NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF

Life Sciences

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BSc in Biochemistry

Remote ischemic conditioning (RIC) in ischemic stroke: functional validation of conditioned plasma in human cells

Master in Molecular Genetics and Biomedicine

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Acknowledgements

Queria começar por expressar a minha enorme gratidão a todas pessoas que me acompanharam e ajudaram na realização desta tese de mestrado.

À minha orientadora, Doutora Helena Vieira, pela oportunidade de crescimento pessoal e profissional, mas também por todo conhecimento e motivação transmitida.

Um agradecimento também ao Nuno Soares pela paciência e bases fornecidas para que eu conseguisse arrancar com esta etapa académica.

Às minhas colegas e amigas de laboratório, Catarina e Diana, pela companhia nas alturas boas e más, pelas conversas de almoço e de lanche e, por terem assegurado o melhor ambiente de trabalho possível.

Aos meus amigos e amigas de faculdade e de Beja, um abraço apertado por estarem comigo todos estes anos e por todos os momentos de diversão e risada. Um obrigado especial à minha eterna colega de casa e amiga, Sara, por toda a companhia não só nestes dois anos, mas no três anteriores, pelas brincadeiras, pelo apoio incondicional, entre outros momentos.

Queria agradecer também à minha família mais próxima que, mesmo por vezes longe, esteve sempre disposta para me ajudar e ouvir quando eu precisava e, por tudo aquilo que não consigo expressar por palavras. Serão sempre a minha base e o meu porto seguro em todas alturas.

Agradeço ao Francisco, por me animar, ouvir, apoiar, por não me deixar sentir só e por outras coisas que parecem pequenas, mas que não são e me fazem sentir feliz.

Um obrigado, mais uma vez, a todos aqueles que mencionei e que possa não ter mencionado aqui, mas aos quais dou muita importância. Sem o vosso apoio esta tese não teria sido possível.

Abstract

Stroke is the first cause of death and disability in Portugal and the second worldwide. The available therapeutic approaches to stroke treatment, thrombolysis, and mechanical thrombectomy promote blood reperfusion but do not directly target the brain parenchyma.

Remote ischemic conditioning (RIC) is a specific sub-type of conditioning that triggers our endogenous mechanisms of defense through the application of a stimulus, below the damage threshold, such as ischemia. In this particular case, a non-vital organ (arm) is submitted to 4 cycles of 5 minutes ischemia followed by 5 minutes reperfusion, the RIC procedure, which may stimulate protective signaling to remote target organs (such as the brain). In this light, our objective was to understand the humoral mechanisms underlying the neuroprotective effects of this procedure, in particular whether and how RIC-conditioned plasma affected neuroinflammation, neuronal function, and blood-brain barrier integrity.

We found that RIC-derived plasma promoted significant anti-inflammatory results since it limits reactive oxygen species generation when human HMC3 microglial cells are challenged with LPS and ATP inflammatory treatment. Therefore, indicating that one of the possible ways for RIC-induced protection occurs *via* inhibition of ROS production.

Additionally, RIC-conditioned plasma also exhibited neuroprotective properties, by increasing synaptophysin expression, a marker for neuronal activity. These results reinforce the protective role of the RIC procedure.

Lastly, slight BBB protective effects were also observed after treating the human brain microvasculature endothelial cells hCMEC/D3 cell line with RIC-derived plasma. However, these results should be further validated.

All in all, besides demonstrating the possible beneficial effects of the RIC procedure, this work also showed a novel way to study these effects, which can be a stepping stone for future studies and the possible clinical application of this novel therapy.

Keywords: neuroinflammation, stroke, neuroprotection, ischemia

Resumo

Os acidentes vasculares cerebrais (AVC's) correspondem à primeira causa de morte e incapacidade em Portugal e a segunda em todo o mundo. As abordagens terapêuticas disponíveis para o tratamento de AVC's, a trombólise e a trombectomia mecânica promovem a reperfusão, mas não atingem o tecido cerebral.

O condicionamento isquémico remoto (RIC) é um subtipo de condicionamento, que ativa os nossos mecanismos endógenos de defesa através da aplicação de um estímulo, abaixo do limiar de dano, tal como a isquemia. Neste caso em particular, um órgão não-vital (braço) é submetido a 4 ciclos de 5 min de isquemia, seguidos de 5 min de reperfusão, o procedimento RIC, que pode estimular a sinalização de carácter protetor para um órgão alvo remoto (tal como o cérebro). Neste contexto, o nosso objetivo foi perceber os mecanismos humorais subjacentes a este procedimento, em particular, se e como o plasma condicionado pelo RIC afeta a neuro-inflamação, a função neuronal e a integridade da barreira hematoencefálica

Nós observámos que o plasma derivado do RIC promoveu resultados anti-inflamatórios significativos, uma vez que limitou a produção de espécies reativas de oxigénio (ROS) quando a linha celular de microglia humana, HMC3, foi submetida a tratamento inflamatório com LPS e ATP. Indicando, assim, que uma das possíveis formas de proteção associada ao RIC poderá ocorrer através da inibição de geração de ROS.

Adicionalmente, o plasma condicionado também exibiu propriedades neuroprotetoras, através do aumento da expressão de sinaptofisina, um marcador de atividade neuronal. Estes resultados reforçam o papel protetor do RIC.

Por fim, também foram observados ligeiros efeitos protetores da barreira após tratar as células endoteliais, hCMEC/D3, com plasma condicionado. Contudo, estes resultados devem ser validados.

Em suma, para além de este trabalho demonstrar os possíveis benefícios do RIC, também mostrou uma nova forma de estudar estes efeitos, o que pode constituir um avanço para futuros estudos e as possíveis aplicações desta terapia inovadora.

Palavras-chave: neuroinflamação, AVC, neuroprotecção, isquemia

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Abbreviations and formulas

AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Propionate
APOC1	Apolipoprotein C1
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CAMs	Cells adhesion molecules
CCL2	CC-chemokine Ligand 2
CNS	Central Nervous System
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
$GABA_B$	γ-Aminobutyric Acid type B
H_2O_2	Hydrogen Peroxide
HIF-1	Hypoxia-inducible factor 1
ICH	Intracerebral Hemorrhage
ICP	Intracerebral Pressure
IFN-γ	Interferon-γ
IGF-1	Insulin-like Growth Factor
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IPC	Ischemic Preconditioning
IRF-1	Interferon regulatory factor 1
LPS	Lipopolysaccharide
MEM	Minimum Essential Media
MHC II	Major Histocompatibility Complex class-II
MMP3	Matrix Metalloproteinase 3
MMP9	Matrix Metalloproteinase 9
MMPs	Matrix Metalloproteases

NF-κB	Nuclear Factor-κB
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NT3	Neurotrophin 3
$\mathbf{O_2^-}$	Superoxide anion
$\mathbf{ONOO^-}$	Peroxynitrite anion
P/S	Penicillin-Streptomycin
PFA	Paraformaldehyde
RA	Retinoic Acid
RIC	Remote Ischemic Conditioning
RIPerC	Remote Ischemic Per-Conditioning
RIPostC	Remote Ischemic Post-Conditioning
RIPreC	Remote Ischemic Pre-Conditioning
RNS	Reactive Nitrogen Species
rtPA	Recombinant Tissue Plasminogen Activator
SHBG	Sex Hormone Binding Globulin
SLPi	Secretory Leukocyte Peptidase Inhibitor
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
VEGF	Vascular Endothelial Growth Factor

I. Introduction

1. Stroke

Stroke, recognized by the "International Classification of Disease 11" as a neurological disorder, corresponds to the world's second leading cause of death, killing approximately 5.5 million patients and leading to neurological disability in around 13.7 million people annually [1,2]. In Portugal, stroke corresponds to the number one cause of death and disability. Due to its great clinical, social, and economic impact, there is a high demand and continued effort from clinicians and scientific researchers to further understand stroke's underlying pathological mechanisms and to develop effective and successful new therapies against it [3]. In this context, ischemic stroke is the most common type of cerebrovascular insult, presenting the highest prevalence (~87%) when compared to hemorrhagic stroke (10-25%) [1,4,5]. Contrasting with its low prevalence, hemorrhagic stroke is far more dangerous than ischemic stroke, with a mortality rate that ranges between 40-50%, while those who survive remain significantly debilitated [3,6]. However, the prevalence, incidence, mortality, and survival rate of both ischemic and hemorrhagic stroke, can be greatly influenced by age, gender, geographic location, race, socioeconomic situation, and risk factors [1]. For instance, the stroke rate in young women is higher than in men; and the mortality rate is also higher in women, whilst in men, stroke incidence increases in proportion with age [1,7].

Hemorrhagic stroke, the less common counterpart to ischemic stroke, occurs when vascular stress causes the cerebral blood vessels to rupture (Figure 1b) [1,3]. As a consequence of this disruption, hypoxia, inflammation, oxidative stress, and increased intracerebral pressure (ICP) can be triggered, which can further impair blood flow in a snowball-like effect [3]. Essentially, hemorrhagic stroke elicits a series of toxic effects in the brain vasculature, that ultimately lead to infarction, which can happen either at the intracerebral level or subarachnoid level [1,3]. Hypertension is one of the most common risk factors in stroke, particularly, hemorrhagic stroke, and it generally induces the rupture of small arteries or arterioles, resulting in blood leakage into the brain parenchyma, otherwise known

as, intracerebral hemorrhage (ICH) [3]. Trauma, amyloid angiopathy, cigarette smoking, and heavy alcohol consumption, also constitute some of the several risk factors for stroke [1,3]. Furthermore, subarachnoid hemorrhage can also occur when there's an accumulation of blood in the subarachnoid brain space, due to the rupture of a cerebral aneurysm or injury-induced vascular malformations [1,3].

Ischemic stroke, can be caused by either a thrombotic or embolic incident, which in turn lead to brain vessel occlusion (Figure 1b) and, subsequently, result in a compromised vascular supply of oxygen to the brain (cerebral ischemia) [1,3,8]. In thrombosis, the blood flow is disrupted due to complications within the blood vessel itself, usually, as a consequence of atherosclerosis, arterial dissection, fibromuscular dysplasia, or inflammation [8]. For example, atherosclerosis or atherosclerotic disease, in a very simplistic way, corresponds to a lipid accumulation within the artery walls, therefore, hypercholesterolemia is one of the major contributing factors to the development of this disease [9]. Atherosclerosis triggers a vast array of cellular and molecular reactions in response to disease-associated lesions, which mainly occur in large and medium-sized elastic and muscular arteries [9]. One of the first lesions, in this context, is the formation of fatty streaks, due to the accumulation of lipid-laden cells, namely, monocyte-derived macrophages and T lymphocytes [9,10]. With time, these fatty streaks often build up and progress into fibrous plaques, resulting in arterial narrowing (stenosis) and clot formation, thusly, causing thrombotic stroke [1,3,10,11]. In the case of an embolic stroke, a clot, or debris from other areas of the body, travel through the bloodstream and can, eventually, block blood vessels [8,11].

The human brain is a highly metabolically demanding organ, with 25% of a human being's whole metabolic processes being solely attributed to this organ [12]. For that reason, there are intricate and highly optimized homeostatic mechanisms that maintain the cerebral blood flow rate at about 50 mL/100g of brain tissue per minute (cerebrovascular autoregulation), therefore, any disruption of this equilibrium compromises the brain and its cells' function [12]. When ischemia ensues, the cerebrovascular tissue can be divided into two areas (Figure 1a): the ischemic core, where blood flow is greatly decreased (7,7-14 mL/100g of brain tissue per minute) and, as consequence, necrosis of neurons and glial cells initiates; and the surrounding tissue, known as the penumbra, where the

perfusion rate is approximately 10-17 mL/100g of brain tissue per minute and, therefore, can still be salvageable, since neurons in this area are functionally impaired but not dead [3,13].

Overall, the severity of the ischemia-induced neuronal injury depends on the degree, area, and duration of the hypoperfusion, thusly, when it comes to stroke treatment, time is of the essence [2,3,12]. Consequently, both currently available therapies for stroke (see section ***Currently available therapies***) focus on the early reestablishment of blood flow to the injured tissue, in order to limit the associated repercussions [2].

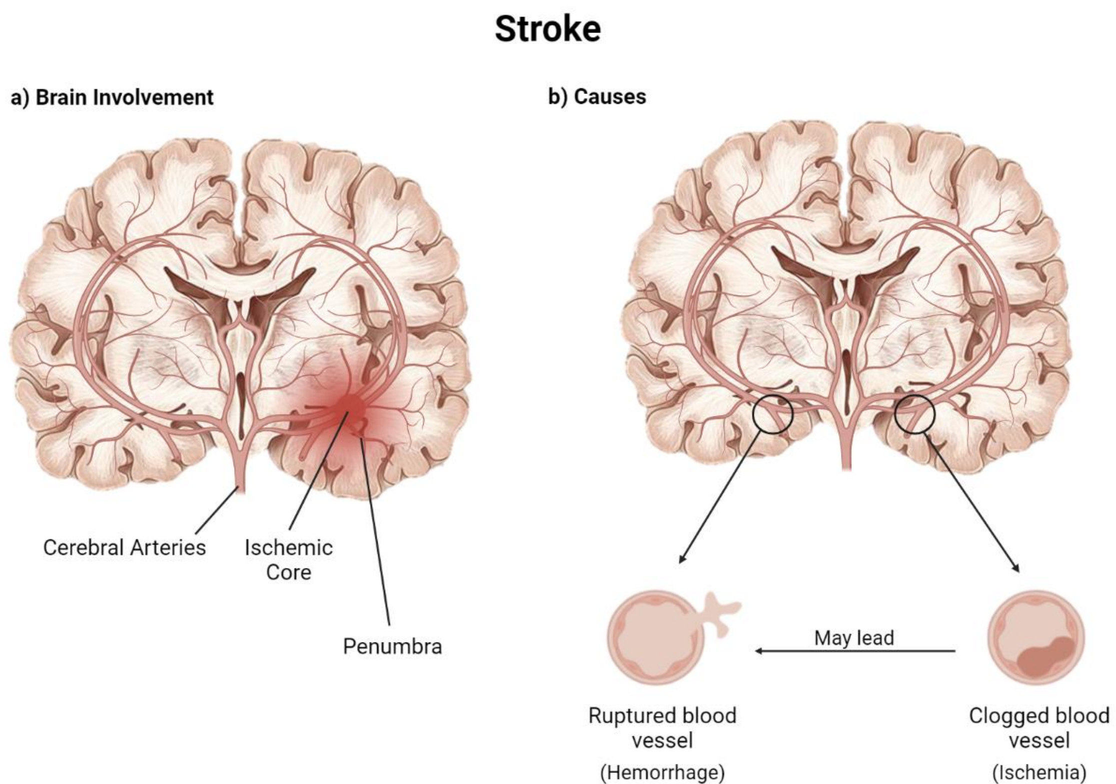


Figure 1 – Depiction of the (a) brain involvement after ischemic stroke and the (b) two major causes of stroke.

1.1 Pathophysiology of ischemic stroke

Stroke is an extremely complex neurological disease with equally complex pathophysiology, which encompasses several cellular and tissue injury mechanisms and interconnected signaling [14]. These processes include energy failure and depletion, disruption of ionic homeostasis, excitotoxicity, generation of oxygen free radicals and reactive oxygen and nitrogen species (ROS/RNS), blood-

brain barrier (BBB) disruption, activation of glial cells, and leukocyte infiltration [2,3]. These events can, ultimately, culminate in both necrotic and apoptotic processes in the ischemic core and penumbra region, respectively [2]. As mentioned before, the ischemic core, as a consequence of cerebral ischemia, suffers the most drastic blood flow decrease and, subsequently, its core tissue becomes irreversibly injured [2]. Surrounding this necrotic core is the ischemic penumbra, which is less severely damaged and still metabolically active but, if left untreated, the cells it comprises, can undergo programmed cell death, otherwise known as, apoptosis [2].

Once ischemia ensues, the local reduction of the oxygen and glucose content greatly impairs the cells' ability to generate high-energy phosphate compounds, such as adenine triphosphate (ATP), due to mitochondrial disturbances [3]. Therefore, if this energy failure progresses, apoptosis may be triggered [3]. For instance, even if an ischemic event does not result in complete occlusion of the vascular supply, prolonged periods of hypoperfusion can be equally harmful [3]. One of the energy-dependent cellular processes that are majorly affected by this energetic deficiency, is the membrane ion pump function [2,3]. This results in the flow of several ionic species (i.e., calcium [Ca^{2+}], sodium [Na^{+}], and chloride [Cl^{-}]) into the cell and, consequently, in a notable increase in water inflow [2,3]. Overall, this causes the rapid swelling of both neurons and glial cells, due to osmosis or membrane depolarization, which often leads to cytotoxic edema [2,3]. Furthermore, ischemia also promotes the release of excitatory neurotransmitters, such as glutamate and aspartate [2,3]. Glutamate, under physiological conditions, is crucial for the maintenance of neuronal plasticity [3]. However, its unrestrained release during an ischemic insult leads to a substantial increase in intracellular Ca^{2+}, which despite being a defensive response, frequently leads to cell death [2,3]. As soon as glutamate is released into the extracellular space, following ischemia, it interacts and activates its receptors (i.e., N-methyl-D-aspartate [NMDA], α-amino-3-hydroxy-5-methyl-4-propionate [AMPA], or kainite receptors) [2,3]. This receptor activation prompts an exacerbated influx of Ca^{2+} e Na^{+}, which can both hinder neuronal survival, with Ca^{2+} inducing cell death at high concentrations [2,3]. Ca^{2+} can cross the neuronal membrane, either through voltage-gated or ligand-gated channels, and activate numerous enzymatic systems, including,

proteases, kinases, lipases, and endonucleases [2,3]. Consequently, their enzymatic activity and metabolic products, such as free oxygen radicals and ROS, can damage cellular membranes, genetic material, and proteins, thusly, disrupting cell homeostasis and activating apoptotic pathways [2,3].

Accordingly, there has been an increasing amount of evidence pointing towards the connection between oxidative stress-related apoptosis and ischemic stroke [2,15–17]. Overall, neurons and other neural cells, are always subjected to basal levels of ROS and free radicals, either of exogenous or endogenous origin [2]. However, when these levels become high, cells activate their anti-oxidant defenses to detoxify these highly reactive compounds, avoiding oxidative stress [18]. Nevertheless, the brain is still highly vulnerable to this process, due to its high concentrations of peroxidizable lipids, high oxygen depletion, and high levels of iron (pro-oxidants) [17]. Once an ischemic insult starts leading to energy failure, lactic acid accumulates in neurons, resulting in acidosis [2,17]. The H^+ concentration promotes the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), for example [17]. The major source of ROS during an ischemic stroke are mitochondria, which generate O_2^- in the electron transport chain process [2]. Furthermore, the arachidonic acid metabolism (cyclooxygenase and lipoxygenase pathways) and the NADPH oxidase of activated microglia and infiltrating peripheral leukocytes also contribute to the overall increase in ROS levels during the ischemic tissue [2,19,20]. Besides ROS and oxidative stress, nitrosative stress can also occur, which is associated with an increased generation of reactive nitrogen species (RNS). RNS induce protein nitrosylation that can alter their integrity and function [17]. In addition, O_2^- can react with nitric oxide (NO), leading to the formation of peroxynitrite anion ($ONOO^-$), which is highly reactive causing DNA fragmentation and lipid peroxidation [17].

Following ischemic stroke, brain cells contribute to the inflammatory environment by the secretion of pro-inflammatory mediators [2]. These cells include endothelial cells, microglia, neurons, and astrocytes (see section ***Brain and the cells***). Activation of transcription factors, such as nuclear factor-κB (NF-κB), hypoxia-inducible factor 1 (HIF-1), and interferon regulatory factor 1 (IRF-1), can result in increased cytokine release and expression of endothelial cell adhesion molecules (CAMs) [2]. Likewise, the major part of the neuroinflammatory process

in stroke is associated with microglia, particularly, microglia in the penumbra region [2,21]. These cells, along with astrocytes, can have both pro-inflammatory and anti-inflammatory/neuroprotective effects, consequently, their role may vary depending on the environment and kinetics after stroke [2,21,22]. Additionally, the brain endothelium that composes the BBB can respond to inflammation and stroke damage, by increasing its permeability and decreasing barrier function [2]. Ischemia also disrupts the overall structural integrity of the brain tissue and blood vessels through the release of proteases, such as matrix metalloproteases (MMPs) [3]. Finally, ischemic injury potentiates the interactions between endothelial cells, brain cells (astrocytes, neurons, microglia), and peripheral cells (leukocytes, platelets), which further disturb BBB integrity and contribute to the progression of brain injury [2,3].

Leukocyte infiltration in the post-ischemic brain has also been indicated as an additional contributing factor to the progression of ischemic stroke injury, particularly, in the context of reperfusion-associated injury [2,23,24]. This leukocyte entry into the brain parenchyma is attributed to its capacity to adhere to the brain endothelium [2,24,25].

1.2 Currently available therapies

Presently, there are two available approaches in stroke treatment: intravenous thrombolysis, using recombinant tissue plasminogen activator (rtPA), and mechanical endovascular thrombectomy [26,27]. The clinical advances that allowed the development of these two therapies greatly contributed to the decrease of ischemic stroke-associated deaths.

Thrombolysis, as the name indicates, is a stroke therapy that induces the lysis of the vascular thrombus or clot responsible for the blockage of cerebral blood supply [14]. Similarly, to what is observed in our endogenous fibrinolytic system, this thrombolytic approach, consists of administering rtPA, most commonly alteplase, which cleaves plasminogen into active plasmin [14,26]. Once plasmin is formed, it can degrade both fibrin and fibrinogen found on the reactive surfaces of thrombi, thusly dissolving the blood clot and allowing the reestablishment of blood flood flow to the ischemic area (reperfusion) [14,26]. This treatment can currently be administered within 12h after ischemic stroke, as opposed to the 4.5h time-

window it used to have. However, it's important to know that early treatment allows a better ischemic stroke prognosis. Alteplase treatment can also result in hemorrhage, one of its more severe side effects, which can increase in proportion with the rtPA administration delay and, eventually, lead to death [26]. Accordingly, hemorrhagic stroke patients must not receive alteplase treatment, therefore, an accurate diagnosis is required before its use.
For this reason, many researchers have focused on the development of more efficient thrombolytics (i.e., Tenecteplase) or even "tPA helpers", to reduce the risk of occurrence of the mentioned side effects [26,28,29]. In addition, whenever a large vessel is involved, it is common practice to treat ischemic stroke patients by the mechanical removal of the clot itself via stent insertion, named thrombectomy [26,27]. In fact, thrombectomy is frequently applied along with thrombolysis.

Mechanical Thrombectomy is a far more recently used therapy for ischemic stroke, which was proven to be effective in 2015, in about 5 randomized controlled clinical trials [26,27]. This therapeutic approach is particularly crucial in patients who suffer large vessel occlusion, which is commonly associated with severe cases of stroke and poor prognosis, and in which thrombolytic therapy has proven to be less effective [26,27]. Overall, mechanical thrombectomy can be successfully used up to 24h after ischemic stroke onset, according to more recent studies [26,30,31]. This therapy is also associated with fewer side effects, which has been even more noticeable with the continuous upgrade of the stent-retriever devices. [26,32]. Nevertheless, the main disadvantage is the need for neuroradiology settings and a central hospital.

Both therapies still have two major drawbacks in common. First, they do not directly target the brain parenchyma and, therefore, are not able to limit the subsequent damage associated with exacerbated inflammation. Secondly, they do not promote any type of regenerative mechanism against neuron loss [26]. For this reason, novel strategies targeting brain cells are of high relevance.

2. Brain and the cells

The brain, along with the spinal cord, comprises the central nervous system (CNS), our own personal control center, that is responsible for the management of all cognitive processes, memory, and movements [33]. In addition to the CNS, the

peripheral nervous system (PNS) extends across the whole body in a complex association of nerves, allowing communication between even the most distal parts of the body and the brain [33]. This communication is carried out in the form of nerve impulses, which are, essentially, messages that either come from the brain (efferent) or the periphery (afferent) [33]. In addition, the brain roughly contains 1000 billion cells, which is a combination of both neurons and the several types of glial cells [33].

2.1 Neurons

The most common way to represent neurons is the one portrayed with an arborized cell body, characterized by an extensive number of dendrites and a single axon [34]. However, neurons have long been recognized as highly polymorphic cells [34]. Neurons are responsible for sending and receiving electrical signals in response to a stimulus [33]. In general, this signal transmission, whether it is chemical or electrical, is carried out by a specialized structure called the axon. After neurons are "fired", the signals can then be received by a variety of structures, most commonly, dendrites, other axons, the soma, and tiny projections that sometimes form on dendrites (dendritic spines) [33]. Interestingly, both of these structures, axons and dendrites, broadly known as neurites, can provide information about neurons' functional importance, since the number of dendrites is proportional to the number of inputs received by neurons [34].

Synapses are the site through which neurons pass these nerve impulses, allowing the formation of neural circuits [33,35]. Furthermore, synapses can be divided into two major types: chemical synapses, and the more rare electrical synapses [35,36].

Chemical synapses consist of three main components, which include, the presynaptic terminal, the synaptic cleft, and the postsynaptic terminal (Figure 2a) [35]. The presynaptic neuron is responsible for the release of specialized chemicals known as neurotransmitters to the synaptic cleft. After their release, these neurotransmitters are recognized by their specific receptors on the postsynaptic side [35]. Chemical synapses are very diversified processes, exhibiting several properties that can vary between them, including, the neurotransmitter in

question, release probability, postsynaptic receptor composition, and presence of neuromodulatory receptors (i.e., γ-aminobutyric acid type B [$GABA_B$] receptor) [35].

The second type of synapse involves specialized connections that enable direct ionic and small metabolite communication between neurons, the so-called electrical synapses (Figure 2b) [36]. In this case and in contrast with chemical synapses, there is a direct physical contact between the presynaptic and the postsynaptic neuron through the formation of a great number of gap junction channels, which tend to cluster themselves [36]. Once this connection is formed, electrical current (ions), rapidly flows from one neuron into the other, allowing a fast signal transmission [36]. Despite their functional relevancy (i.e., contribution to initial neural circuit function and cortical processing), electrical synapses are often only thought of as a simple and temporary process necessary to pave the way for their more complex counterpart, chemical synapses [36]. However, more recent studies have proposed that electrical and chemical synapses most likely work together to configure brain function and its neural circuits [36,37].

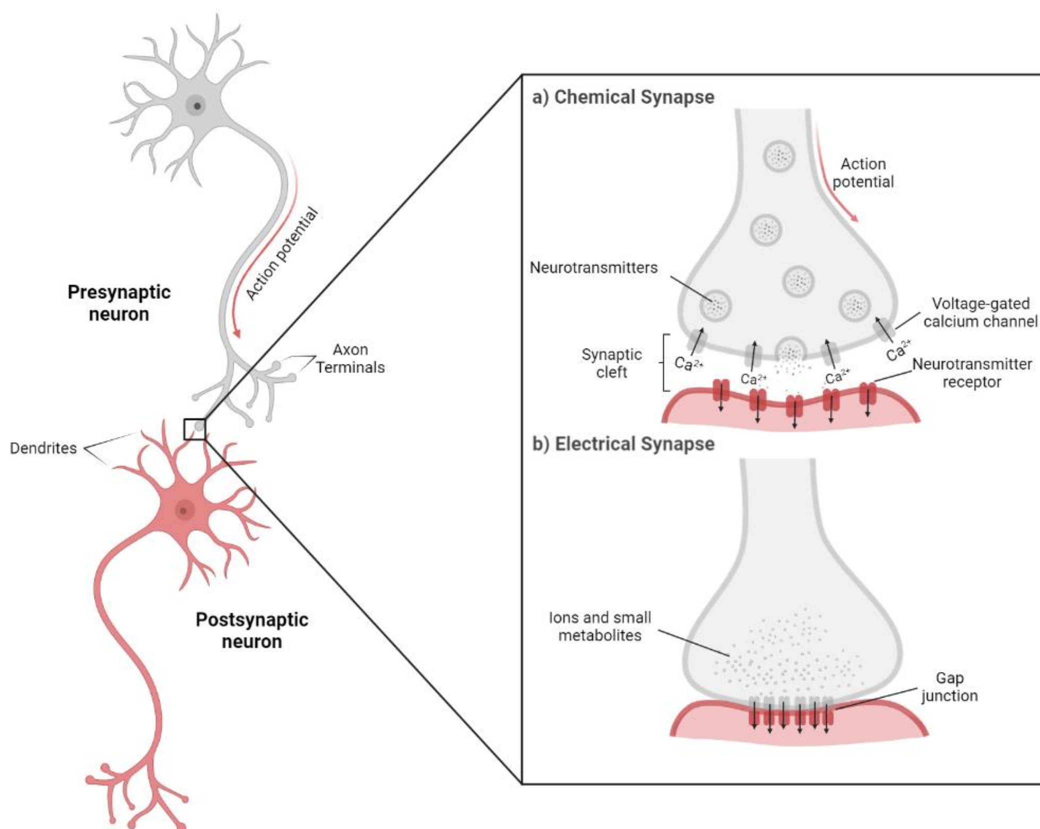


Figure 2 – Illustration of the two types of synaptic interactions between two neurons. a) **Chemical synapse**, the most common type of synapse. It occurs when Ca^{2+} enters the presynaptic axon, in response to the action potential, which prompts the release of neurotransmitters, stored in synaptic vessels. b) **Electrical synapse** is

rarer than the chemical synapse and is only exerted by specific neurons. Despite this, the fast nerve pulse transmission provided by electrical synapses is still an important process.

2.2 Glial cells

The brain's tissue is also populated by glial cells, which are often referred to as the main physical supporters of neurons, which is even evidenced by their name since "glia" in ancient Greek, means "glue" [33,38]. Despite this, we now know that these cells exert many other functions during development and in the mature nervous system (Figure 3), some of which have not yet been fully disclosed [38,39]. The term glial cell is broad and comprises several cellular subtypes, found in both the CNS and PNS [39]. In general, the CNS is mainly populated by astrocytes, oligodendrocytes, and microglia, while the major glial subtypes in the PNS are Schwann cells and satellite cells [40,41].

Microglia are immunocompetent cells, that can adapt their phenotype upon brain homeostasis disruption, which can be due to a mechanical or biochemical injury [38]. In short, these cells can rapidly switch and adapt their phenotype from a more "dormant" state and become "activated" (see section ***Microglial activation and neuroinflammation***).

Astrocytes are known to be the most abundant glial cell type in the adult CNS [38]. Astrocytes are key cells for the metabolic support of neuronal function, along with antioxidant defense and pH buffering. Similar to microglia, astrocytes also display a reactive behavior and, consequently, suffer morphological and functional shifts (astrogliosis) [38]. In addition, reactive astrocytes often release a series of inflammatory factors, that can strongly dictate the outcome of a given pathology [38,42]. Therefore, these cells are responsible for a great variety of roles both in the healthy and diseased CNS, ranging from the maintenance of ion, water, and BBB homeostasis to the preservation of diseased and damaged tissue, by blocking inflammation [38].

Oligodendrocytes and **Schwann cells** are responsible for neuron myelination in the CNS and PNS, respectively [39,43]. This role is highly important since axon myelinization allows the rapid propagation of information between neurons [40]. Moreover, these two types of glial cells are also able to provide trophic support and influence axon structure and their electrical properties by adjusting axon diameter [40].

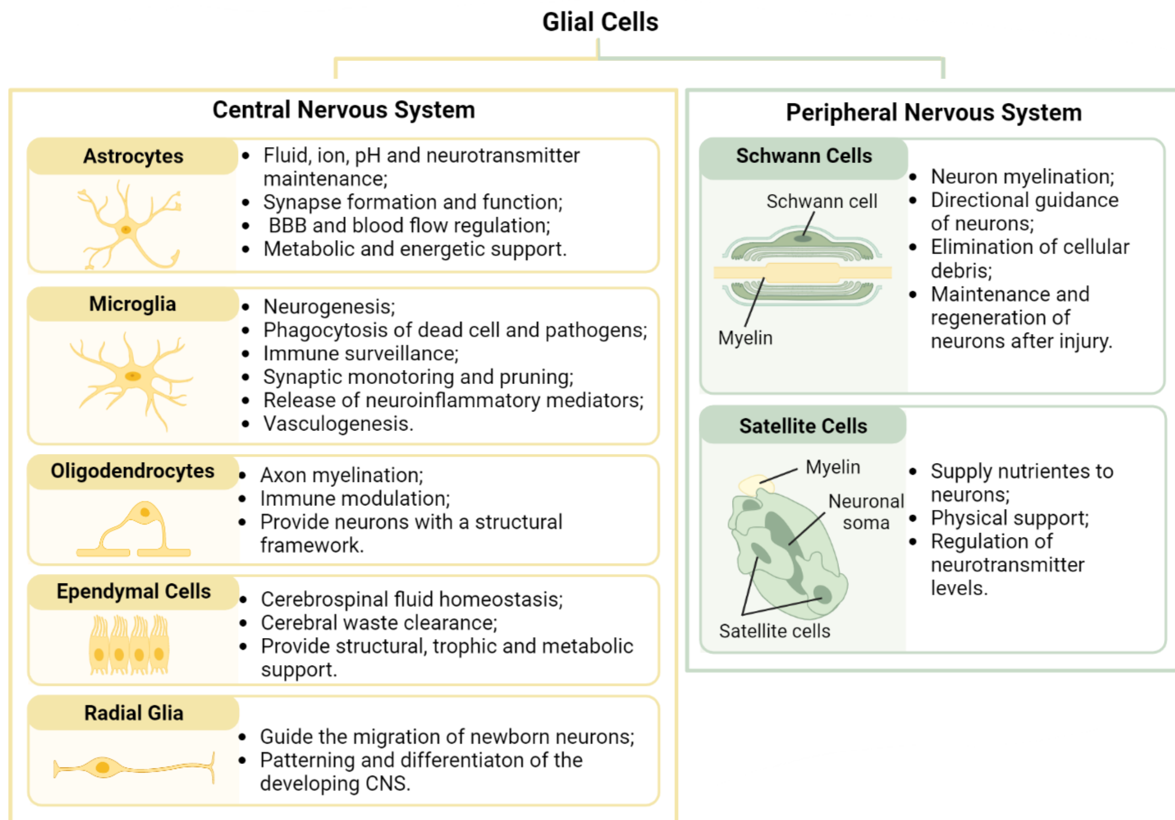


Figure 3 – Summary of the major types of glial cells of the CNS and PNS and some their corresponding functions.

Lastly, it is important to mention CNS **endothelial cells**, which along with other glial cells, constitute a specialized barrier structure called the **blood-brain barrier** [44–46]. This barrier separates the CNS from the periphery by tightly regulating the movement of plasmatic proteins, ions, inflammatory mediators, and immune cells between the circulatory system and the brain parenchyma [44,46]. All of this is possible due to the characteristic properties of the cerebral endothelial cells [45,47]. For instance, the tight , gap and adherens junction proteins, connect cell membranes and are responsible for the high electrical resistance and low paracellular permeability associated with BBB function (Figure 4) [44,46]. In a diseased state, these functions and properties become disrupted, leading to BBB dysfunction, a highly critical event in various neurological disorders (i.e., stroke, multiple sclerosis, Alzheimer's disease, etc.) [44,45].

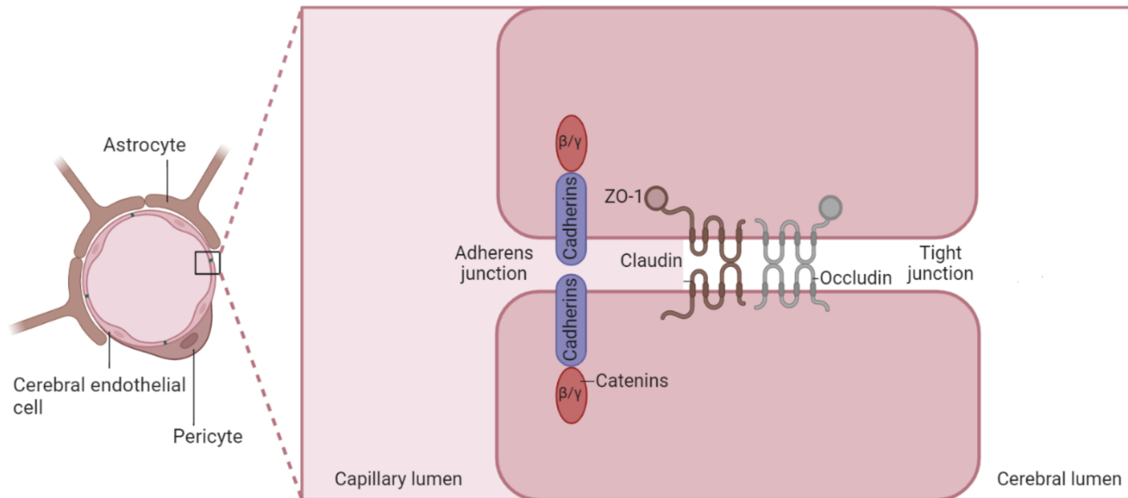


Figure 4 – Schematic representation of adherens and tight junction proteins responsible for blood-brain barrier integrity (Adapted from Ronaldson and Davis 2011).

In summary, it's always important to take into account that the complexity of the brain processes is not solely due to neurons, glial cells, or even endothelial cells separately, but due to the extensive crosstalk between these cells [40,47]. These interactions, along with glia-glia interaction, regulate a vast array of processes, including, neurogenesis, synapse formation, neuronal migration, proliferation, differentiation, neuronal signaling, and overall maintenance of neuronal function [47].

3. Microglia and stroke-induced neuroinflammation

Microglial cells, the brain's resident immune cells, are particularly unique small myeloid cells. Despite their similar ancestry to macrophages, microglia play very specific and important roles both under physiological and pathological conditions [48,49]. Interestingly, even though microglia possess distinctive and fixed molecular signatures, they still constitute a quite heterogenic and morphologically adaptable cell population, which allows them to adjust their responses to different stimuli, injurious or otherwise [50,51]. Microglia are important in the context of neuroinflammation since these cells are involved in the production of several pro- and anti-neuroinflammatory mediators, such as cytokines, chemokines, reactive oxygen species, and secondary messengers [52,53].

3.1 Microglial activation and neuroinflammation

Under physiological conditions, microglia are characterized by a ramified morphology with short and fine processes [50]. Once disruption of the healthy brain environment is detected, microglia rapidly become activated to counteract the damage [49,54]. During the activation, microglial soma undergo hypertrophy and process thickening, assuming a rounder and more amoeboid-like shape [49]. This transition allows these activated microglial cells to promptly migrate to the site of the lesion in a process known as microgliosis [49,52,53]. Along with this ATP-dependent cytoskeletal restructuring, there's also an upregulation of cell surface receptors, namely, CD45, CD68, and the major histocompatibility complex class II (MHC-II) [49,52,53].

In the context of infection, ischemia, or neurodegenerative diseases, several pro-inflammatory stimuli released and/or generated by surrounding cells, mark the start of microglial activation [55]. These stimulatory molecules can, generally, be grouped into two major categories: damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) [49,53]. For instance, in ischemic or hemorrhagic stroke, the ischemic/hypoxic environment can directly provoke neuronal death. Consequently, neuronal cell death leads to the release of a series of proteic DAMPs, such as high mobility group box protein 1 (HMCB1), proteins from the peroxiredoxin family (Prx), and galectin-3 (Gal3), amplifying tissue damage [49]. In addition, some PAMPs might include, lipopolysaccharide (LPS) and other bacterial cell wall components [55]. In general, a large number of these stimuli can be recognized by Nod-like receptors (NLRs) and Toll-like receptors (TLRs), which have been largely implicated in the coordination of the inflammatory response [49,53]. Interestingly, the latter has been found to play a crucial role in stroke-induced neuroinflammation [49,53]. In fact, microglial activation and the subsequent release of cytokines and chemokines are expected to protect and benefit the CNS [52]. However, when this response becomes exaggerated and persists during long periods (chronic microglial activation), it leads to secondary tissue damage [52,56].

Neuroinflammation, a coordinated cellular response to tissue damage, is a normal physiological process that, usually, is mentioned with a negative undertone [52,56]. Nevertheless, the severity of neuroinflammation depends on the context,

duration, and progression of the initial stimulus or insult, and is tightly linked with the robustness of microglial activation [52,56]. For instance, neuroinflammation is mainly mediated by microglia, which are responsible for the production and release of several key molecules, namely pro-inflammatory cytokines, nitric oxide, prostaglandins, and ROS [52,56,57]. When these mediators are uncontrollably produced, they can greatly hinder brain tissue repair [57]. Likewise, this uncontrolled microglial activation and cytokine production can increase BBB permeability, which in turn leads to edema and circulating immune cell infiltration, associated with a severe pathological state [52]. Moreover, vascular ischemia, cell death, and other secondary inflammatory repercussions can further aggravate the primary mechanical and physical damage of the insult [52]. These life-threatening occurrences, which can result in both primary and secondary damage, usually characterized by acute and transient neuroinflammation, may develop a more chronic-like neuroinflammatory profile [52,56].

Neuroinflammation, similarly to peripheral inflammation, can be divided into two major interconnected categories: acute neuroinflammation and chronic neuroinflammation [58]. The first is characterized by an immediate glial response (gliosis), in which there is an accumulation of glial cells, namely microglia, and astrocytes, as well as the release of pro-inflammatory molecules [58]. This sudden shift from practically nonexistent cellular activity to robust cytokine expression and inflammatory response can be viewed as a harmful reaction [56]. Whenever the injurious insult does not damage the BBB, the immune response is less aggressive. Therefore, one can argue that in acute neuroinflammation, microglia and other glial cells fulfill their reparative and protective roles, mitigating the cause of the lesion [56,58]. In contrast, chronic neuroinflammation occurs when certain harmful stimuli persist and is usually associated with neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease, among others [58]. Nonetheless, inflammation and, consequently, neuroinflammation independently of type, can always be deleterious when exacerbated.

Interestingly, in the particular case of ischemic stroke, neuroinflammation can be subdivided into several phases, according to different time points after injury, that includes hyperacute, acute, subacute, and chronic stages. Furthermore, this disease progression may be linked with dynamic changes in microglial polarization

(see section ***Microglia and disease***), since acute stroke seems to induce a more anti-inflammatory phenotype in activated microglia, whilst chronic stroke is associated with a more pro-inflammatory phenotype [59].

3.2 Microglial roles under physiological and pathological conditions

The definition and nomenclature of microglial phenotype and its associated functions have long been splitting researchers' opinions. For years, the so-called "resting" microglia were viewed as merely dormant cells waiting to be activated by any minimum disruption of the surrounding microenvironment. Once this happened, these activated microglia were usually only associated with their cytotoxic and inflammatory properties [60,61]. However, this is a misrepresentative and simplistic subdivision, since "resting" microglia and their processes are highly motile. Therefore, microglia actively scan the brain parenchyma, assuring the maintenance of the cellular, synaptic, and myelin homeostasis of the CNS [62]. In contrast, activated microglia have the ability to adapt their response and phenotype to several environmental cues, that prompt them to limit detrimental effects and also positively affect tissue repair and recovery [53,62].

3.2.1 Microglia and homeostasis

Recently, regardless of their name, "resting" microglial cells have been attributed to more active and crucial roles in the healthy central nervous system (Figure 5). In fact, microglia actively monitor the brain microenvironment, whilst carrying out several fine processes that contribute to the maintenance of the brain's homeostasis and, consequently, its neural networks [60]. Thusly, this constant immunosurveillance carried out by non-activated microglia, allows them to recognize certain factors that might be indicative of disease, such as microbial structures, serum components, and protein aggregates. When this happens, these surveillant and resting microglia change to an activated state (see section ***Microglial activation and neuroinflammation***), which is characterized by a switch from a more ramified phenotype to an amoeboid-like morphology [49,53,60].

Moreover, microglia are also involved in the cellular organization and synaptic wiring of the developing and mature CNS (i.e., synaptic pruning and synapse maturation), the regulation of cell survival, proliferation, and differentiation, and neurogenesis [50,63].

During early postnatal development, neurons and the corresponding synapses are constantly being formed and remodeled. In fact, a large percentage (~50%) of these newborn neurons undergo programmed cell death or apoptosis, which corresponds to a key process to achieve the typical cellular organization of the mature nervous system [60,63,64]. Subsequently, the resulting dead or dying cells need to be cleared, which occurs by microglial phagocytose of these cells [63,65]. In addition, this phagocytic role regulating neuron cell number regulation is not only important during development, but also throughout an individual's life span. Moreover, it is also observed during neurogenesis, where most newborn cells undergo apoptosis during their transition from neural progenitors to neuroblasts and are, thereafter, rapidly phagocytized [50,62]. Likewise, microglial cells also support neuronal maturation, by releasing neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), fibroblast growth factor (FGF), and insulin-like growth factor 1 (IGF-1), which are essential for the survival, proliferation, and differentiation of the brain parenchymal cells [50,64].

Similarly, to the excess of neurons generated in early development, an excess of synaptic contacts is also observed in the mature CNS, many of which are also eliminated by phagocytic microglia [50,63]. This process occurs in an activity-dependent manner, meaning that microglia selectively engulf a large portion of less active and immature synapses, while the remaining synapses are maintained and strengthened [63,65]. Within this context, two of the possible molecular pathways involved in this microglia-synapse interaction and pruning are the classical complement cascade and a CX3CR1-dependent pathway. Regarding the first, studies have shown that C3 and C1q are found in the synaptic compartments, suggesting that these proteins may target synapses for microglia-mediated phagocytosis by binding to the neuronal cell components [63,64]. The latter pathway is associated with protein CX3CL1 (fractalkine), expressed by neurons, and its receptor, which is expressed by microglia [53,63]. In the adult brain, this

CX3CL1-CX3CR1 pair allows the constant neuron-microglia communication, which mediates the response to damaged neurons [66]. In the developing CNS, it is also associated with neuronal plasticity and synaptic transmission, possibly through the recruitment of microglia to the synaptic sites [63,66].

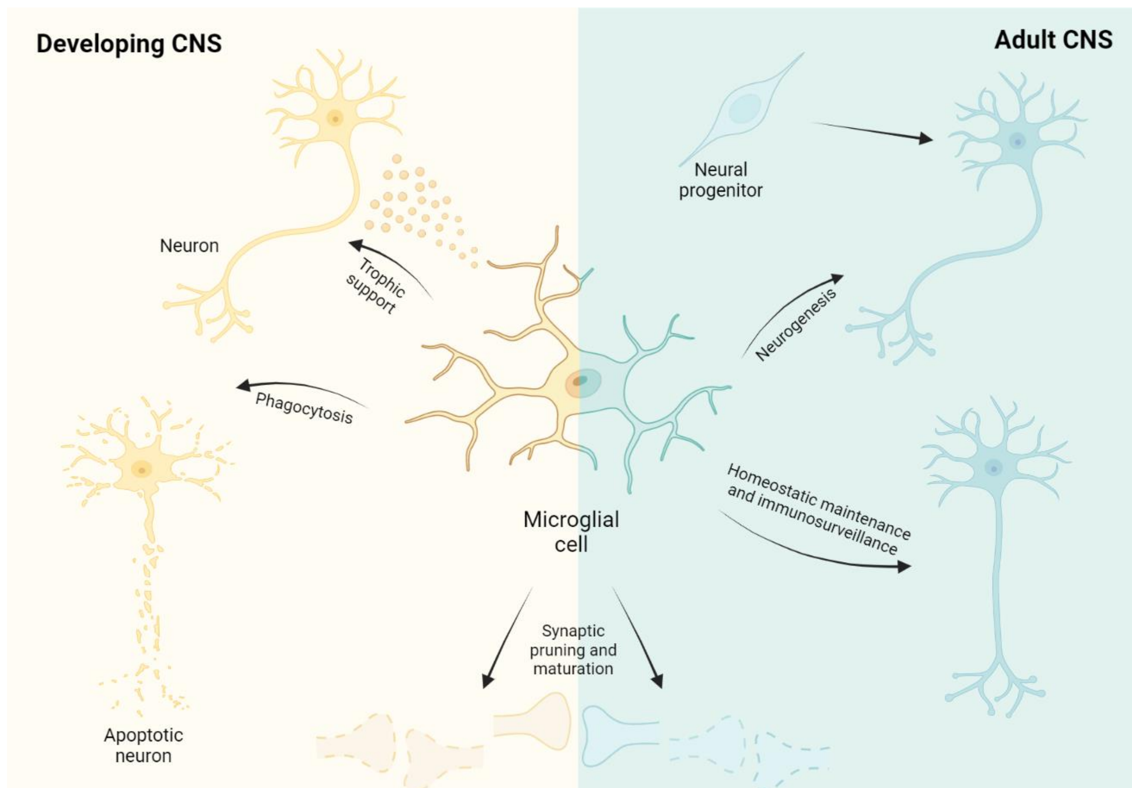


Figure 5 - Homeostatic roles of microglia in both the developing and mature CNS. Besides their immunosurveillance-related functions, microglia also play crucial roles in the maintenance of tissue homeostasis, which includes phagocytosis of apoptotic neurons, promotion of cell proliferation and differentiation, and the modulation of the synaptic wiring (Adapted from Kierdorf and Prinz 2017).

3.2.2 Microglia and disease

Upon brain homeostasis disruption, microglial activation is highly influenced by the local brain environment [60]. For instance, proximity to blood vessels, exposure to neurotransmitters, and/or changes in the blood-brain barrier may result in local microglial adaptions [60]. When activated, these cells can be both pro-inflammatory and anti-inflammatory and, these differential responses are highly influenced by their surrounding microenvironment, including interactions with other neural cells, namely, neurons, astrocytes, oligodendrocytes, and brain endothelial cells [49,53,68].

After infection or injury, microglia can vary between two different phenotypes, the M1-phenotype, and the M2-phenotype (Figure 6), which are based on the

phenotypic classification of macrophages [49,69]. On one hand, there's the M1-phenotype or classically activated phenotype, which is associated with a pro-inflammatory response, accompanied by the upregulation of proteins such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), interferon γ (IFN-γ), interleukin (IL-6), inducible nitric oxide synthase (iNOS) and proteolytic enzymes (i.e., MMP9 and MMP3)[53]. On the other hand, there's the M2-phenotype or alternatively activated phenotype, characterized by the production and release of interleukin (IL-10), transforming growth factor β (TGF-β), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF), which are either pro-angiogenic or anti-inflammatory mediators [49,53]. However, this binary classification is an over-simplification, since the M1 and M2 microglia should not be seen as two different cellular subtypes, but more as polar opposites of a phenotypic spectrum, through which microglia can transition, adapting their functions to the task at hand [53,61].

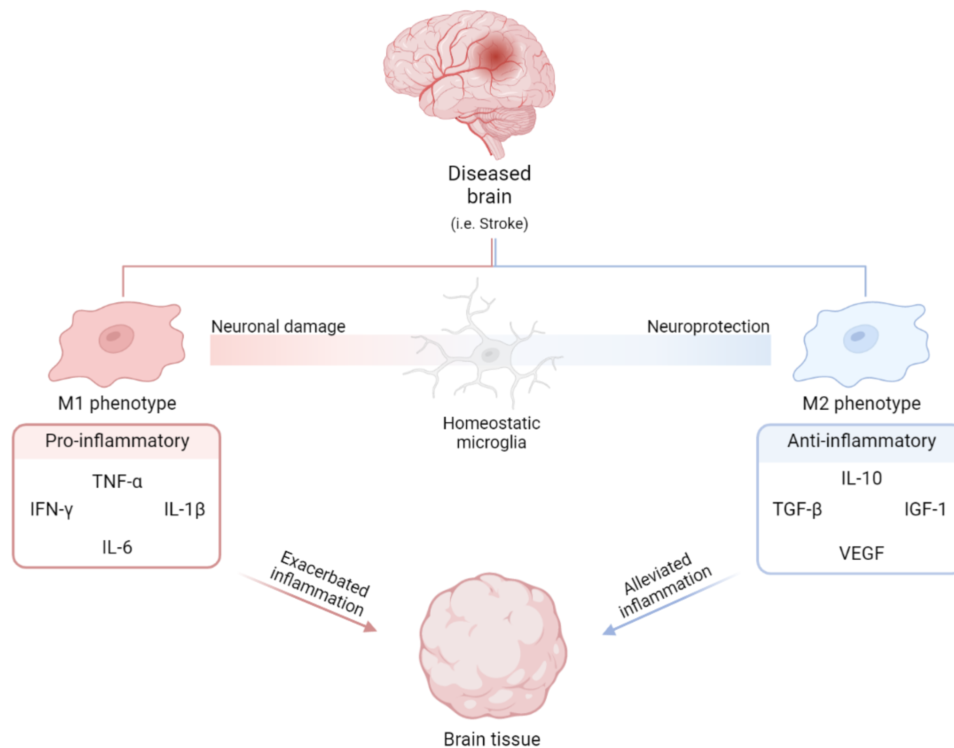


Figure 6 – Adaptive microglial profile in response to disease. Once disease or injury occurs, resident microglia rapidly respond to reestablish homeostasis. During this response, microglial cells dynamically adapt their phenotype depending on the signal they receive. These signals can push microglial polarization towards a more damaging or neuroprotective phenotype (Adapted from Xiong et al., 2016).

In the context of various diseased states, namely, glioma, ischemic stroke, Alzheimer's disease, and many others, microglia are often attributed the label of "professional" phagocytes, that can rapidly and efficiently respond to "eat-me" and "don't-eat-me" signals expressed by neurons and other neural cells, through a great variety of signaling pathways (e.g., TREM2, MERTK, and MFG-E8) [60,63]. For instance, TREM2 is a receptor mainly expressed at the cell surface in both osteoclasts and microglia. TREM2 activation is implicated in the phagocytosis of apoptotic cell debris, suppression of pro-inflammatory factors, and upregulation of chemokine synthesis [60]. Furthermore, microglia produce and release a series of low molecular weight proteins, named cytokines, which are expressed at significantly increased levels under pathological conditions [60]. One of the most crucial cytokines in an immune stimulatory and pro-inflammatory environment is IL-1β. This cytokine is mainly secreted by activated microglia in the CNS, during infection, ischemia, stroke, and excitatory and mechanic lesions, while also playing an important role in the regulation of cell proliferation and differentiation [60]. Along with IL-1β, microglia secret many other cytokines that are implicated in different battlefronts in disease, which may be responsible for neuroprotective and immunosuppressive effects or exacerbated inflammation (Table 1) [53,60].

Lastly, microglia also participate in immune cell recruitment (i.e., monocytes) into the brain, in a chemokine-dependent manner. Some of the involved chemokines include CC-chemokine ligand 2 (CCL2), interferon-γ inducible protein 10 (IP-10), and macrophage inflammatory protein 1α (MIP-1α) [49,50,68]. These circulating monocytes are particularly important for the neuroinflammatory process and are, therefore, recruited by microglial levels of chemokine expression, occurring during disease [50].

Table 1 - Summary of a few known cytokines produced and released by activated microglial cells in response to an injurious stimulus.

Cytokines	Functions	Reference
IL-10	Neuronal homeostasis modulator Anti-inflammatory Neuroprotective Glial activation modulator	[70–72]
IL-4	Regulates brain homeostasis Supports oligodendrogenesis and neurogenesis Neuroprotective	[73–75]
IL-6	Pro- and anti-inflammatory Stimulation of acute phase responses Neurogenesis Neuroprotective	[76]
TNF-α	Pro-inflammatory Regulation of microglial activation Induces oxidative stress	[77,78]
IFN-γ	Pro-inflammatory Microglial priming	[79]

4. Remote ischemic conditioning

To understand what remote ischemic conditioning (RIC) is, it's necessary to first understand the bigger picture, particularly the concept of conditioning itself. Conditioning (also described as preconditioning or hormesis) consists of procedures that take advantage of the complex defense mechanisms that organisms have developed to protect, compensate and, sometimes, even regenerate occurring injuries [80,81]. This protective effect can be achieved in cells, tissues, organs, or even whole living beings by submitting them to low levels of noxious stimuli [80,82]. Overall, conditioning promotes the development of tolerance or resistance against higher levels of harmful events [80].

The first scientific description of ischemic preconditioning (IPC) occurred in 1986, in the context of cardiac ischemia [83]. In these experiments, a dog's heart was directly treated with four 5-minute cycles of alternating occlusion and reperfusion, earlier to the initiation of cardiac ischemia, which showed a significant reduction in myocardial infarct size [83]. Ischemic preconditioning occurs in two phases of protection: the early phase and the late or delayed phase [81,84]. Essentially, the early phase occurs as soon as the conditioning process

ends and it lasts ~3h [84]. This phase is thought to be associated with rapid protein kinase signaling, which promotes the mitochondria-induced halt of the apoptotic pathways [84]. The late phase, in contrast, starts 18-24h after ischemic conditioning and lasts for about 4 days [84]. This delayed conditioning, is most likely dependent on *de novo* protein synthesis, for example, proteins that are implicated in inflammation after ischemia [80,84,85]. It was later, in 1997, when the concept of remote ischemic conditioning was first mentioned, particularly, limb RIC [81,84]. All in all, RIC refers to a form of IPC that can be applied directly to an organ or tissue (i.e., heart, kidney, liver, brain, and limb) and have a systemic effect [81,86]. However, in this study, the main focus will be the non-invasive, safe, and feasible limb RIC, which will be henceforth mentioned only as RIC [84].

Keeping in mind the definition of conditioning mentioned above, RIC is a procedure in which cyclic inflation and deflation of a cuff around a given limb, such as the arm, simulate an attenuated ischemic event [82]. This RIC-induced ischemia can, subsequently, activate protective pathways and induce tolerance against a stronger injurious insult [81,82]. This form of conditioning is defined as "remote" due to its ability to protect distant organs, including the heart, kidney, lung, gastrointestinal tract, brain, skeletal muscle, liver, and pancreas [82,86]. In addition, conditioning can be subcategorized into pre-, per-, and post-conditioning, depending on whether this procedure is applied before, during (between ischemia and reperfusion), or after the injurious episode [81,84]. Out of these three time-based categories, remote ischemic preconditioning (RIPreC), may be considered the less practical approach, since, in normal conditions, stroke onset can't be predicted [84]. Nevertheless, it could be used in a preventive manner, in situations where patients undergo surgeries that may lead to postoperative ischemic complications [81,82,84]. Still, RIC could also be used as a preventive treatment in individuals with stroke-associated risk factors [81,82,84]. In contrast, remote ischemic per-conditioning (RIPerC) and remote ischemic post-conditioning (RIPostC), correspond to the more promising strategies in hospital settings. In fact, it can be administered while the patients are in transport or even in conjunction with the currently available therapies [82,84]. As a whole, RIC introduces a new perspective to stroke treatment, but also other neurological diseases[80–82]. In contrast to thrombolysis and mechanical thrombectomy (see section **Stroke and**

currently available therapies), RIC targets the brain parenchyma, particularly, the penumbra area [80–82].

4.1 Inter-organ communication mechanisms underlying RIC

The RIC-induced effect is not limited to a specific type of organ, injury, or disease, thus this technique may be adaptable to many clinical contexts [80,84]. In the particular case of the CNS, RIC can be beneficial in the context of both acute neurodegeneration and chronic degeneration [80,84]. Taking all of this into consideration, the consensus is that, there may be three major mechanisms (Figure 7) involved in the protective signaling from the periphery to the target organ, namely, the brain [81,82,84]. These potential mechanisms are: (i) the neuronal (autonomous nervous system), (ii) humoral (circulating factors), and the (iii) immune-mediated mechanisms (immune cells) [82,84]. Moreover, the underlying mechanisms of RIC might function in a synergetic manner, thusly providing neurovascular protection, anti-inflammatory influence, reduced excitotoxicity, and metabolic protection [87]. Consequently, the inflammatory milieu, mitochondrial processes, and/or the upregulation of protective pathways are activated, which, in turn, allows a decrease in tissue damage [87].

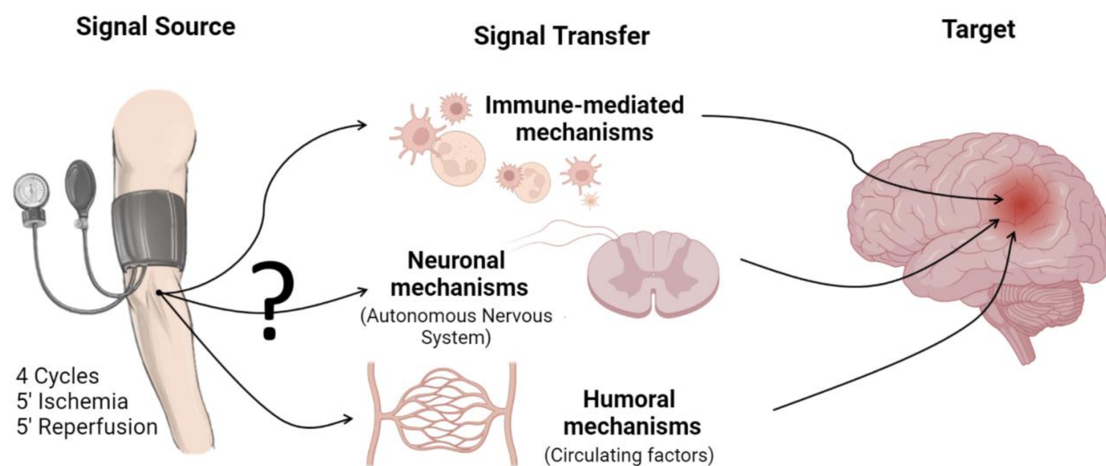


Figure 7 – Representation of the three possible mechanisms through which the RIC-induced protective signal may be transferred to the target organ (Adapted from Hess et al. 2015).

4.1.1 Autonomous Nervous System

When it comes to the neuronal aspect of RIC, there have been several experimental and clinical studies that focused on understanding the role of the autonomous nervous systems on RIC-induced protective signal transmission [88–92]. For instance, in an experimental study where autonomic ganglia were blocked using hexamethonium, a diminished level of RIC-induced neuroprotection was observed [90]. Moreover, another study to disclose the cross-talk between the vagus nerve and muscarinic receptors found that RIPreC and its cardioprotective effect depend on the vagus nerve signal transmission to the heart and subsequent activation of muscarinic receptors by acetylcholine [91]. In general, both the sympathetic and parasympathetic nervous systems seem to participate in this procedure [89]. Despite these results, the neuroprotective effect provided by RIC has been shown to demand an interlinked action between both the neural constituents and the blood circulating biomolecules [81,93]. For example, the release of blood-borne factors into the circulation may be dependent on the vagal nerves' contribution and, this subsequent release may even activate afferent nerves [84,93].

4.1.2 Humoral mechanisms

The second hypothesis for inter-organ communication is based on circulating molecules that are released into the bloodstream when RIC is applied [84]. This idea is supported by the RIC protocol itself since the four cycles of ischemia are accompanied by four other cycles of reperfusion. Reperfusion, thusly, allows the secreted molecules to flow throughout the whole circulatory system towards the target organ [84,94]. Furthermore, this mechanism was experimentally reinforced by the cross-species blood transfer, in which an unconditioned subject (rabbit) received blood from a conditioned one (human), which promoted protection against cardiomyocyte necrosis [94,95]. Furthermore, it was even observed that plasma obtained from a conditioned animal was able to positively affect isolated hearts [95].

As mentioned before, the neural and humoral pathways seem to be tightly connected, therefore, the release of blood-borne factors, which are secreted in the conditioned limb, may be induced by peripheral nerves [96]. These molecules

include adenosine, bradykinin, opioids, calcitonin gene-related peptide, catecholamines, and prostaglandins, which function mainly as neurostimulators [82,84]. Besides autacoids, chemokine CXCL12, IL-10, microRNA-144, nitrite, and many other factors, seem to also mediate RIC-induced cytoprotection. For instance, nitrite is an oxidation byproduct of nitric oxide oxidation, that essentially functions as a storage of NO [81,82]. When hypoxemia occurs, nitrite is reduced to nitric oxide which, subsequently, acts as a vasodilator [82,97]. Furthermore, nitrite also protects mitochondria against oxidative damage, a common consequence of hypoxia, acute ischemia, and ischemia-reperfusion injury [82,97]. In the context of RIC, increased nitrite levels have been observed, which may account, at least partially, for its cytoprotective effects [82,98–101].

All things considered, RIC might induce the secretion of specific or even multiple protective factors into the bloodstream, which seems to confer cross-species and cross-individual protection through blood and/or plasma transfusion [102,103]. However, the contribution of these circulating humoral factors to the RIC-induced cytoprotection remains inconclusive, even though several proteomic analysis of both animal and human plasma have been performed [104–107]. Considering the several experimental studies using animal models, further research should be done on humans, to effectively use these molecules as biomarkers. This includes the validation and analysis of their efficacy, mechanisms/pathways, and how RIC influences both the associated genomics and proteomics [104].

4.1.3 Immune-mediated mechanisms

Neuroinflammation, in general, is associated with the activation and migration of both the brain's resident immune cells (i.e., microglia and astrocytes) and peripheral immune cells (i.e., neutrophils, monocytes, and T cells) [84]. After their activation, these cells also secrete a variety of proinflammatory mediators, which further exacerbate inflammation (see section ***Microglial activation and neuroinflammation***). In this context, RIC has been shown to inhibit microglial and astrocytic activation once acute ischemic stroke ensued, as well as the recruitment of peripherally-located immune cells [84,108]. Therefore, emerging evidence suggests RIC-induced endogenous protection is, at least, partially due to the modulation of immune-inflammatory interactions [104]. This cellular component,

along with the humoral and neural ones, allows RIC to promote a response that, in the neuroinflammation spectrum, falls closer to the "resolution" and "repair" phases [109].

Starting with the **brain's resident immune cells**, microglia, and astrocytes, which upon the conditioning stimulus, seem to exhibit an immunosuppressive effect on neuroinflammation, most likely through the release of anti-inflammatory cytokines [84,109]. Moreover, astrocytes also promote repair by generating and releasing trophic factors (i.e., IGF-1, VEGF), thusly supporting neuronal growth and angiogenesis [84,109]. Besides this molecular aspect, microglia also highly express TLR4, which confers them the ability to start an innate immune response [84]. Therefore, even if the exacerbated activation of these receptors usually is harmful, their attenuated activation, like the one observed after RIC, may prepare the brain against severe and sustained ischemia [84,110]. In fact, when TLR4 receptors are genetically deleted, the neuroprotection provided by IPC was completely absent [110]. In addition to this, proteins commonly known to present a pro-inflammatory profile were upregulated (i.e., TNF-α, iNOS, and the p65 subunit of NF-κB) [110].

On top of the role of microglia and astrocytes, growing evidence also indicates points to the participation of the **peripheral circulating immune cells** in RIC neuroprotection [84,109]. For example, the molecular and cellular aspects of inflammation have been demonstrated to suffer changes after the conditioning procedure, particularly, the subtypes of activated immune cells and the cytokines they released [84,111]. On one hand, in this study, RIPreC presented an immunomodulatory effect that favored the infiltration of non-inflammatory monocytes as opposed to the monocytes with an inflammatory phenotype [111]. On the other hand, the expression levels of TNF-α and IL-6, pro-inflammatory cytokines, were notably elevated [111].

Overall, RIC may induce an immunomodulatory effect on both brain and peripheral immune cells and their responses, which may explain its effect.

II. Objectives

Ischemic stroke, a high incidence and high mortality neurological disorder, is still treated with therapeutic strategies based on blood reperfusion. Currently, there is a therapy that directly targets the brain tissue, namely neuroinflammation and cell death.

RIC is a promising and pioneering therapeutic approach, which can be administered in a non-pharmaceutical and non-invasive way, comprising an affordable therapy. RIC is based on endogenous defense mechanisms and may allow multiple organ protection [104]. The neuroprotective and anti-inflammatory effects of this procedure have been extensively experimentally proven using animal models, where it was shown to reduce overall oxidative stress, inflammatory cytokine release, inhibit brain edema [81,112–114]. In contrast, much less clinical trials have studied RIC in stroke patients [81,115,116]. Only a few studies have targeted the underlying mechanisms of RIC in healthy human subjects [89,117].

Thusly, the main goal of this thesis is to understand the underlying endogenous mechanisms of RIC responsible for the inter-organ communication and the subsequent possible neuroprotective and anti-inflammatory effects. Our research group has already studied the autonomous nervous system [89] and immune associated mechanisms [117]. Therefore, the present work will focus on the functional validation of human plasma derived from healthy volunteers subjected to the RIC procedure. We aim to functionally test the conditioned plasma for assessing circulating molecules, namely proteins, organic molecules, that may be released and altered in the blood after the RIC procedure. This is a translational study, since plasma samples from healthy young individuals were used to treat human cell cultures to assess their potential beneficial effects.

This functional assessment of the RIC-conditioned plasma will be divided in three strategies:

(i) Neuroinflammation assay, using a human microglial HMC3 cell line;

(ii) Neuroprotection/neuronal function assay, using a human neuroblastoma derived SH-SY5Y cell line;

(iii) BBB integrity assay in human brain microvasculature endothelial cells, using the hCMEC/D3 cell line.

With these cellular models, we aim to answer specific questions, such as: Does RIC have an anti-inflammatory and neuroprotective effect? Can it preserve BBB integrity?

If our hypothesis is true, then other long-term objectives emerge: (i) which are the humoral factors involved in the protective function and (ii) whether the RIC procedure can be used as a therapy against ischemic stroke or even against other diseases?

III. Materials and Methods

1. Participants

A total of 10 young healthy volunteers (5:5 female/male proportion) were recruited from the local population, all older than 18 years-old, with normal body mass index and arterial blood pressure. The following exclusion criteria were used: previous neurological disease or neurosurgical procedure, severe heart failure, peripheral artery disease, skin ulcer, or other severe dermatological diseases. Informed consent was signed by all of the participants before the beginning of the study. The study was approved by the Nova Medical School, NOVA University of Lisbon, Lisbon, Portugal (no10/2016/CEFCM) Independent Ethical Committee.

2. RIC procedure

The RIC protocol consisted of four cycles of 5-minute ischemia followed by 5-minute reperfusion, applied to the upper limb (Figure 8a). Ischemia was performed with a blood pressure cuff inflated to above 220 mmHg or at least 20 mmHg above the subject's systolic arterial pressure. Adverse reactions to this procedure were screened during the entire time.

3. Blood collection and plasma preparation

Peripheral blood samples were collected from all the participants before RIC (RIC1), immediately after RIC (RIC2) and then 4h (RIC3) and one day (RIC4) after the RIC procedure (Figure 8b). All of the samples were collected in EDTA-coated tubes. For plasma preparation, the collected blood samples were centrifuged at 800g for 15 min to obtain the plasma. After separation, plasma samples were aliquoted and frozen at −80°C until further use.

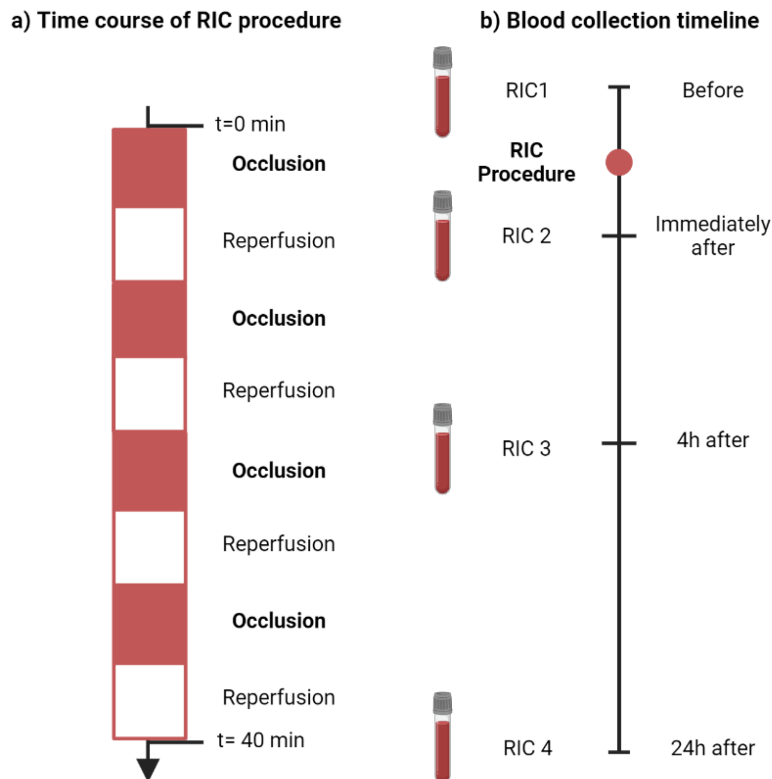


Figure 8 – Schematic representation of the remote ischemic conditioning procedure time course (b) and subsequent blood collection timeline (a).

4. Cell lines and Reagents

4.1 HMC3 cell line

For microglia and inflammatory response tests, the HMC3 microglial cells kindly supplied by Dr. Inês Figueira (Molecular nutrition and health lab, CEDOC) were used. These cells were kept in Minimum Essential Media (MEM), as the basal media, which was supplemented with 11% (v/v) of non-inactivated Fetal Bovine Serum (FBS), and 1% (v/v) of Penicillin/Streptomycin (Pen/Strep), provided by Sigma and Gibco Life Technologies, respectively. Cells were maintained in 75 cm^2 T-flasks (Corning) and 25cm^2 T-flasks (Corning), while passages were performed once to two times a week in 1/3 dilution and 1/2 dilution, depending on cell confluency.

4.2 SH-SY5Y cell line

For neuronal viability and function assessment, the neuronal SH-SY5Y neuroblastoma cell line was used. These cells were maintained in DMEM-F12 (Biowest) as the basal media, supplemented with 10% (v/v) of inactivated FBS,

and 2% (v/v) Pen/Strep. Cells were maintained in 75 cm^2 T-flasks and cells were subcultured two times a week in 1/3 dilution.

For analysis, SH-SY5Y cells were differentiated by culturing them in DMEM/F12 differentiation medium, characterized by a reduced FBS percentage of 1% (v/v), 2% (v/v) Pen/Strep, and supplemented with 10 μM of *all-trans* retinoic acid (RA).

4.3 hCMEC/D3 cell line

Lastly, the hCMEC/D3 cerebral endothelial cell line was also used, to assay BBB integrity. The hCMEC/D3 cell line was kindly supplied by Dr. Pierre-Olivier Couraud (INSERM U, 567, Univeristé René Descartes, Paris, France). Cells were grown in an EBM-2 medium supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, as recommended by the manufacturer, 2,5% FBS, and 1% (v/v) of Pen/Strep. The cell medium was changed every 48h, and the cells reached confluence after 5-6 days of culture. All of the cell lines were kept in a humified incubator at 37°C and 5% CO_2.

5. HMC3 Treatment and ROS Quantification Protocol (DCF Assay)

HMC3 cells were plated onto 96-well plates according to an optimized seeding of $8,5\times10^3$ cells/well. For the functional validation of the RIC-derived plasma, HMC3 cells underwent media change after 48h of cell growth. Control cells were maintained in normal culture media (11% FBS), while experiment cells were treated with conditioned media (5% RIC plasma + 6% FBS). The total "serum" percentage remained the same in all groups. After 24 hours, cells were stimulated with LPS (Sigma), with a concentration of 500 ng/mL, and 24 hours after this, ATP (Sigma), with a concentration of 5mM, was also administered to promote inflammation. After 30 minutes, the DCF Assay was initiated. Cell media was discarded, and cells were washed with PBS. Then, HMC3 cells were incubated with 5 μM of DCFDA (Invitrogen), and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Tecan Infinite F200 Pro microplate reader. At the end of the DCF Assay, the reagent was discarded from each well, and protein extraction and quantification were performed.

The same protocol was used for optimizing the inflammatory stimuli used in this study, with the exception that no cells were treated with the RIC-conditioned media.

Table 2 - Composition of PBS commonly used for washing steps and solution making.

Reagent	Composition
PBS	1,54mM NaCl; 20mM KH_2PO_4; 34mM Na_2HPO_4 pH 7,2

6. SH-SY5Y and hCMEC/D3 differentiation protocol

SH-SY5Y neuroblastoma cells were plated at an optimized seeding of $6,3 \times 10^3$ cells/well in 24-well plates. On the following day, the differentiation protocol was initiated by changing the cell medium to a differentiation medium (FBS, Pen/Strep, and *all-trans* retinoic acid). Differentiation occurred over 7 days, the media was changed on the fourth day, and on the sixth day, the media was changed to conditioned media. After 24 hours, the protein was extracted (see section ***Protein Extraction and Quantification***) for Western blot analysis. For Immunofluorescence analysis, the same protocol was followed, except cells were plated onto coverslips previously treated with PDL at concentration 0,1 mg/mL and washed three times with Milli-Q water.

hCMEC/D3 cerebral endothelial cells were differentiated for 11 days in 12-well plates, using a differentiation medium (EBM-2 medium supplemented with Hepes, basic FGF, hydrocortisone, 1% Pen/Strep, and 2,5% FBS as recommended by the manufacturer).

7. Protein Extraction and Quantification

For the protein extraction, HMC3 and SH-SY5Y cells were lysed with RIPA buffer. In some cases, HMC3 cells were lysed with ELISA lysis buffer. Cell extracts were then transferred onto 96-well plates, at a final volume of 10 µL per well. 100 µL of working reagent from Pierce BCA Protein Assay kit (Thermo Scientific) were added, followed by a 30-minute incubation step at 37°C. The absorbance values from each well were then measured at 560 nm, using a Tecan Infinite F200 Pro microplate reader. Protein concentration values were calculated using a standard BSA curve with known concentration values (1000 to 7,8 µg/mL).

Table 3 - Composition of the above-mentioned buffers and their corresponding applications.

Buffers	Composition
RIPA Buffer	50 mM Tris-HCl; 150mM NaCl; 0,1% SDS; 1% Sodium Deoxycholate; 1% Triton X-100; 1% protease inhibitors

8. Western Blot

Equal amounts of protein from SH-SY5Y and hCMEC/D3 cell extracts, respectively, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels of 15 and 10%, for 1h30min, along with the NZYColour Protein Marker II (NZYTech). The proteins were then transferred onto nitrocellulose membranes (1h30min), blocked with 5% BSA (Sigma) solution, and incubated with the primary antibodies, mouse anti-synaptophysin (Thermofisher) dil. 1/1000 in BSA (for SH-SY5Y) and rabbit anti-beta catenin (Invitrogen) dil. 1/125 in BSA (for hCMEC/D3), at RT. Next, the membranes were treated with secondary antibodies, sheep anti-mouse HRP conjugated (Thermofisher) dil. 1/5000 in BSA (for SH-SY5Y) and donkey anti-rabbit HRP conjugated (Thermofisher) dil. 1/5000 in BSA (for hCMEC/D3), for 1 hour at RT. Between blocking and incubation with both antibodies, the membranes were always washed steps with TTBS. Rouge Ponceau (Biotium) was used as the control for total protein loading.

Immunoblots were revealed by exposing the membranes to electrochemiluminescence western blot detection reagent (Bio-Rad) for approximately 1 minute and the reactive bands were detected after the membranes contacted an X-ray film (Cytia Amersham Hyperfilm). The resulting area and intensity of the bands were quantified using ImageJ software and are present as a percentage relative to the control (100%).

Table 4 - Composition of the buffers commonly used in the western blot analysis.

Buffers	Composition
Running Buffer	29,8g Tris base; 141,95g Glicine; 10g of SDS; pH 8,3
Transfer Buffer	144g Glicine; 30g Tris base; pH 8,3
Stacking Buffer	0,5 M Tris-HCl; 0,4% (w/v) SDS; 1L H_2O
Separating Buffer	1,5 M Tris-HCl; 0,4% (w/v) SDS; 1L H_2O
TTBS	3 M NaCl; 1M Tris-HCl
Loading Buffer	1,5 mM Tris-HCl; 10% (w/v) SDS, 25 mL glycerol, 2,5 mL bromophenol blue; pH 8,8

9. Immunofluorescent Microscopy

SH-SY5Y cells were fixed with 4% paraformaldehyde (PFA) + 4% Sucrose for 20 min. Fixed cells were washed with PBS and permeabilized with 0,3% Triton X100 in PBS for 15 min. After permeabilization cells were blocked with BlockPerm (0,1% Triton X100, 1% BSA in PBS) for 30 min at RT. The cells were washed with PBS and incubated in a humified box at RT with the primary antibodies, mouse anti-synaptophysin dil. 1/400 in BlockPerm and chicken anti-neurofilament M dil. 1/400, for 2 hours. After washing, cells were labeled for 1 hour with fluorescent secondary antibodies, goat anti-mouse conjugated to Alexa Fluor 488 dil. 1/1000 and goat anti-chicken conjugated to Alexa Fluor 647 dil. 1/1000, from Invitrogen. Coverslips were washed with PBS and mounted onto glass slides with Prolong with DAPI (Thermofisher). Fluorescence imaging was done using a Zeiss AxioImager D2 fluorescence microscope.

10. ELISA

HMC3 cells were plated with a 3×10^4 cells/well seeding value in 24-well plates. The same treatment protocol used for the DCF Assay was followed. After protein extraction and quantification, the cell extracts were used to measure TNF-α concentration levels. The Human TNF-α Standard ABTS ELISA Development Kit (Prepotech) was used, and the manufacturer’s instructions were followed throughout. In the end, the absorbances were measured at 415 nm with

wavelength correction set at 560 nm, using a Tecan Infinite F200 Pro microplate reader. Absorbance data was thereafter converted to concentration values using standard curves constructed by following the kit's instructions.

This protocol was used for optimizing the inflammatory stimuli used in this study.

11. Statistical analysis

The data present throughout is the mean ± standard deviation. Comparisons between distinct groups of conditions and treatments were analyzed using the two-tailed and unpaired Student's t-test through Excel, with p-values of less than 0,05 being considered statistically significant.

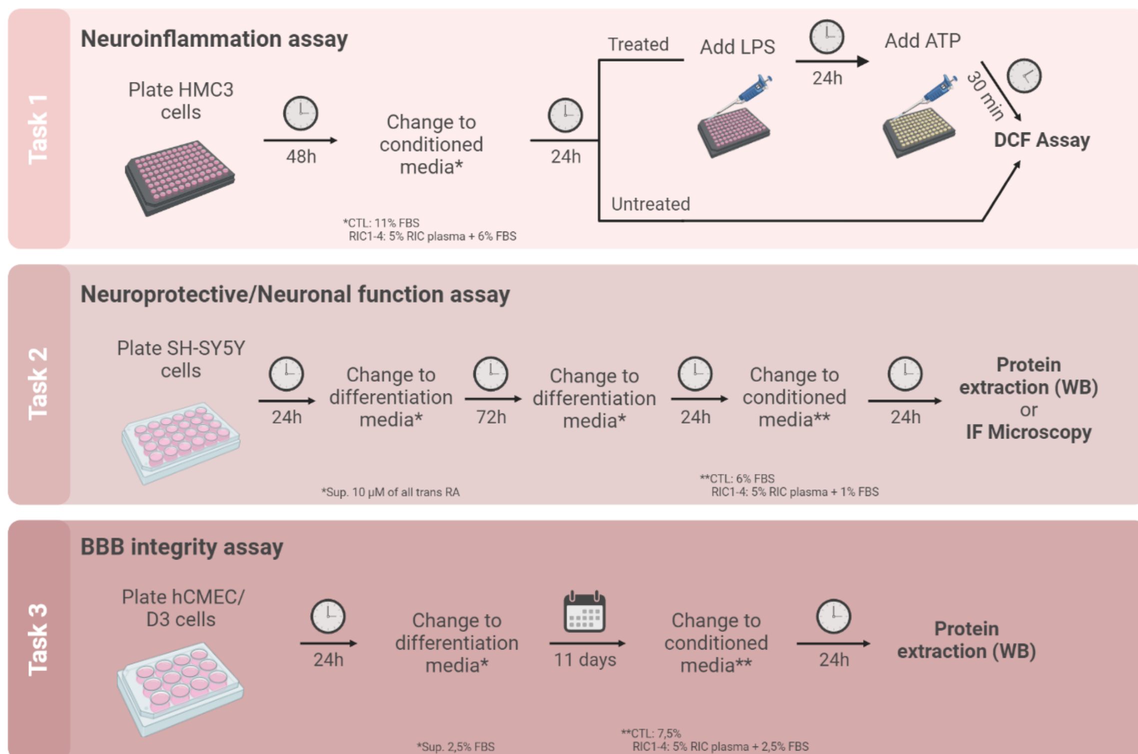
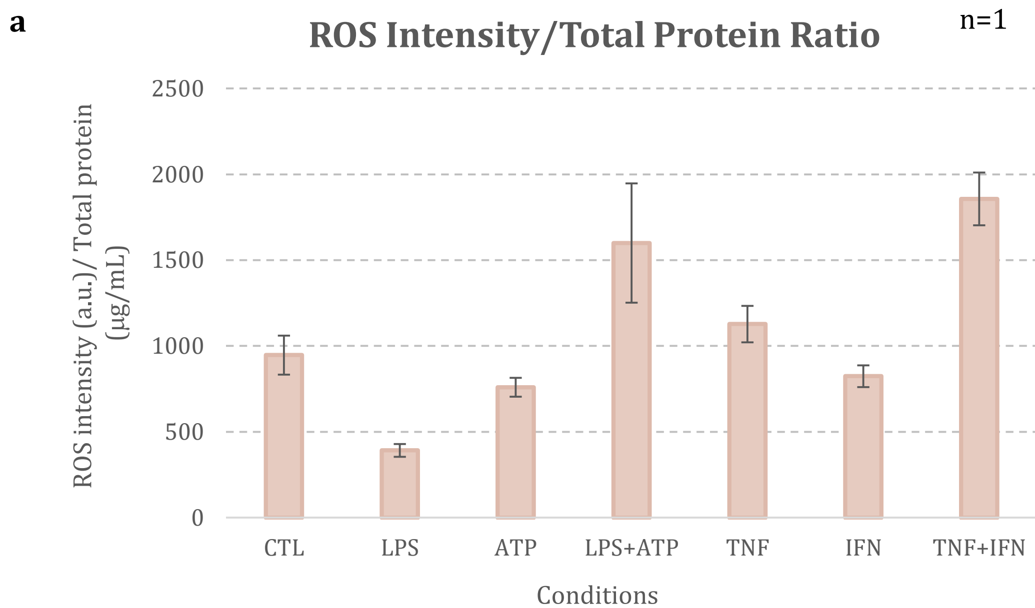


Figure 9 **–** Simplified schematic representation of the protocols used in this study.

IV. Results

1. Inflammatory stimuli optimization

To proceed with the neuroinflammation assay, it was necessary to first establish the most appropriate inflammatory stimuli for inducing inflammation in HMC3 cells. For this, techniques such as ROS and TNF-α quantification (DCF Assay and ELISA) were used. The inflammatory stimuli in question were lipopolysaccharide (LPS), adenosine triphosphate (ATP), the combination of the two, TNF-α, IFN-γ, and the combination of these two.



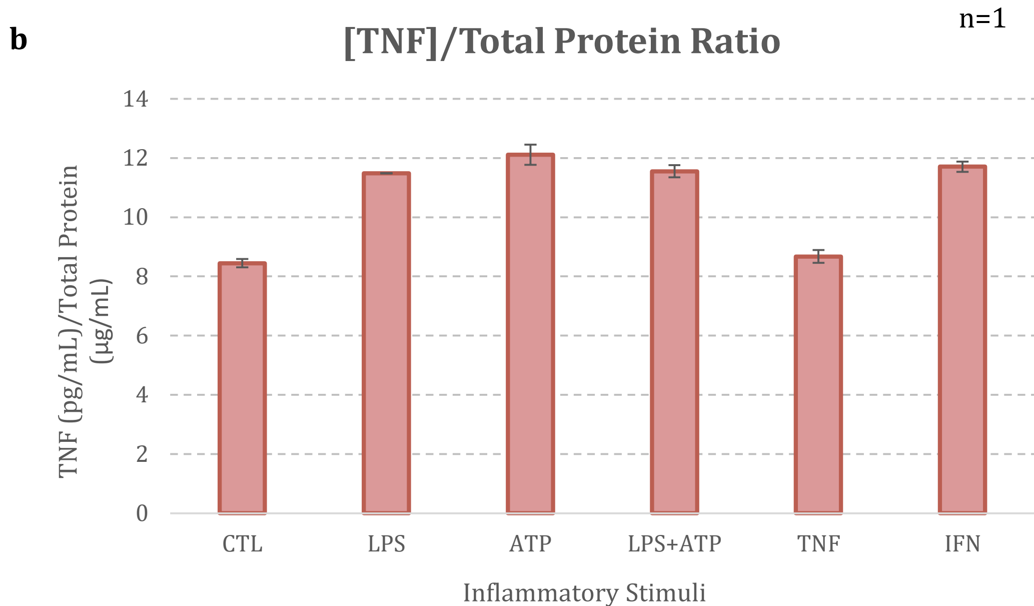


***Figure 10* – Representative data of the effects of various inflammatory stimuli on ROS generation (a) and TNF-α release (b).** HMC3 cells were treated with the inflammatory stimuli for 24h and 30 min in the case of ATP. ROS intensity and TNF-α levels were normalized with intracellular protein content. Data represents the median ± standard deviation of three technical replicates of one experiment.

As both figures, 10a and 10b illustrate, all of the analyzed inflammatory stimuli induced inflammation on the HMC3 cell line when compared to the control. In the DCF assay, the highest ROS intensity percentage was observed after treatment with TNF+IFN and LPS+ATP. While in the ELISA assay, the highest TNF-α concentrations resulted from ATP (12,1 pg/mL) LPS+ATP (11,6 pg/mL), and IFN-γ (11,7 pg/mL) treatment.

Based on this data, we concluded that the combined stimuli LPS+ATP were a valid trigger for inducing inflammation and activation of the HMC3 microglial cells. Thusly, these were the stimuli used in the subsequent neuroinflammatory studies.

2. Neuroinflammation assay – effect of the conditioned plasma on ROS generation

Neuroinflammation and oxidative stress are definitive signs of neurodegeneration and neurological disease, often contributing to disease evolution [17,118]. In fact, after ischemic stroke, the accumulation of lactic acid (acidosis), contributes to the generation of H_2O_2, among other ROS [17]. In that regard, we decided to assess the effect of the RIC-conditioned plasma cell treatment in the generation of this ROS. For this, ROS production was quantified through the DCF Assay.

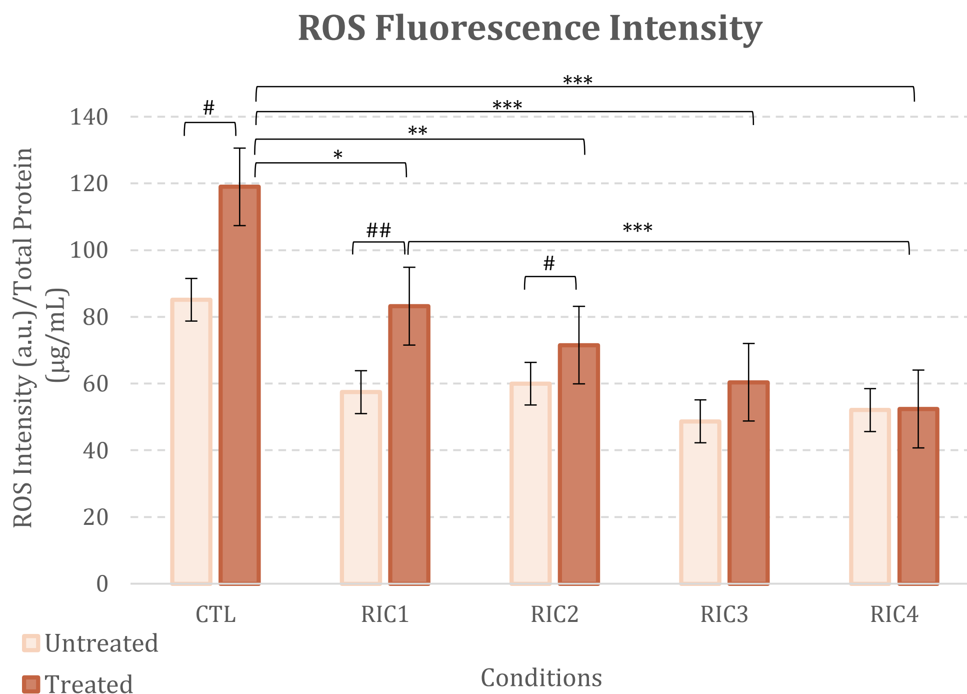


***Figure 11* – Effect of RIC-conditioned plasma treatment in LPS and ATP induced inflammation.** Cells were either treated or not with conditioned plasma for 24 hours and then an additional 24 hours with LPS (500 ng/mL) and 30 minutes with ATP (5mM). After this, cell media was discarded, and cells were treated with DCFDA for 15 minutes. Differences between experimental conditions were analyzed by the two-tailed and unpaired Student's t-test, with the result being considered statistically significant when p-value < 0,05 (n=3). The asterisk indicates statistical significance when comparing pairs of the treated conditions. The cardinal indicates statistical significance when comparing pairs of untreated and treated conditions.

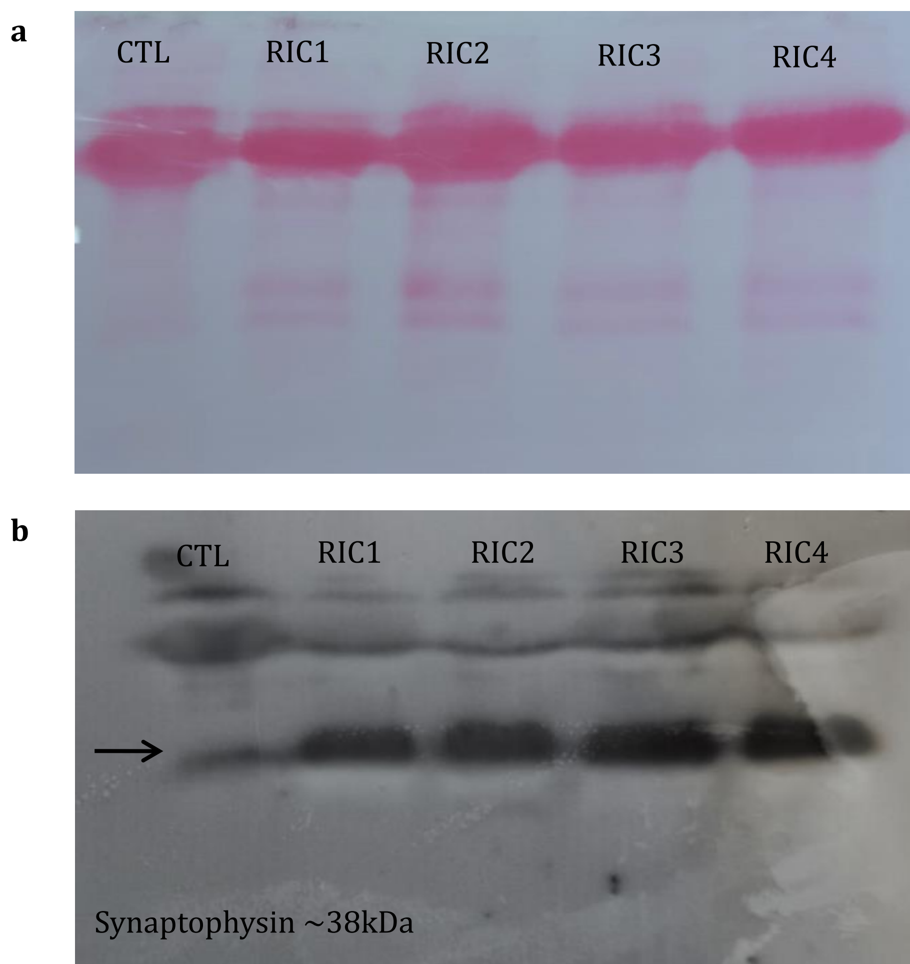
As shown in figure 11, HMC3 microglial ROS generation, namely, H_2O_2 increases in response to LPS+ATP treatment, per the optimization done previously and, it gradually decreases after treatment with not-conditioned human plasma (RIC1) and conditioned human plasma (RIC2-4). The highest decrease in ROS generation is observed in the RIC4 condition, in which ROS levels seem to reach similar levels to the same condition, but without inflammatory induction.

These results indicate that RIC treatment may have an anti-inflammatory effect on exacerbated ROS generation, particularly, 4h and one day after the RIC procedure.

3. **Neuroprotection/Neuronal function assay – effect of the conditioned plasma on synapse formation**

Synaptophysin is a specific presynaptic marker for neurons [119]. Thus, an indirect way of assessing neuronal function is by the expression of synaptophysin. In fact, during neurological diseases, including ischemic stroke, there is a decrease in its expression, which is associated with damage to the brain's neural networks and synaptic functions [120].

In this context, synaptophysin can indicate neuronal viability and neuronal function. Considering this, we tested the hypothesis of whether the RIC procedure can improve neuronal function. Therefore, the effect of the RIC-conditioned plasma on synaptophysin expression was analyzed in the SH-SY5Y cell line.



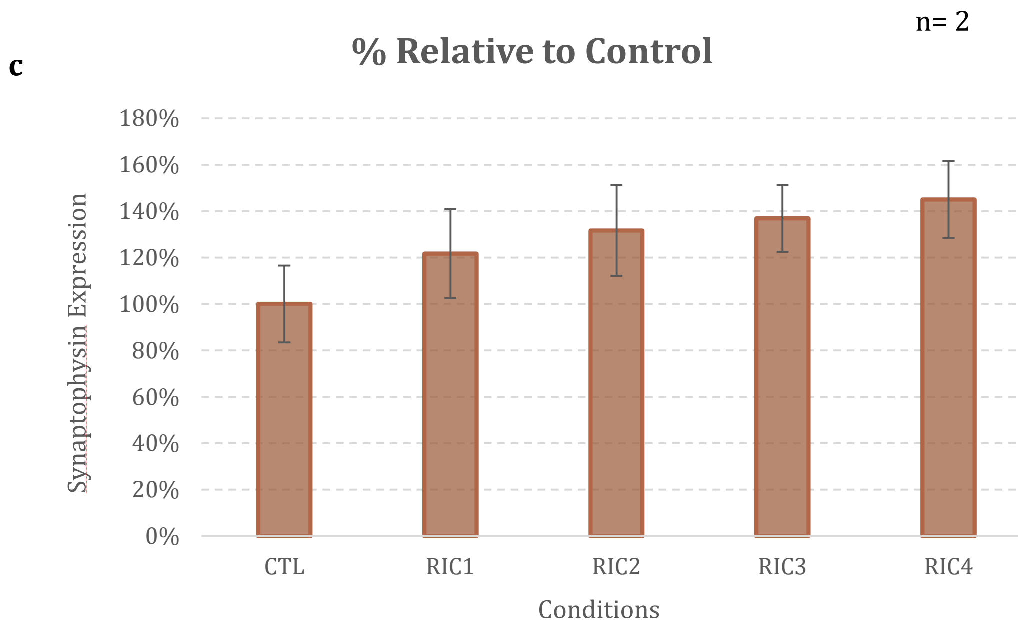


***Figure 12* – Preliminary data of the effects of conditioned plasma on synaptophysin expression.** Differentiated SH-SY5Y cells were treated or not with RIC-conditioned plasma for 24h. Protein extracts were obtained through RIPA buffer lysis. Protein was separated using SDS-PAGE on a 15% polyacrylamide gel. Mouse anti-synaptophysin , Thermofisher (1/1000) was the primary antibody used, while the secondary was sheep anti-mouse, Thermofisher (1/5000). **(a)** Ponceau red was used as control for checking total protein loading. In order to obtain the results, present in **(c)**, the quantification of **(b)** was done using ImageJ software and are presented as percentage relative to control (100%).

The pre-treatment with the conditioned plasma in SH-SY5Y promotes upregulation of the presynaptic marker synaptophysin (Figure 12c). This effect is observed in all of the RIC procedure time points (RIC1, RIC2, RIC3, and RIC4). Firstly, human plasma per se improves synaptophysin expression, as indicated by the increased expression in RIC conditions compared to FBS control. More interestingly, there is a tendency of plasma derived from all time points after the RIC procedure to upregulate synaptophysin. Out of these four time points, RIC4 is the one that promotes a more noticeable increase when compared to the control, in which synaptophysin expression is increased by 45%.

This data suggest that RIC treatment may promote neuroprotection through increased neuronal function. However, this preliminary data needs to be further reproduced, to validate the tendency and to assure its accuracy.

In addition to quantification of synaptophysin protein expression through western blot, an immunofluorescence assay was also conducted.

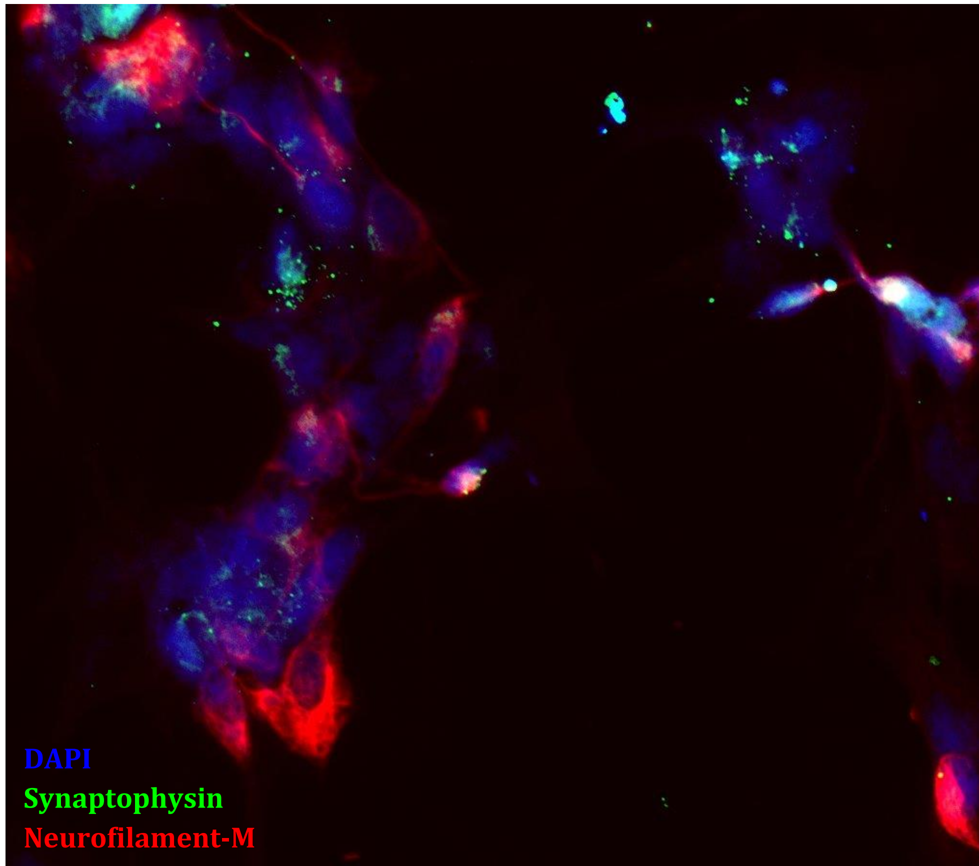


***Figure 13* – Immunofluorescence staining for synaptophysin.** SH-SY5Y cells were treated with not-conditioned and conditioned human plasma for 24h. Cells were labeled for synaptophysin (green) and neurofilament-M (red). Nuclei were stained using prolong mounting media with DAPI (blue). The image corresponds to the control group (original magnification 400x).

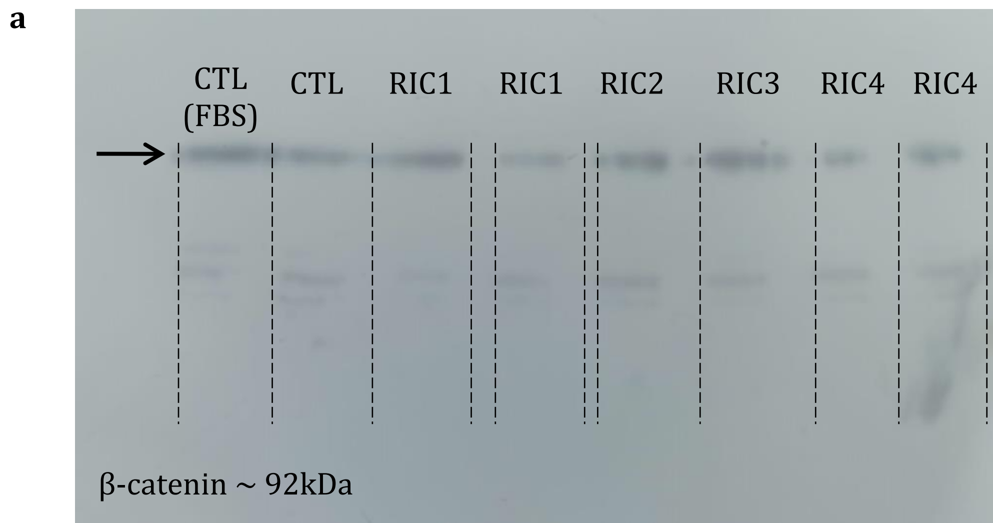
As shown in figure 13, it is possible to observe a few green speckles that correspond to synaptophysin expressed by cells cultured with normal cell medium with only FBS. This image may indicate that SH-SY5Y cells do, in fact, express synaptophysin in normal conditions. However, no further conclusion can be made, since it was not possible to render images corresponding to RIC1-4 samples. Moreover, image and sample quality must be improved for a better observation of neuronal cell body and neurites.

Still, this assay may lead to promising results that may support our findings obtained by western blot (Figure 12c). With this in mind, it is necessary to do additional experiments studying synaptophysin expression in response to RIC-conditioned plasma, through immunofluorescent microscopy.

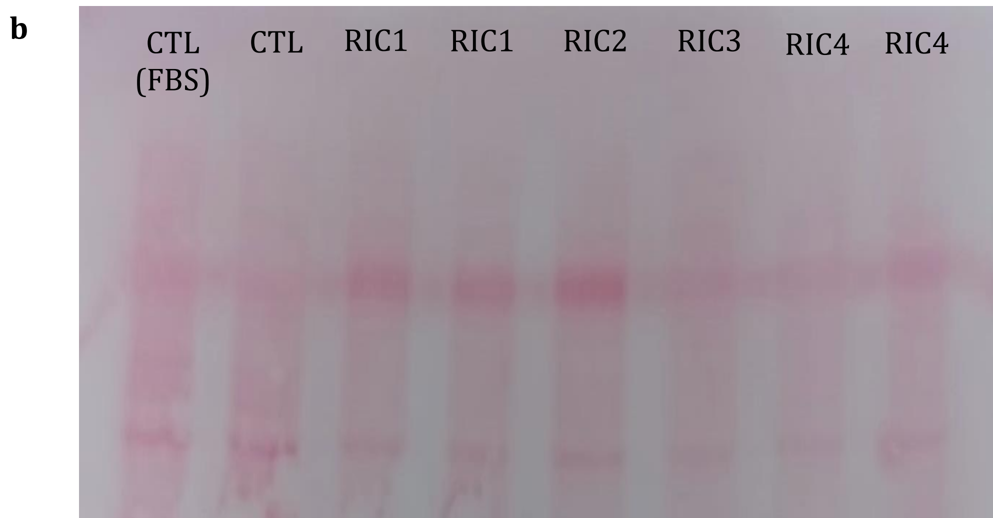
4. BBB integrity assay – effect of the conditioned plasma on adherens junction protein expression

One of the possible aftermaths of ischemic stroke is the disruption of the blood-brain barrier. This disruption is associated with an altered expression of tight, adherens, and gap junction proteins, consequently exacerbating stroke-associated injury [121,122]. Knowing this, the expression of β-catenin, an adherens junction protein, was analyzed through western blot. Therefore, the RIC-derived protection of the BBB was tested by the assessment of β-catenin expression following RIC-conditioned plasma treatment of the human brain microvasculature endothelial cells hCMEC/D3 cell line.

a



b



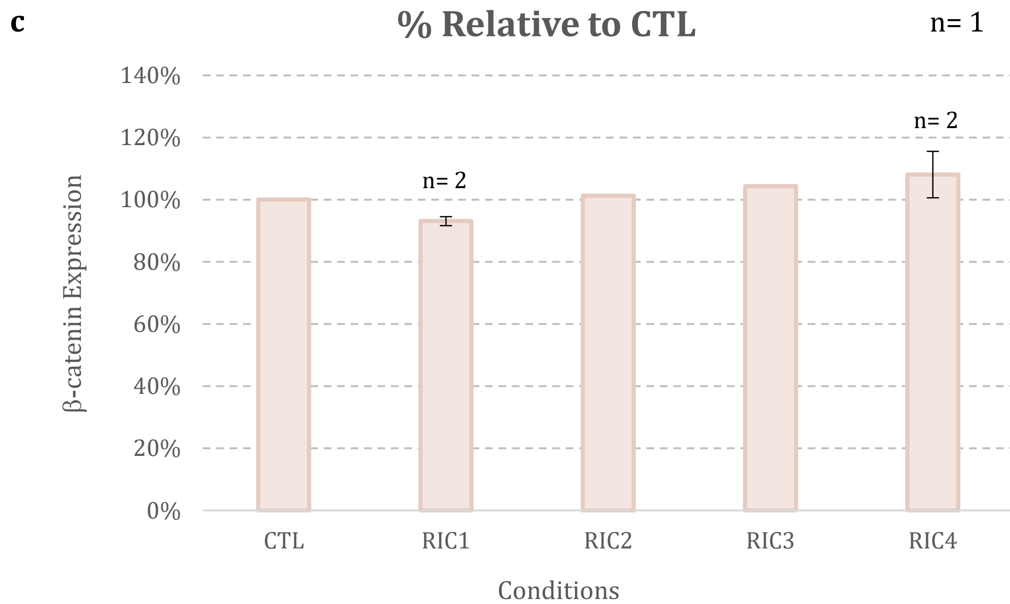


Figure 14 **- β-catenin expression in hCMEC/D3 cells treated with conditioned plasma.** Differentiated hCMEC/D3 cells were treated or not with RIC-conditioned plasma for 24h. Protein extracts were obtained through Loading buffer lysis. Protein was separated using SDS-PAGE on a 10% polyacrylamide gel. Rabbit anti-beta catenin, Invitrogem (1/125) was the primary antibody used, while the secondary was donkey anti-rabbit, Thermofisher (1/5000). **(a)** Rouge red was used as control for checking total protein loading. In order to obtain the results, present in **(c)**, the quantification of **(b)** was done using ImageJ software and are presented as percentage relative to control (100%).

β-catenin protein expression, as observed in figure 14c, appears to alter in response to RIC-conditioned plasma cell treatment by increasing slightly and gradually. The RIC 4 time-point exhibited the highest percentage relative to control, with 108% of β-catenin expression.

Based on these results no concrete conclusions concerning the influence of RIC-conditioned plasma treatment on β-catenin expression can be made. Therefore, further analysis of β-catenin protein expression should be done.

V. Discussion and Conclusions

Ischemic stroke is a worldwide concern, due to its prevalence, high mortality, and morbidity rate. In this neurological disorder, the propagation of the damage in the CNS provoked by ischemia is associated with various events, such as exacerbated neuroinflammation, neuronal death, and increased BBB permeability, among others. In this context, microglia, neurons, and cerebral endothelial cells play major roles, whether they are detrimental to disease progression or beneficial.

Considering this, a therapy that targets the brain's cells and their possible damaging effects is of most interest. In this context, remote ischemic conditioning procedure could correspond to a potential therapy that can tackle all of these areas.

Thusly, the main goal of this work was to understand if RIC-conditioned plasma collected from healthy human volunteers could limit inflammation, protect neurons, and prevent an increase in BBB permeability, by the maintenance of its integrity. For this purpose, three human cell models were used: the HMC3 cell line (neuroinflammation study), SH-SY5Y cell line (neuronal function/neuroprotection study), and the hCMEC/D3 cell line (BBB integrity study).

RIC-conditioned plasma significantly attenuated LPS+ATP-induced oxidative stress, while RIC4 plasma samples, specifically, prevented this oxidative stress (Figure 9). Interestingly, human plasma alone (RIC1 samples) also decreased ROS generation by HMC3 cells, which may be explained by the fact that human cells respond preferably to human plasma as opposed to the frequently used bovine serum. Therefore, RIC treatment may directly attenuate tissue injury by reducing ROS generation. Furthermore, this antioxidant and anti-inflammatory effect may be more noticeable in a later stage (i.e., 4h or one day after RIC), which may indicate that de novo protein synthesis is needed for this effect. In fact, the RIC-induced protective effects were prevented, in an experimental study, in which a protein synthesis inhibitor (cycloheximide) was administered to rats after RIC treatment [92]. Although there are several studies in animal models proving the anti-inflammatory capabilities of this procedure [112,113,123], this is the first

time that this effect has been observed using human samples from healthy individuals on human cell lines.

To further validate this anti-inflammatory effect due to RIC-conditioned plasma treatment, HMC3 cells should also be assessed for other inflammatory markers. One of the possible markers could be TNF-α, which is one of the key cytokines produced by cultured brain cells and also one of the key cytokines expressed after stroke [124]. Therefore, it would be interesting to understand how its expression would be altered in HMC3 cells after treating them with the inflammatory stimuli (LPS+ATP) and with the RIC-conditioned media. This could be achieved, in progress future work, through human TNF-α ELISA. However, the fact that RIC-derived plasma has shown this potential is a hopeful step toward a better understanding of the RIC effects and its possible applicability in a clinical context. Additionally, the microglial inflammatory response may also be tested, through the cell migration assay. Thusly, we could observe if HMC3 cell became more or less activated in response to the inflammatory stimuli and after treating them with RIC-derived plasma.

Another interesting result obtained with RIC-conditioned media is the indirect neuroprotection, by increased neuronal function, exhibited by SH-SY5Y cells (Figure 10). In this neuronal function assay, the gradual increase of synaptophysin expression reaches its peak one day (RIC4) after the RIC procedure, indicating once again that the RIC-associated protection increases over time, most likely, due to protein synthesis, instead of the immediate kinase activity that might occur.

In this context, it would also be interesting to quantify this synaptophysin expression increase by determining the number of synaptophysin "spots" through immunofluorescent microscopy, as observed in figure 11. For this, more careful labeling of the different cell components should be done, to render more clear images that can be quantified through image analysis software, such as ImageJ. Moreover, it would also be interesting to understand if microglial HMC3 cells, in response to both LPS+ATP and RIC-conditioned plasma treatment, release factors that protect SH-SY5Y neurons, by co-culturing both cell lines.

Finally, to further characterize the protective role of the RIC procedure, an analysis of β-catenin expression in response to conditioned and not-conditioned plasma was performed. This assay would allow us to understand, in an indirect

way, if RIC treatment could also promote the maintenance of BBB permeability, by securing the integrity of its junctions. Although the results presented in figure 12, were not conclusive, despite the very slight increase in β-catenin expression, this is still a promising approach. Therefore, other junction proteins, such as ZO-1 (tight junction protein), should be analyzed. In the future, β-catenin and ZO-1 could also be analyzed by microscopy in order to evaluate its organization in the plasma membrane, since permeability can be affected not only by expression levels but also by special organization.

Overall, this was the first time that plasma collected from healthy young individuals submitted to RIC was analyzed in human cell lines, for assessing its potential to modulate microglial and neuronal activity in a protective manner. In addition to this, our lab has also identified differently expressed circulating proteins (i.e., secretory leukocyte peptidase inhibitor (SLPi), Apolipoprotein C1 (APOC1), Sex hormone binding globulin (SHBG), etc), whose functions have been associated with neuroinflammation, neuroprotection, and the BBB [125,126].

The results here presented, contribute to the hypothesis that this procedure may ameliorate ischemic stroke-associated tissue damage, and as such, further studies should be performed to evaluate its application in hospital settings. Furthermore, taking these results into account, a clinical trial where patients were submitted to 5 cycles daily over 6 months with clear cognition improvements [127], and the fact that RIC exerts mild short-term effects, we can speculate that it could be more effective when applied chronically. In this context, we can draw parallels between RIC and physical exercise, since RIC may also have long-term effects once routinely applied. Besides this, when it comes to its clinical application, several other parameters may influence RIC efficacy, which includes sex and age. For instance, animal experiments have shown that the alterations associated with age (i.e., exacerbated oxidative stress, higher pro-inflammatory cytokine expression, etc) may reduce the RIC-associated protective effects [128]. Therefore, RIC may have more limited effects on aged patients, with subsequently lower recovery and survival rates.

Additional studies are required, to fully confirm the results obtained in this work, to reveal and understand the molecular mechanisms underlying the RIC procedure, and how these can contribute to stroke patients' survival and quality of

life. The findings obtained in this thesis contribute to a better understanding of the novel therapeutic approach to ischemic stroke treatment and other neurological diseases.

VI. References

[1] D. Kuriakose, Z. Xiao, Pathophysiology and treatment of stroke: Present status and future perspectives, Int. J. Mol. Sci. 21 (2020) 1–24. https://doi.org/10.3390/ijms21207609.

[2] T.M. Woodruff, J. Thundyil, S.C. Tang, C.G. Sobey, S.M. Taylor, T. V. Arumugam, Pathophysiology, treatment, and animal and cellular models of human ischemic stroke, Mol. Neurodegener. 6 (2011) 1–19. https://doi.org/10.1186/1750-1326-6-11.

[3] P. Deb, S. Sharma, K.M. Hassan, Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis, Pathophysiology. 17 (2010) 197–218. https://doi.org/10.1016/j.pathophys.2009.12.001.

[4] V.L. Roger, A.S. Go, D.M. Lloyd-Jones, E.J. Benjamin, J.D. Berry, W.B. Borden, D.M. Bravata, S. Dai, E.S. Ford, C.S. Fox, H.J. Fullerton, C. Gillespie, S.M. Hailpern, J.A. Heit, V.J. Howard, B.M. Kissela, S.J. Kittner, D.T. Lackland, J.H. Lichtman, L.D. Lisabeth, D.M. Makuc, G.M. Marcus, A. Marelli, D.B. Matchar, C.S. Moy, D. Mozaffarian, M.E. Mussolino, G. Nichol, N.P. Paynter, E.Z. Soliman, P.D. Sorlie, N. Sotoodehnia, T.N. Turan, S.S. Virani, N.D. Wong, D. Woo, M.B. Turner, Heart disease and stroke statistics-2012 update: A report from the American heart association, Circulation. 125 (2012) 2–220. https://doi.org/10.1161/CIR.0b013e31823ac046.

[5] S. Ojaghihaghighi, S.S. Vahdati, A. Mikaeilpour, A. Ramouz, Comparison of neurological clinical manifestation in patients with hemorrhagic and ischemic stroke, World J. Emerg. Med. 8 (2017) 34. https://doi.org/10.5847/wjem.j.1920-8642.2017.01.006.

[6] B. Kojic, A. Burina, R. Hodzic, Z. Pasic, O. Sinanovic, Risk Factors Impact on the Long-Term Survival After Hemorrhagic Stroke, Acta Medica Salin. 41 (2013). https://doi.org/10.5457/ams.101.10.

[7] P. Appelros, B. Stegmayr, A. Terént, Go Red for Women Sex Differences in Stroke Epidemiology A Systematic Review, Am. Hear. Assoc. (2009). https://doi.org/10.1161/STROKEAHA.108.540781.

[8] C. Hui, P. Tadi, L. Patti, Ischemic Stroke, StatPearls [Internet] StatPearls Publ. (2021). https://www.ncbi.nlm.nih.gov/books/NBK499997/.

[9] D.E. Andreou, I. Andreadou, Atherosclerosis: An inflammatory disease, Pharmakeftiki. 22 (2009) 83–96. https://doi.org/10.2174/138161212802481237.

[10] M.A. Crowther, Pathogenesis of Atherosclerosis, Am. Soc. Hematol. (2005).

[11] S.A. Randolph, Current Topics: Ischemic Stroke, Workplace Health Saf. (2016). https://doi.org/10.1161/CIR.0000000000000350.

[12] C. DeSai, A.H. Shapshak, Cerebral Ischemia, StatPearls [Internet]StatPearls Publ. (2021). https://www.ncbi.nlm.nih.gov/books/NBK560510/.

[13] A. Frendl, L. Csiba, Pharmacological and non-pharmacological recanalization strategies in acute ischemic stroke, Front. Neurol. MAY (2011). https://doi.org/10.3389/fneur.2011.00032.

[14] J.C. Grotta, G.W. Albers, J.P. Broderick, S.E. Kasner, E.H. Lo, D.A. Mendelow, R.L. Sacco, L.K.S. Wong, Stroke: Pathophysiology, Diagnosis and Management, 6th ed., Elsevier, 2016.

[15] W.M. Elsayed, E.H.A. Abdel-Gawad, D.I.A. Mesallam, T.S. El-Serafy, The relationship between oxidative stress and acute ischemic stroke severity and functional outcome, Egypt. J. Neurol. Psychiatry Neurosurg. 56 (2020) 4–9. https://doi.org/10.1186/s41983-020-00206-y.

[16] S. Shaafi, F. Hadisi, M. Mahmoudinezhad, H. Razmi, S.A. Nejadghaderi, M. Khalili, The significance of the oxidative stress markers in the one-year prognosis of patients with acute ischemic stroke: a case-control study, BMC Neurol. 21 (2021) 1–8. https://doi.org/10.1186/s12883-021-02257-x.

[17] C.L. Allen, U. Bayraktutan, Oxidative stress and its role in the pathogenesis of ischaemic stroke, Int. J. Stroke. 4 (2009) 461–470. https://doi.org/10.1111/j.1747-4949.2009.00387.x.

[18] G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla, A. Bitto, Oxidative Stress : Harms and Benefits for Human Health, Oxid. Med. Cell. Longev. 2017 (2017). https://doi.org/10.1155/2017/8416763.

[19] J.Y. Im, D. Kim, S.G. Paik, P.L. Han, Cyclooxygenase-2-dependent neuronal death proceeds via superoxide anion generation, Free Radic. Biol. Med. 41 (2006) 960–972. https://doi.org/10.1016/j.freeradbiomed.2006.06.001.

[20] G. Stoll, S. Jander, M. Schroeter, Inflammation and glial responses in ischemic brain lesions, Prog. Neurobiol. 56 (1998) 149–171. https://doi.org/10.1016/S0301-0082(98)00034-3.

[21] M. Gelderblom, F. Leypoldt, K. Steinbach, D. Behrens, C.U. Choe, D.A. Siler, T. V. Arumugam, E. Orthey, C. Gerloff, E. Tolosa, T. Magnus, Temporal and spatial dynamics of cerebral immune cell accumulation in stroke, Stroke. 40 (2009) 1849–1857. https://doi.org/10.1161/STROKEAHA.108.534503.

[22] R. Jin, G. Yang, G. Li, Inflammatory mechanisms in ischemic stroke: role of inflammatory cells, J. Leukoc. Biol. 87 (2010) 779–789.

https://doi.org/10.1189/jlb.1109766.

[23] F.C. Barone, D.B. Schmidt, L.M. Hillegass, W.J. Price, R.F. White, G.Z. Feuerstein, R.K. Clark, E. V. Lee, D.E. Griswold, H.M. Sarau, Reperfusion increases neutrophils and leukotriene b4 receptor binding in rat focal ischemia, Stroke. 23 (1992) 1337–1347. https://doi.org/10.1161/01.STR.23.9.1337.

[24] G. Yilmaz, N. Granger, Leukocyte Recruitment and Ischemic Brain Injury, Neuromolecular Med. (2010). https://doi.org/10.1007/s12017-009-8074-1.Leukocyte.

[25] A. Liesz, W. Zhou, É. Mracskó, S. Karcher, H. Bauer, S. Schwarting, L. Sun, D. Bruder, S. Stegemann, A. Cerwenka, C. Sommer, A.H. Dalpke, R. Veltkamp, Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke, Brain. 134 (2011) 704–720. https://doi.org/10.1093/brain/awr008.

[26] M. Daniel, I. Goel, Y. Sakai, Y. Teramura, Current status of ischemic stroke treatment : From thrombolysis to potential regenerative medicine, Regen. Ther. 18 (2021) 408–417. https://doi.org/10.1016/j.reth.2021.09.009.

[27] S. El Tawil, K.W. Muir, Thrombolysis and thrombectomy for acute ischaemic stroke, Clin. Med. J. R. Coll. Physicians London. 17 (2017) 161–165. https://doi.org/10.7861/clinmedicine.17-2-161.

[28] B.C. V. Campbell, Thrombolysis and Thrombectomy for Acute Ischemic Stroke: Strengths and Synergies, Semin. Thromb. Hemost. 43 (2017) 185–190. https://doi.org/10.1055/s-0036-1585078.

[29] J.S. Kim, tPA Helpers in the Treatment of Acute Ischemic Stroke: Are They Ready for Clinical Use?, J. Stroke. 21 (2019) 160–174. https://doi.org/10.5853/jos.2019.00584.

[30] T.G. Jovin, A. Chamorro, E. Cobo, M.A. de Miquel, C.A. Molina, A. Rovira, L. San Román, J. Serena, S. Abilleira, M. Ribó, M. Millán, X. Urra, P. Cardona, E. López-Cancio, A. Tomasello, C. Castaño, J. Blasco, L. Aja, L. Dorado, H. Quesada, M. Rubiera, M. Hernandez-Pérez, M. Goyal, A.M. Demchuk, R. von Kummer, M. Gallofré, A. Dávalos, Thrombectomy within 8 Hours after Symptom Onset in Ischemic Stroke, N. Engl. J. Med. 372 (2015) 2296–2306. https://doi.org/10.1056/nejmoa1503780.

[31] M. Inoue, T. Yoshimoto, K. Tanaka, J. Koge, M. Shiozawa, T. Nishii, Y. Ohta, T. Fukuda, T. Satow, H. Kataoka, H. Yamagami, M. Ihara, M. Koga, M. Mlynash, G.W. Albers, K. Toyoda, Mechanical Thrombectomy Up to 24 Hours in Large Vessel Occlusions and Infarct Velocity Assessment, J. Am. Heart Assoc. 10 (2021) 1–9. https://doi.org/10.1161/JAHA.121.022880.

[32] N. Liaw, D. Liebeskind, C. Nijasri, C. Suwanwela, A.M. Buchan, T.I. Nathaniel, Open Peer Review Emerging therapies in acute ischemic stroke [version 1; peer review: 3 approved] 1 2 3, (2020). https://doi.org/10.12688/f1000research.21100.1.

[33] R. Carter, The Human Brain Book: An Illustrated Guide to Its Structure, Function, and Disorders, DK Publishing (Dorling Kindersley), 2019.

[34] S.T. Brady, L. Tai, Cell Biology of the Nervous System, Eighth Edi, Elsevier Inc., 2012. https://doi.org/10.1016/B978-0-12-374947-5.00001-8.

[35] T.C. Südhof, The cell biology of synapse formation, J. Cell Biol. 220 (2021) 1–18. https://doi.org/10.1083/jcb.202103052.

[36] E.A. Martin, A.M. Lasseigne, A.C. Miller, Understanding the Molecular and Cell Biological Mechanisms of Electrical Synapse Formation, Front. Neuroanat. 14 (2020) 1–13. https://doi.org/10.3389/fnana.2020.00012.

[37] A.E. Pereda, Electrical synapses and their functional interactions with chemical synapses, Nat. Rev. Neurosci. 15 (2014) 250–263. https://doi.org/10.1038/nrn3708.

[38] S. Jäkel, L. Dimou, Glial cells and their function in the adult brain: A journey through the history of their ablation, Front. Cell. Neurosci. 11 (2017) 1–17. https://doi.org/10.3389/fncel.2017.00024.

[39] K.R. Jessen, Glial cells, Int. J. Biochem. Cell Biol. 36 (2004) 1861–1867. https://doi.org/10.1016/j.biocel.2004.02.023.

[40] F.A. Court, J. Alvarez, Glial Cells in Health and Disease of the CNS, 2016. https://doi.org/10.1007/978-3-319-40764-7.

[41] M.E. Kastriti, I. Adameyko, Specification, plasticity and evolutionary origin of peripheral glial cells, Curr. Opin. Neurobiol. 47 (2017) 196–202. https://doi.org/10.1016/j.conb.2017.11.004.

[42] M. V. Sofroniew, Astrocyte barriers to neurotoxic inflammation, Nat. Rev. Neurosci. (n.d.). https://doi.org/10.1038/nrn3898.

[43] K.K. Bercury, W.B. Macklin, Dynamics and mechanisms of CNS myelination, Dev. Cell. 32 (2015) 447–458. https://doi.org/10.1016/j.devcel.2015.01.016.

[44] C.P. Profaci, R.N. Munji, R.S. Pulido, R. Daneman, The blood–brain barrier in health and disease: Important unanswered questions, J. Exp. Med. 217 (2020) 1–16. https://doi.org/10.1084/jem.20190062.

[45] S. Liebner, R.M. Dijkhuizen, Y. Reiss, K.H. Plate, D. Agalliu, G. Constantin, Functional morphology of the blood–brain barrier in health and disease, Acta Neuropathol. 135 (2018) 311–336. https://doi.org/10.1007/s00401-018-1815-1.

[46] P. Ludewig, J. Winneberger, T. Magnus, The cerebral endothelial cell as a key regulator of inflammatory processes in sterile inflammation, J. Neuroimmunol. 326 (2019) 38–44. https://doi.org/10.1016/j.jneuroim.2018.10.012.

[47] F.C.A. Gomes, T.C.L.S. Spohr, R. Martinez, V.M. Neto, Cross-talk between neurons and glia: highlights on soluble factors, 2001.

[48] C. Dello Russo, N. Cappoli, I. Coletta, D. Mezzogori, F.

Paciello, G. Pozzoli, P. Navarra, A. Battaglia, The human microglial HMC3 cell line: Where do we stand? A systematic literature review, J. Neuroinflammation. 15 (2018) 1–24. https://doi.org/10.1186/s12974-018-1288-0.
[49] X.Y. Xiong, L. Liu, Q.W. Yang, Functions and mechanisms of microglia/macrophages in neuroinflammation and neurogenesis after stroke, Prog. Neurobiol. 142 (2016) 23–44. https://doi.org/10.1016/j.pneurobio.2016.05.001.
[50] O. Butovsky, H.L. Weiner, Microglial signatures and their role in health and disease, Nat. Rev. Neurosci. 19 (2018) 622–635. https://doi.org/10.1038/s41583-018-0057-5.
[51] K. Borst, M. Schwabenland, M. Prinz, Microglia metabolism in health and disease, Neurochem. Int. 130 (2019) 104331. https://doi.org/10.1016/j.neuint.2018.11.006.
[52] D. Disabato, N. Quan, J.P. Godbout, Neuroinflammation : The Devil is in the Details, J. Neurochem. 139 (2017) 136–153. https://doi.org/10.1111/jnc.13607.Neuroinflammation.
[53] C. Qin, L.Q. Zhou, X.T. Ma, Z.W. Hu, S. Yang, M. Chen, D.B. Bosco, L.J. Wu, D.S. Tian, Dual Functions of Microglia in Ischemic Stroke, Neurosci. Bull. 35 (2019) 921–933. https://doi.org/10.1007/s12264-019-00388-3.
[54] F.C. Detterbeck, The Devil Is in the Details, Ann. Surg. Oncol. 28 (2021) 11–13. https://doi.org/10.1245/s10434-020-09263-2.
[55] M.E. Lull, M.L. Block, Microglial Activation and Chronic Neurodegeneration, Neurotherapeutics. 7 (2010) 354–365. https://doi.org/10.1016/j.nurt.2010.05.014.
[56] A.D. Kraft, G.J. Harry, Features of Microglia and Neuroinflammation Relevant to Environmental Exposure and Neurotoxicity, J. Environ. Res. Public Heal. (2011) 2980–3018. https://doi.org/10.3390/ijerph8072980.
[57] W. Zhang, T. Tian, S. Gong, W. Huang, Q. Zhou, A. Wang, Y. Tian, Microglia-associated neuroinflammation is a potential therapeutic target for ischemic stroke, Neural Regen. Res. 16 (2021) 6–11.
[58] W.J. Streit, R.E. Mrak, W.S.T. Griffin, Microglia and neuroinflammation: A pathological perspective, J. Neuroinflammation. 1 (2004) 1–4. https://doi.org/10.1186/1742-2094-1-14.
[59] L. Lian, Y. Zhang, L. Liu, L. Yang, Y. Cai, J. Zhang, S. Xu, Neuroinflammation in Ischemic Stroke: Focus on MicroRNA-mediated Polarization of Microglia, Front. Mol. Neurosci. 13 (2021). https://doi.org/10.3389/fnmol.2020.612439.
[60] C. Arcuri, C. Mecca, R. Bianchi, I. Giambanco, R. Donato, The pathophysiological role of microglia in dynamic surveillance, phagocytosis and structural remodeling of the developing CNS, Front. Mol. Neurosci. 10 (2017) 1–22. https://doi.org/10.3389/fnmol.2017.00191.
[61] J.D. Cherry, J.A. Olschowka, M.K. O'Banion, Are "resting" microglia more "M2"?, Front. Immunol. 5 (2014) 2–5. https://doi.org/10.3389/fimmu.2014.00594.
[62] C. Sundal, Microglia: Multiple roles in surveillance, circuit shaping, and response to injury, Neurology. 82 (2014) 1846. https://doi.org/10.1212/WNL.0000000000000399.
[63] D.P. Schafer, B. Stevens, Microglia function in central nervous system development and plasticity, Cold Spring Harb. Perspect. Biol. 7 (2015) 1–18. https://doi.org/10.1101/cshperspect.a020545.
[64] C. Kaur, G. Rathnasamy, E.A. Ling, Biology of microglia in the developing brain, J. Neuropathol. Exp. Neurol. 76 (2017) 736–753. https://doi.org/10.1093/jnen/nlx056.
[65] M.È. Tremblay, B. Stevens, A. Sierra, H. Wake, A. Bessis, A. Nimmerjahn, The role of microglia in the healthy brain, J. Neurosci. 31 (2011) 16064–16069. https://doi.org/10.1523/JNEUROSCI.4158-11.2011.
[66] S. Rivas-Fuentes, A. Salgado-Aguayo, J. Arratia-Quijada, P. Gorocica-Rosete, Regulation and biological functions of the CX3CL1-CX3CR1 axis and its relevance in solid cancer: A mini-review, J. Cancer. 12 (2021) 571–583. https://doi.org/10.7150/jca.47022.
[67] K. Kierdorf, M. Prinz, Microglia in steady state, J. Clin. Invest. 127 (2017) 3201–3209. https://doi.org/10.1172/JCI90602.
[68] M.A. Lynch, The multifaceted profile of activated microglia, Mol. Neurobiol. 40 (2009) 139–156. https://doi.org/10.1007/s12035-009-8077-9.
[69] M.W. Salter, B. Stevens, Microglia emerge as central players in brain disease, Nat. Med. 23 (2017) 1018–1027. https://doi.org/10.1038/nm.4397.
[70] S. Ceruti, M.A. Panaro, M. Kasper, B. Laffer, D. Bauer, S. Wasmuth, M. Busch, T.V. Jalilvand, S. Thanos, G. Meyer Zu Hörste, K. Loser, T. Langmann, A. Heiligenhaus, Loss of IL-10 Promotes Differentiation of Microglia to a M1 Phenotype, (2019). https://doi.org/10.3389/fncel.2019.00430.
[71] A. Cianciulli, T. Dragone, R. Calvello, C. Porro, T. Trotta, D.D. Lofrumento, M.A. Panaro, IL-10 plays a pivotal role in anti-inflammatory effects of resveratrol in activated microglia cells, Int. Immunopharmacol. 24 (2015) 369–376. https://doi.org/10.1016/j.intimp.2014.12.035.
[72] D. Lobo-Silva, G.M. Carriche, A.G. Castro, S. Roque, M. Saraiva, Balancing the immune response in the brain: IL-10 and its regulation, J. Neuroinflammation. 13 (2016) 1–10. https://doi.org/10.1186/s12974-016-0763-8.
[73] C. Rossi, M. Cusimano, M. Zambito, A. Finardi, A. Capotondo, J. Manuel Garcia-Manteiga, G. Comi, R. Furlan, G. Martino, L. Muzio, Interleukin 4 modulates microglia homeostasis and attenuates the early slowly progressive phase of amyotrophic lateral sclerosis,

(n.d.). https://doi.org/10.1038/s41419-018-0288-4.

[74] O. Butovsky, Y. Ziv, A. Schwartz, G. Landa, A.E. Talpalar, S. Pluchino, G. Martino, M. Schwartz, Microglia activated by IL-4 or IFN-γ differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells, Mol. Cell. Neurosci. 31 (2006) 149–160. https://doi.org/10.1016/j.mcn.2005.10.006.

[75] N.C. Derecki, A.N. Cardani, C.H. Yang, K.M. Quinnies, A. Crihfield, K.R. Lynch, J. Kipnis, Regulation of learning and memory by meningeal immunity: A key role for IL-4, J. Exp. Med. 207 (2010) 1067–1080. https://doi.org/10.1084/jem.20091419.

[76] M. Erta, A. Quintana, J. Hidalgo, Interleukin-6, a Major Cytokine in the Central Nervous System Ivyspring International Publisher, Int. J. Biol. Sci. 8 (2012) 1254–1266. https://doi.org/10.7150/ijbs.4679.

[77] X. Kong, Z. Zhang, T. Fu, J. Ji, J. Yang, Z. Gu, TNF-α regulates microglial activation via the NF-κB signaling pathway in systemic lupus erythematosus with depression, Int. J. Biol. Macromol. 125 (2019) 892–900. https://doi.org/10.1016/j.ijbiomac.2018.12.146.

[78] S. Raffaele, M. Lombardi, C. Verderio, M. Fumagalli, cells TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions, (n.d.). https://doi.org/10.3390/cells9102145.

[79] S.D. Rao, H.Z. Yin, J.H. Weiss, Disruption of Glial Glutamate Transport by Reactive Oxygen Species Produced in Motor Neurons, 2003.

[80] U. Dirnagl, K. Becker, A. Meisel, Preconditioning and tolerance against cerebral ischaemia : from experimental strategies to clinical use, Lancet Neurol. 8 (2009) 398–412. https://doi.org/10.1016/S1474-4422(09)70054-7.

[81] I. Mollet, J.P. Marto, M. Mendonça, M.V. Baptista, H.L.A. Vieira, Remote but not Distant: a Review on Experimental Models and Clinical Trials in Remote Ischemic Conditioning as Potential Therapy in Ischemic Stroke, Springer US, 2022. https://doi.org/10.1007/s12035-021-02585-6.

[82] D.C. Hess, R.A. Blauenfeldt, G. Andersen, K.D. Hougaard, N. Hoda, Remote ischaemic conditioning — a new paradigm of self-protection in the brain, (2015) 1–13. https://doi.org/10.1038/nrneurol.2015.223.

[83] C.E. Murry, R.B. Jennings, K.A. Reimer, Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium, Circulation. 74 (1986) 1124–1136. https://doi.org/10.1161/01.CIR.74.5.1124.

[84] S. Abbasi-Habashi, G.C. Jickling, I.R. Winship, Immune Modulation as a Key Mechanism for the Protective Effects of Remote Ischemic Conditioning After Stroke, Front. Neurol. 12 (2021) 1–20. https://doi.org/10.3389/fneur.2021.746486.

[85] F.C. Barone, R.F. White, P.A. Spera, J. Ellison, R.W. Currie, X. Wang, G.Z. Feuerstein, N.J. Rothwell, Ischemic Preconditioning and Brain Tolerance, Stroke. 29 (1998) 1937. http://stroke.ahajournals.org/cgi/content/abstract/29/9/1937.

[86] S.Y. Lim, D.J. Hausenloy, Remote ischemic conditioning: From bench to bedside, Front. Physiol. 3 FEB (2012) 1–11. https://doi.org/10.3389/fphys.2012.00027.

[87] G. Zhou, M.H. Li, G. Tudor, H.T. Lu, R. Kadirvel, D. Kallmes, Remote ischemic conditioning in cerebral diseases and neurointerventional procedures: Recent research progress, Front. Neurol. 9 (2018). https://doi.org/10.3389/fneur.2018.00339.

[88] E.A. Lambert, C.J. Thomas, R. Hemmes, N. Eikelis, A. Pathak, M.P. Schlaich, G.W. Lambert, Sympathetic nervous response to ischemia-reperfusion injury in humans is altered with remote ischemic preconditioning, Am. J. Physiol. - Hear. Circ. Physiol. 311 (2016) H364–H370. https://doi.org/10.1152/ajpheart.00369.2016.

[89] D. Noronha Osório, R. Viana-Soares, J.P. Marto, M.D. Mendonça, H.P. Silva, C. Quaresma, M. Viana-Baptista, H. Gamboa, H.L.A. Vieira, Autonomic nervous system response to remote ischemic conditioning: Heart rate variability assessment, BMC Cardiovasc. Disord. 19 (2019) 1–9. https://doi.org/10.1186/s12872-019-1181-5.

[90] S. Malhotra, I. Naggar, M. Stewart, D.M. Rosenbaum, Neurogenic pathway mediated remote preconditioning protects the brain from transient focal ischemic injury, Brain Res. 1386 (2011) 184–190. https://doi.org/10.1016/j.brainres.2011.02.032.

[91] M. Donato, B. Buchholz, M. Rodríguez, V. Pérez, J. Inserte, D. García-Dorado, R.J. Gelpi, Role of the parasympathetic nervous system in cardioprotection by remote hindlimb ischaemic preconditioning, Exp. Physiol. 98 (2013) 425–434. https://doi.org/10.1113/expphysiol.2012.066217.

[92] C. Ren, Z. Yan, D. Wei, X. Gao, X. Chen, H. Zhao, Limb remote ischemic postconditioning protects against focal ischemia in rats, Brain Res. 1288 (2009) 88–94. https://doi.org/10.1016/j.brainres.2009.07.029.

[93] J.M.J. Pickard, S.M. Davidson, D.J. Hausenloy, D.M. Yellon, Co-dependence of the neural and humoral pathways in the mechanism of remote ischemic conditioning, Basic Res. Cardiol. 111 (2016) 1–12. https://doi.org/10.1007/s00395-016-0568-z.

[94] K.E. Salmeron, K. Poinsatte, T.J. Parker, A.M. Stowe, G.J. Bix, Extracellular matrix peptides as theranostic mediators of ischemic stroke neuroprotection and repair : lessons from studies in remote ischemic preconditioning and exercise, Cond. Med. 1 (2018).

[95] M. Shimizu, M. Tropak, R.J. Diaz, F. Suto, H. Surendra, E. Kuzmin, J. Li, G. Gross, G.J. Wilson, J. Callahan, A.N. Redington, Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: Evidence suggesting cross-species protection, Clin. Sci. 117 (2009) 191–200. https://doi.org/10.1042/CS20080523.

[96] K.L. Redington, T. Disenhouse, S.C. Strantzas, R. Gladstone, C. Wei, M.B. Tropak, X. Dai, C. Manlhiot, J. Li, Remote cardioprotection by direct peripheral nerve stimulation and topical capsaicin is mediated by circulating humoral factors, Basic Res. Cardiol. 107 (2012). https://doi.org/10.1007/s00395-011-0241-5.

[97] M.B. Amdahl, A.W. DeMartino, M.T. Gladwin, Inorganic nitrite bioactivation and role in physiological signaling and therapeutics, Biol. Chem. 401 (2019) 201–211. https://doi.org/10.1515/hsz-2019-0349.

[98] D.C. Hess, M.N. Hoda, M.B. Khan, Humoral mediators of remote ischemic conditioning: Important role of eNOS/NO/nitrite, Acta Neurochir. Suppl. 121 (2016) 45–48. https://doi.org/10.1007/978-3-319-18497-5_8.

[99] T. Rassaf, M. Totzeck, U.B. Hendgen-Cotta, S. Shiva, G. Heusch, M. Kelm, Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning, Circ. Res. 114 (2014) 1601–1610. https://doi.org/10.1161/CIRCRESAHA.114.303822.

[100] H.G. Zhao, X.C. Sun, X.H. Xian, W. Bin Li, M. Zhang, Q.J. Li, The role of nitric oxide in the neuroprotection of limb ischemic preconditioning in rats, Neurochem. Res. 32 (2007) 1919–1926. https://doi.org/10.1007/s11064-007-9381-2.

[101] M.B. Khan, S. Hafez, M.N. Hoda, B. Baban, J. Wagner, M.E. Awad, H. Sangabathula, S. Haigh, M. Elsalanty, J.L. Waller, D.C. Hess, Chronic Remote Ischemic Conditioning Is Cerebroprotective and Induces Vascular Remodeling in a VCID Model, Transl. Stroke Res. 9 (2018) 51–63. https://doi.org/10.1007/s12975-017-0555-1.

[102] al W. Eric Dickson, C.P. Reinhardt, F.P. Renzi, R.C. Becker, W.A. Porcaro, S.O. Heard, Ischemic Preconditioning May Be Transferable via Whole Blood Transfusion: Preliminary Evidence, J. Thromb. Thrombolysis. (1999).

[103] Y. Zhao, Z.N. Zheng, C.W. Cheung, Z.Y. Zuo, S.Q. Jin, Transfusion of plasma collected at late phase after preconditioning reduces myocardial infarct size induced by ischemia-reperfusion in rats in vivo, Chin. Med. J. (Engl). 130 (2017) 303–308. https://doi.org/10.4103/0366-6999.198933.

[104] D. Zhou, J. Ding, J. Ya, L. Pan, Y. Wang, X. Ji, R. Meng, Remote ischemic conditioning: A promising therapeutic intervention for multi-organ protection, Aging (Albany. NY). 10 (2018) 1825–1855. https://doi.org/10.18632/aging.101527.

[105] P. Hibert, D. Prunier-Mirebeau, O. Beseme, M. Chwastyniak, S. Tamareille, Modifications in Rat Plasma Proteome after Remote Ischemic Preconditioning (RIPC) Stimulus: Identification by a SELDI-TOF-MS Approach, PLoS One. 9 (2014) 85669. https://doi.org/10.1371/journal.pone.0085669.

[106] E. Helgeland, L.E. Breivik, M. Vaudel, Ø.S. Svendsen, H. Garberg, J.E. Nordrehaug, F. Steingrimsen Berven, A.K. Jonassen, Exploring the Human Plasma Proteome for Humoral Mediators of Remote Ischemic Preconditioning-A Word of Caution, PLoS One. (2014). https://doi.org/10.1371/journal.pone.0109279.

[107] S. Song, L. Guo, D. Wu, J. Shi, Y. Duan, X. He, Y. Liu, Y. Ding, X. Ji, R. Meng, Quantitative Proteomic Analysis of Plasma after Remote Ischemic Conditioning in a Rhesus Monkey Ischemic Stroke Model, Biomolecules. (2021). https://doi.org/10.3390/biom11081164.

[108] J. You, L. Feng, L. Bao, M. Xin, D. Ma, J. Feng, Potential applications of remote limb ischemic conditioning for chronic cerebral circulation insufficiency, Front. Neurol. 10 (2019) 1–17. https://doi.org/10.3389/fneur.2019.00467.

[109] A. McDonough, J.R. Weinstein, Neuroimmune Response in Ischemic Preconditioning, Neurotherapeutics. 13 (2016) 748–761. https://doi.org/10.1007/s13311-016-0465-z.

[110] J.M. Pradillo, D. Fernández-López, I. García-Yébenes, M. Sobrado, O. Hurtado, M.A. Moro, I. Lizasoain, Toll-like receptor 4 is involved in neuroprotection afforded by ischemic preconditioning, J. Neurochem. (2009). https://doi.org/10.1111/j.1471-4159.2009.05972.x.

[111] Z.-J. Liu, C. Chen, X.-R. Li, Y.-Y. Ran, T. Xu, Y. Zhang, X.-K. Geng, Y. Zhang, H.-S. Du, R.K. Leak, X.-M. Ji, X.-M. Hu, Remote Ischemic Preconditioning-Mediated Neuroprotection against Stroke is Associated with Significant Alterations in Peripheral Immune Responses, Neurosci. Ther. (2015). https://doi.org/10.1111/cns.12448.

[112] M. Xia, Q. Ding, Z. Zhang, Q. Feng, Remote Limb Ischemic Preconditioning Protects Rats Against Cerebral Ischemia via HIF-1α/AMPK/HSP70 Pathway, Cell. Mol. Neurobiol. 37 (2017) 1105–1114. https://doi.org/10.1007/s10571-016-0444-2.

[113] S. Hu, H. Dong, H. Zhang, S. Wang, L. Hou, S. Chen, J. Zhang, L. Xiong, Noninvasive limb remote ischemic preconditioning contributes neuroprotective effects via activation of adenosine A1 receptor and redox status after transient focal cerebral ischemia in rats, Brain Res. 1459 (2012) 81–90. https://doi.org/10.1016/j.brainres.2012.04.017.

[114] D. Wei, C. Ren, X. Chen, H. Zhao, Inflammatory Factors in Rat Stroke, PLoS One. 7 (2012) 30892. https://doi.org/10.1371/journal.pone.0030892.

[115] F. Pico, B. Lapergue, M. Ferrigno, C. Rosso, E. Meseguer, M.-L. Chadenat, F. Bourdain, M. Obadia, C. Hirel, D. Long Duong, S. Deltour, P. Aegerter, J. Labreuche, A. Cattenoy, D. Smadja, H. Hosseini, B. Guillon, V. Wolff, Y. Samson, C. Cordonnier, P. Amarenco, C. Author, Effect of In-Hospital Remote Ischemic Perconditioning on Brain Infarction Growth and Clinical Outcomes in Patients With Acute Ischemic Stroke The RESCUE BRAIN Randomized Clinical Trial Supplemental content, JAMA Neurol. 77 (218AD) 725–734. https://doi.org/10.1001/jamaneurol.2020.0326.

[116] W. Zhao, R. Che, S. Li, C. Ren, C. Li, C. Wu, H. Lu, J. Chen, J. Duan, R. Meng, X. Ji, Remote ischemic conditioning

for acute stroke patients treated with thrombectomy, Ann. Clin. Transl. Neurol. 5 (2018) 850–856. https://doi.org/10.1002/acn3.588.

[117] I. Mollet, C. Martins, M. Ângelo-Dias, A.S. Carvalho, K. Aloria, R. Matthiesen, M. Viana-Baptista, L.M. Borrego, H.L.A. Vieira, Pilot study in human healthy volunteers on the mechanisms underlying remote ischemic conditioning (RIC) – Targeting circulating immune cells and immune-related proteins, J. Neuroimmunol. 367 (2022). https://doi.org/10.1016/j.jneuroim.2022.577847.

[118] D.S.A. Simpson, P.L. Oliver, antioxidants ROS Generation in Microglia: Understanding Oxidative Stress and Inflammation in Neurodegenerative Disease, (n.d.). https://doi.org/10.3390/antiox9080743.

[119] X. Bai, R. Strong, Expression of synaptophysin protein in different dopaminergic cell lines, J. Biochem. Pharmacol. Res. 2 (2014) 185–190.

[120] M.K. Sun, J. Hongpaisan, T.J. Nelson, D.L. Alkon, Poststroke neuronal rescue and synaptogenesis mediated in vivo by protein kinase C in adult brains, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13620–13625. https://doi.org/10.1073/pnas.0805952105.

[121] K. Nian, I.C. Harding, I.M. Herman, E.E. Ebong, Blood-Brain Barrier Damage in Ischemic Stroke and Its Regulation by Endothelial Mechanotransduction, Front. Physiol. 11 (2020). https://doi.org/10.3389/fphys.2020.605398.

[122] A. Hartsock, W.J. Nelson, Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton, Biochim. Biophys. Acta - Biomembr. 1778 (2008) 660–669. https://doi.org/10.1016/j.bbamem.2007.07.012.

[123] S. Ramagiri, R. Taliyan, Remote limb ischemic post conditioning during early reperfusion alleviates cerebral ischemic reperfusion injury via GSK-3β/CREB/ BDNF pathway, Eur. J. Pharmacol. 803 (2017) 84–93. https://doi.org/10.1016/j.ejphar.2017.03.028.

[124] A. Tuttolomondo, D. Di Raimondo, R. di Sciacca, A. Pinto, G. Licata, Inflammatory Cytokines in Acute Ischemic Stroke, Curr. Pharm. Des. 14 (2008) 3574–3589. https://doi.org/10.2174/138161208786848739.

[125] D.A. Elliott, C. Shannon Weickert, B. Garner, Clinical Lipidology Apolipoproteins in the brain: implications for neurological and psychiatric disorders, (2017). https://doi.org/10.2217/clp.10.37.

[126] S. Doumas, A. Kolokotronis, P. Stefanopoulos, Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor, Infect. Immun. 73 (2005) 1271–1274. https://doi.org/10.1128/IAI.73.3.1271-1274.2005.

[127] Z. Liao, Y. Bu, M. Li, R. Han, N. Zhang, J. Hao, W. Jiang, Remote ischemic conditioning improves cognition in patients with subcortical ischemic vascular dementia, BMC Neurol. 19 (2019) 1–8. https://doi.org/10.1186/s12883-019-1435-y.

[128] V.A. Dinapoli, S.A. Benkovic, X. Li, K.A. Kelly, D.B. Miller, C.L. Rosen, J.D. Huber, J.P. O'callaghan, J.D. Huber, Age exaggerates proinflammatory cytokine signaling and truncates STAT3 signaling following ischemic stroke in the rat, 170 (2010) 633–644. https://doi.org/10.1016/j.neuroscience.2010.07.011.