.003	0.489
TOI	1.146	0.517 to 2.541	0.737	0.185	-0.001 to 0.001	0.545
TOAST	1.286	0.864 to 1.915	0.215	-0.146	-0.002 to 0.001	0.337
NHISS at admission	0.953	0.863 to 1.054	0.351	-0.177	-0.001 to 0.000	0.246
mRS at admission	1.367	0.364 to 5.128	0.644	-0.088	-0.006 to 0.004	0.569
HTA	0.481	0.133 to 1.737	0.264	-0.107	-0.007 to 0.004	0.484
Diabetes	1.241	0.329 to 4.678	0.749	-0.166	-0.009 to 0.003	0.276
Dyslipidaemia	1.282	0.419 to 3.921	0.663	0.109	-0.003 to 0.007	0.476
Hyperuricemia	1.241	0.329 to 4.678	0.749	-0.015	-0.008 to 0.007	0.920
Atrial fibrillation	0.351	0.114 to 1.083	0.068*	0.112	-0.003 to 0.007	0.463
Cardiac insufficiency	1.059	0.177 to 6.348	0.950	0.120	-0.004 to 0.010	0.434
Coronopathy	5.067	0.586 to 43.786	0.140	-0.101	-0.009 to 0.004	0.511
Previous stroke	1.056	0.090 to 12.400	0.966	-0.070	-0.012 to 0.008	0.648
Smoking	2.235	0.233 to 21.456	0.486	-0.124	-0.014 to 0.006	0.417
Alcohol	0.771	0.118 to 5.042	0.786	0.053	-0.007 to 0.009	0.727
Migraine	1.056	0.090 to 12.400	0.966	-0.092	-0.013 to 0.007	0.549
Peripheral arterial disease	2.235	0.233 to 21.456	0.486	0.027	-0.009 to 0.011	0.858
Obesity	1.077	0.332 to 3.490	0.902	0.068	-0.004 to 0.007	0.657
Intravenous thrombolysis	0.600	0.200 to 1.799	0.362	0.304	0.000 to 0.010	0.042*
K^{trans} ratio	0.544	0.904 to 1.114	0.264	0.401	0.000 to 0.001	0.007*
Stroke volume (m^3)	-0.002	0.996 to 1.000	0.001*	0.149	0.000 to 0.000	0.360
Stroke area	-0.011	0.968 to 1.011	0.051*	-0.290	0.000 to 0.000	0.337

4.3. Final outcome

We evaluated the association of each clinical variable with the final outcome at three months represented by NIHSS and mRS (Table 4). As shown with univariate analysis, age, NIHSS at admission, K^{trans} ratio, collateral flow and ipsilateral K^{trans} were associated with functional outcome (mRS). Univariate analysis for the neurological outcome revealed significant association between stroke etiology (TOAST), NIHSS at admission, previous stroke risk factor and K^{trans} ratio. Subsequent multivariate analyses

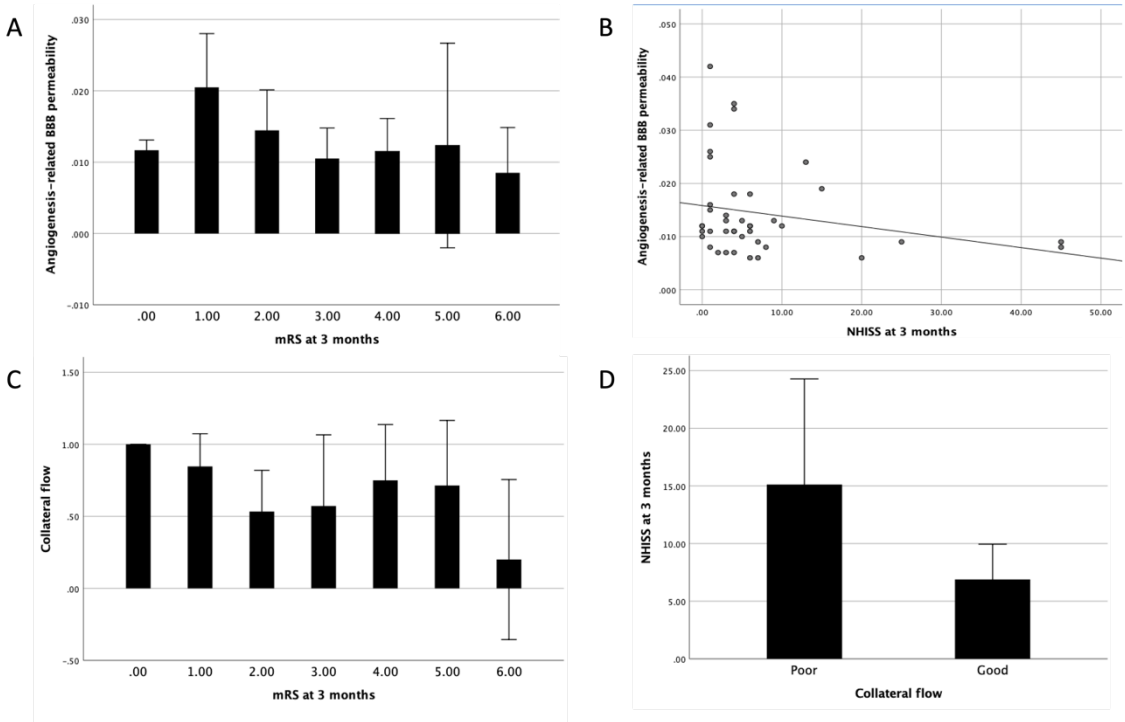

Figure 7. Significant associations between BBB permeability and final outcome at 3 months measured with mRS (A) and NIHSS (B), and between collateral flow and mRS (C) and NIHSS (D).

for associations between mRS and NIHSS at 3 months and pinpointed variables did not show any significant results.

Table 4. Coefficients of univariate analyses between clinical variables and final outcome represented by NIHSS and mRS

Factor	NIHSS (at 3 months)			mRS (at 3 months)		
	B	95% CI	P-value	B	95% CI	P-value
Age	0.210	-39.900 to 16.314	0.131	0.049	-0.006 to 0.103	0.079*
Sex	0.094	-4.800 to 9.625	0.505	-0.124	-0.037 to 0.789	0.790
TOI	-0.060	-0.998 to 0.832	0.845	0.301	-0.195 to 0.797	0.234
TOAST	-0.239	-4.579 to 0.308	0.085*	-0.112	-0.427 to 0.202	0.483
NHISS at admission	0.393	0.313 to 1.510	0.004*	0.184	0.090 to 0.278	0.000*
mRS at admission	-0.092	-0.035 to 0.018	0.514	-0.002	-0.006 to 0.002	0.286
HTA	0.101	-4.917 to 10.456	0.473	0.574	-0.435 to 1.578	0.265
Diabetes	-0.159	-14.312 to 3.887	0.256	-0.484	-1.578 to 0.614	0.387
Dyslipidaemia	0.037	-6.527 to 8.500	0.793	0.355	-0.594 to 1.300	0.464
Hyperuricemia	0.168	-3.356 to 13.635	0.231	-0.049	-1.145 to 1.044	0.929
Atrial fibrillation	0.145	-3.421 to 10.856	0.300	0.303	-0.612 to 1.212	0.514
Cardiac insufficiency	0.064	-8.756 to 13.956	0.648	0.169	-1.321 to 1.657	0.824

Coronopathy	-0.062	-11.689 to 7.478	0.661	-0.624	-1.887 to 0.653	0.648
Previous stroke	0.231	-2.851 to 33.967	0.096*	1.293	-0.778 to 3.357	0.222
Smoking	-0.012	-14.222 to 13.078	0.933	0.484	-1.131 to 2.100	0.557
Alcohol	-0.129	-17.889 to 6.567	0.375	-0.570	-2.200 to 1.063	0.495
Migraine	-0.124	-27.121 to 10.435	0.377	-1.818	-4.012 to 0.384	0.104
Peripheral arterial disease	-0.144	-17.256 to 7.257	0.417	-0.550	-2.182 to 1.079	0.510
Obesity	0.085	-5.552 to 10.400	0.544	0.376	-0.60 to 1.363	0.454
Intravenous thrombolysis	0.048	-5.978 to 8.432	0.734	0.026	-0.881 to 0.933	0.955
K^{trans} ratio	-0.432	-1.61-(-.33)	0.004*	-0.147	-0.243 to -0.053	0.003*
Stroke volume (m^3)	0.230	-.002-.018	0.125	0.001	0.000 to 0.003	0.128
Stroke area	0.219	-.021-.042	0.473	0.001	-0.015 to 0.017	0.899
Collateral flow	-0.299	-15.60-(-.86)	0.029*	-0.918	-1.889 to 0.060	0.066*
K^{trans} ipsilateral	-0.234	-648.1-95.87	0.141	-66.889	-133.534 to -0.231	0.049*

Chapter 5

| Discussion

The current study aimed to investigate the association between collateral flow and angiogenesis in ischemic stroke. The main finding of our study suggests that there is no direct association between collateral flow development and BBB permeability at day 7 after stroke that represents angiogenesis.

Collateral flow was associated with atrial fibrillation which, as it has been previously shown, is associated with poorer functional outcomes. Previous studies have shown that in strokes related to atrial fibrillation and cardioembolism there is more severe hypoperfusion and lower collateral score graded on CTA compared to carotid atherosclerosis strokes (Yang et al., 2022). The correlation between atrial fibrillation and poorer collaterals can be possibly explained by the type of collateral flow that is being measured. Collateral flow from the circle of Willis has more potential to adapt to arterial stenosis, while leptomeningeal collateralization is more genetic-dependent. Stroke area and stroke volume were also associated with collateral flow. With stroke area representing anatomical location of the lesion, its relationship with collateral flow is most likely due to physical proximity of collateral vasculature to the particular brain area. When occlusion of the main artery happens in its distal segment, collateral arteries have little to none use. Stroke volume represents the volume of ischemic damage. Better collateralization improves blood perfusion of the penumbra, which is why it is associated with lesser stroke volume.

We used MRI-measured BBB permeability (K^{trans}) at day 7 after stroke as the measurement of angiogenesis. In line with previous research, our findings suggest association of permeability with age, intravenous thrombolysis and K^{trans} ratio, that accounts for the differences in BBB permeability between the ischemic and healthy tissues. While the relationship between ipsilateral K^{trans} and K^{trans} ratio can be easily explained as the latter is calculated based on the prior, association with thrombolysis is of more interest. It is known from animal studies that recombinant tPA-induced thrombolysis significantly increase the chance to detect BBB disruption within 1-4 hours after it is being performed (Niego & Medcalf, 2014). Early intraparenchymal hyperdense areas that are detected in NECT scans after intravenous thrombolysis may predict haemorrhagic transformation triggered by the pharmacological intervention. However, since in our study angiogenesis-related permeability is measured at day 7 after stroke, plasminogen activity is unlikely to fully explain this relationship. Probably, flow restoration can contribute to subsequent angiogenesis in the subacute phase of stroke.

To measure the final outcome two grading systems were used, namely NIHSS for neurological evaluation and mRS for the functional. Even though we did not detect direct association between angiogenesis and vascular collateralization, a positive association between better mRS outcome and both good collateral flow and increased ipsilateral permeability at day 7, as well as the association between better NIHSS outcome with good collateral flow indicate that collateralization and angiogenesis may have independent effects on stroke outcomes. Functional outcome at 3 months but not the neurological score was also associated with age. This could be explained by the fact that with age compensation of neurologic deficit induced by stroke is less due to the lack of general strength, which is why elderly people are less independent in their daily life after an ischemic event compared to younger stroke patients. Higher K^{trans} ratio also turned out to be associated with better outcome. Since it represents the difference in permeability, a higher ratio would mean a bigger difference from the patients' baseline permeability in the non-affected hemisphere, suggesting enhanced angiogenesis. Speaking of neurological outcome, apart from collateral flow it was also associated with stroke etiology, neurologic score at admission, previous stroke history and K^{trans} ratio. There is significant association of NIHSS scores with the location of vessel occlusion, which most likely explains the relationship of the score with stroke etiology (Fischer et al., 2005).

One of the major goals of stroke research and experimental stroke treatment is avoiding excessive opening of the barrier at the early stage of stroke to prevent aggravation. Good collateral flow is associated with lesser infarction. Hence, promoting collateralization immediately after an ischemic event is a potential protective mechanism. Previous animal studies have pinpointed the link between collateralization development and angiogenesis (Wei et al., 2001). Moreover, a study of human EPCs revealed that properties that regulate angiogenesis are associated with hemodynamic properties of patients as expressed by flow diversion in the acute stage of stroke (Sargento-Freitas et al., 2018b). The mechanisms that underlie these vascular processes differ significantly. Collateral arteries do not differ morphologically from the usual arteries. The stress caused by increased blood flow through the collateral artery triggers a cascade of reactions that activate endothelial cells and expand collateral artery for it to improve blood supply (Scholz et al., 2015). This process is alternatively referred to as arteriogenesis. When the main artery is occluded the blood pressure difference turn a slow bidirectional blood flow into a fast unidirectional flow. The pressure that increased blood flow imposes on vessel

walls is termed the shear stress. Shear stress activates BECs in the same way as some cytokines do through adhesion proteins like E-Selectin, ICAM-1, and VCAM-1 (Scholz et al. 2000). Further on, a number of growth factors that originate from blood-derived monocytes and promote endothelial proliferation and arteriogenesis. This is the point when mechanisms of collateralization and angiogenesis converge the most because, as we discussed previously, vascular remodelling required increased BBB permeability. Matrix metalloproteinases actively contribute to the local degradation of basal lamina that precedes collateral arterial growth. Probably, what makes the difference is the degree of lamina degradation because in case of collateralization it is not enough to be detected as contrast extravasation on CT scans. Still, our findings in patients indicate that a more complex relationship may exist between collateral circulation and angiogenesis.

Some limitations of our study should be mentioned. First, this is a single-centre study which limits the sample size. Second, we used the dichotomized variable for collateral flow to make TCCD and CTA data compatible. This results in some generalisation and oversimplification that could reduce the chance to detect associations between different extensions of collateral vasculature development and clinical variables, including angiogenesis-related BBB permeability. There might be some limitations related to the technique used. For example, single-phase CTA is easier to use and interpret but it is not as accurate as multiphase CTA since it could underestimate pial arterial filling due to the variability of the acquisition and contrast arrival timing (Sousa et al., 2022).

Overall, our data suggests that collateral flow is not directly associated with angiogenesis-related BBB permeability measured with conventional imaging methods in stroke patients. On the other hand, independent associations between final outcome, collateralization and BBB permeability support the idea that both mechanisms are important for stroke recovery. Probably, there is a more complex interaction that is not possible to detect in the current sample size.

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