UNIVERSIDADE
BEIRA INTERIOR

Papel da argonauta-2 no secretoma microglial

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Dissertação para obtenção do Grau de Mestre em
Medicina
(Mestrado integrado)

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dezembro de 2020

“The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.”

Santiago Ramón y Cajal

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor, Dr. Raquel Ferreira, for her scientific guidance, knowledge, dedication, and constant assistance. I am very grateful for the opportunity to work in a laboratory and experience the process of an investigation project.

I would also like to thank my co-supervisor, Dr. Ana Cristovão, for her valuable suggestions.

I thank João Pais for his time available to help improve my laboratory knowledge and skills and for the patience to answer all my questions.

I would like to thank God for the strength, guidance and blessing that allowed me to finish this project.

I sincerely thank my parents, Dulce and Domingos, grandparents, Luísa and Manuel, aunt Judite, for all for the unconditional support. I would like to especially thank my mom for always being there with her kind, wise words and her warm hugs. Thank you for always believing in me and always accompanying me in all the struggles and joys.

To Rita and Nuno, I am grateful for their unlimited friendship throughout the years and for being always present in the best and worst moments. To my friends, Ana Catarina, Beatriz, Catarina, Jéssica, Joana, Nicole Foreman, Nicole Silva and Filipa, who made my life in Covilhã happier, thank you for the companionship and all the moments shared.

To the rest of my family and friends that have indirectly contributed to my professional training and writing of my dissertation, I also thank you.

Finally, I would like to thank The Health Sciences Research Centre for providing me with the resources to write this project and acknowledge the University of Beira Interior for their participation in my medical training.

Resumo

O acidente cerebral vascular (AVC) é a principal causa de morte em Portugal, continuando a estar associado a elevadas taxas de morbilidade e potenciais anos de vida perdidos dentro das doenças cardiovasculares. No Mundo, estima-se que a cada segundo uma pessoa sofra desta patologia.

O AVC ocorre devido a um bloqueio no suprimento sanguíneo cerebral (AVC isquémico) ou rompimento de um vaso sanguíneo (AVC hemorrágico) que resulta na morte ou disfunção das células cerebrais pela ausência de oxigénio e nutrientes. Esta lesão culmina em inflamação local com ativação de células cerebrais, a microglia, astrócitos e oligodendrócitos e recrutamento de leucócitos periféricos. Estas células produzem uma variedade de mediadores que podem, por um lado, exacerbar a degradação tecidular e, por outro, estimular a reparação. A neuroinflamação observada na isquémia é modulada por microRNA, pequenos RNA responsáveis pela regulação génica pós-transcricional através da clivagem de um RNA mensageiro-alvo ou da repressão da sua tradução. Neste processo, a Argonauta-2, uma proteína componente-chave do complexo de silenciamento induzido por RNA com atividade catalítica, liga-se ao microRNA e identifica o RNA mensageiro-alvo resultando no silenciamento de genes.

Neste sentido, o objetivo deste projeto foi determinar o papel da Argonauta-2 no secretoma microglial através da aferição da sua expressão em células de microglia e avaliação do seu impacto na resposta inflamatória num modelo experimental de isquemia.

Utilizámos culturas primárias de microglia de murganhos, onde medimos os níveis de expressão da Argonauta-2 em células expostas a um período de privação de oxigénio e glucose e a lipopolissacarídeo, tido como o controlo positivo. Nesta fase, verificou-se um aumento da proteína e um eficaz silenciamento da mesma após privação de oxigénio e glucose e estímulo inflamatório, em comparação com o controlo. Em seguida, quantificámos a expressão de mediadores pro-inflamatórios (fator de necrose tumoral alfa, interleucina-6 e oxido nítrico) em células com silenciamento da proteína num contexto inflamatório, tendo-se observado uma diminuição de todas estas moléculas, embora nem todas de uma forma significativa.

Os resultados obtidos sugerem um papel importante da Argonauta-2 nos processos subjacentes responsáveis pela modulação da resposta inflamatória e isquémica. No entanto, será necessária mais investigação nesta área pois, a Argonauta-2 pode tornar-se num novo alvo de estudo para potenciais abordagens terapêuticas.

Palavras-chave

Argonauta-2; secretoma; isquémia; inflamação; microglia

Resumo Alargado

O acidente cerebral vascular (AVC) é a principal causa de morte em Portugal, continuando a estar associado a elevadas taxas de morbilidade e potenciais anos de vida perdidos dentro das doenças cardiovasculares. No Mundo, estima-se que a cada segundo uma pessoa sofra desta patologia. Existem vários fatores de risco, sendo alguns não modificáveis tais como a idade, género, história familiar, e ainda outros modificáveis como a diabetes, a hipertensão arterial, o colesterol, a obesidade, o sedentarismo, as arritmias, a displasia fibromuscular, o consumo de tabaco e de álcool. Apesar de ser uma área bastante estudada e com planos de atuação eficientes, os tratamentos disponíveis não são 100% eficazes e abrangem apenas uma percentagem limitada de doentes, levando a graves incapacidades e efeitos adversos.

O AVC resulta de um bloqueio no suprimento sanguíneo cerebral (AVC isquémico), de origem embólica ou trombótica, ou de um rompimento de um sanguíneo (AVC hemorrágico). Esta interrupção do fluxo sanguíneo provoca lesão das células cerebrais que levam à sua disfunção e morte pela privação de oxigénio e de nutrientes. Posteriormente, gera-se uma resposta inflamação local desencadeada pela ativação de células cerebrais, a microglia, astrócitos e oligodendrócitos e recrutamento de leucócitos periféricos, que produzem uma variedade de mediadores que podem, por um lado, exacerbar a degradação tecidual e, por outro, estimular a sua reparação. A microglia é a primeira célula a reagir numa situação de perigo e, quando ativa, age de forma semelhante aos macrófagos, com capacidade de fagocitose e produção de citoquinas, que comprometem a barreira hematoencefálica. A neuroinflamação observada na isquémia é modulada por microRNA, pequenos RNA responsáveis pela regulação génica pós-transcricional através da clivagem de um RNA mensageiro-alvo ou da repressão da sua tradução. Os microRNA são capazes de controlar o início e a manutenção da inflamação através de microRNA com função pro e anti-inflamatória. A Argonauta-2 desempenha, assim, um papel fundamental neste processo, uma vez que é um componente-chave do complexo RISC e possui atividade catalítica. A Argonauta-2 liga-se ao microRNA e identifica o RNA mensageiro-alvo através da complementaridade de sequência resultando na sua clivagem ou inibição da tradução, que leva à inibição da expressão de genes específicos. Por isso, uma manipulação nos níveis da Argonauta-2 pode influenciar a função dos microRNA, levando a uma exacerbação ou diminuição da inflamação.

Neste seguimento, o objetivo deste projeto foi determinar qual o papel da Argonauta-2 no secretoma microglial através da aferição da sua expressão em células de microglia e avaliação do seu impacto na resposta inflamatória num modelo experimental de isquémia.

Assim, foram medidos os níveis de expressão da Argonauta-2 em células de microglia expostas a um período de privação de oxigénio e glucose e a lipopolissacarídeo, com e sem o silenciamento da proteína. Verificou-se um aumento dos níveis da proteína e um eficaz silenciamento da mesma após privação de oxigénio e glucose e estímulo inflamatório, em comparação com o controlo. Em seguida, foi quantificada a expressão de mediadores pro-inflamatórios produzidos pela microglia (fator de necrose tumoral alfa, interleucina-6 e oxido nítrico) em células com silenciamento da proteína num contexto inflamatório. Em situações inflamatórias, estes mediadores inflamatórios estão aumentados e após o silenciamento, verificou-se uma diminuição de todas estas moléculas mediadoras da inflamação, embora nem todas de uma forma significativa.

Em suma, os resultados obtidos sugerem um papel importante da Argonauta-2 nos processos subjacentes responsáveis pela modulação da resposta inflamatória e isquémica mediada por microRNA. O silenciamento da Argonauta-2 pode ser pertinente em novas abordagens terapêuticas, uma vez que pode resultar numa redução da inflamação, promovendo a reparação do tecido cerebral. No entanto, embora os resultados sejam promissores, há ainda um longo caminho a percorrer, pois será necessário futuramente mais investigação nesta área para que o uso da Argonauta-2 nesta patologia seja uma realidade.

Abstract

Stroke is the leading cause of death in Portugal, continuing to be associated with high rates of morbidity and potential years of life lost among cardiovascular diseases. Worldwide, it is estimated that every second a person suffers from this pathology.

Stroke is caused by a blockage of the cerebral blood supply (ischaemic stroke) or rupture of a blood vessel (haemorrhagic stroke) resulting in death or dysfunction of brain cells due to lack of oxygen and nutrients. After this lesion, local inflammation occurs with activation of cell brains, microglial cells, astrocytes and oligodendrocytes, and leukocyte recruitment. All these cells produce a variety of mediators that can, on the one hand, exacerbate tissue deterioration and, on the other, stimulate tissue repair. The neuroinflammation observed in ischaemia is modulated by microRNA, small RNA responsible for post-transcriptional gene regulation, through the cleavage of a target messenger RNA or the repression of its translation. In this process Argonaute-2, a vital component of the RNA-induced silencing complex with catalytic activity, binds with microRNA and identifies the target messenger RNA, resulting in gene silencing.

Based on the above, the goal of this project was to determine the role of Argonaute-2 in the microglial secretome by measuring its expression in microglia cells and evaluating its impact on the inflammatory response in an experimental model of ischaemia.

We used primary microglial cultures from mice, in which we measured the expression levels of Argonaute-2 in cells exposed to a period of oxygen and glucose deprivation and lipopolysaccharide. An increase in protein levels and its effective silencing was observed after oxygen and glucose deprivation and inflammatory stimulus, compared to the control. We then quantified the expression of pro-inflammatory mediators (tumour necrosis factor-alpha, interleukin-6, and nitric oxide) in cells with silencing of the protein in an inflammatory context and we detected that all molecules decreased, although not all of them in a significant way.

Thus, the results suggest an essential role of Argonaute-2 in the underlying processes responsible for the modulation of inflammatory and ischaemic response. However, more research is needed in this area, as Argonaute-2 may be a new target for potential therapeutic approaches.

Keywords

Argonaute-2; secretome; ischaemia; inflammation; microglia

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List of abbreviations

Ago	Argonaute
Ago2	Argonaute-2
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CT	Noncontrast computed tomography
DMEM-HG	Dulbecco's modified Eagle's medium-high glucose
dsRNA	Double-stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IL-23	Interleukin-23
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
miRNA	Micro ribonucleic acid
MMP-9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor k-light-chain-enhancer of activated B cells
NO	Nitric oxide
NO2-	Nitrite
NOS	Nitric oxide synthase
OGD	Oxygen and glucose deprivation
OGD/R	Oxygen and glucose deprivation with 24 hours of recovery
P/S	Penicillin/streptomycin
P-bodies	Cytoplasmic processing bodies
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
piRNA	Piwi-interacting ribonucleic acid
pre-miRNA	Micro ribonucleic acid precursor
RIPA	Radioimmunoprecipitation
RISC	Ribonucleic acid-induced silencing complex
RNAi	Ribonucleic acid interference
RT	Room temperature

SG	Stress granules
siAgo2	Short interfering ribonucleic acid for Argonaute-2
siRNA	Short interfering ribonucleic acid
TBST	Tris-buffered saline containing 0.05% Tween 20
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor

Chapter 1 – Introduction

Stroke overview

Globally, stroke is the second leading cause of death and a significant cause of disability [1]. One in four adults over the age of 25 will have a stroke in their lifetime. Annually, 15 million people suffer from stroke worldwide, according to the World Health Organisation, and 2/3 of those people die or become permanently disabled [2, 3]. In Portugal, the number one cause of death is stroke [4], affecting all ages, from neonates to older people, increasing the risk of occurrence with age. The incidence of stroke is declining in developed countries like Portugal, due to effective health programs that focus on lowering blood pressure and reducing smoking. Nevertheless, the overall incidence of stroke remains high because of population ageing [3].

Stroke is defined as an occlusion (ischaemic stroke) or by the rupture (haemorrhagic stroke) of a cerebral artery resulting in the sudden death of brain cells. Hence, there are two types of stroke, with the ischaemic being the most common [5]. Additionally, when the blood flow is blocked temporarily, a transient ischaemic attack (TIA) occurs. People that suffer a TIA have a higher risk to have a stroke within a year [6].

Ischaemic stroke is caused by acute occlusion of the blood vessels in the brain or neck, secondary to an embolism (cardioembolic or arteroembolic), thrombosis or stenosis of an artery in or leading to the brain [5, 7, 8]. If the occlusion occurs in a small artery following an atherothrombotic or lipohyalinosis occlusion, the term lacunar stroke is used [5]. Nowadays, the main causes of stroke are atherosclerosis and atrial fibrillation. However, other causes include small vessel disease, arterial dissection, infective endocarditis, cerebral vasculitis, reversible cerebral vasoconstriction syndrome, reversible cerebral vasoconstriction syndrome [9].

When an ischaemic stroke occurs, there is a deprivation of oxygen and glucose to areas supplied by the occluded vessel that causes energy depletion, excitotoxicity, and ion imbalances across the plasma membrane. Those events result in loss of cellular integrity and cell death by necrosis or apoptosis that leads to an inflammatory response that lasts for several days. Microglia, the primary innate immune cells of the central nervous system (CNS), rapidly react to those signals and become activated with macrophage-like capabilities, gaining the ability to weaken the blood-brain barrier (BBB) and facilitate the infiltration of circulating immune cells into the injured brain [10, 11]. Infiltrating

neutrophils release pro-inflammatory mediators, while infiltrating monocytes and macrophages perform a dual function, expressing both anti- and pro-inflammatory mediators [12]. Additionally, astrocyte and oligodendrocytes can release trophic factors like cytokines that contribute to inflammation. The excess of excitatory amino acids reactivates astrocytes into pro-inflammatory or immunomodulatory phenotypes that also regulate glial scar, interfering in this way with neuronal regeneration. The oligodendrocytes also suffer dysfunction leading to white matter loss, demyelination, and Wallerian degeneration. Pericytes are responsible for capillary constriction during stroke. In case of its death, this constriction is irreversible contributing to BBB dysfunction and infiltration of peripheral inflammatory cells to the brain parenchyma [9]. All these processes and cells contribute to the inflammatory response. Inflammation is considered a double-edged sword, albeit linked to poor clinical outcomes in CNS diseases when exacerbated [13]. So microglial cells, the initial responders to ischaemia, can play a harmful role, promoting inflammation (previously known as M1 microglia) or a protective role (M2), reducing inflammation [14]. Even though the initial function of microglial activation is the protection of neurons, overactivation of the microglia results in dangerous inflammation, which raises the risk of neuronal death [15].

When a patient is suspected of having a stroke, it is crucial to determine the time of the onset of symptoms or the last normal time seen, including risk factors, i.e. hypertension, diabetes, smoking, obesity, hyperlipidaemia, atrial fibrillation, and drug use [5]. Based on the artery that is affected, different symptoms are presented, and areas of the brain compromised. Common stroke symptoms in the left hemisphere consist of aphasia, right hemiparesis and right hemianopia, and in the right hemisphere, left hemispatial neglect, left hemiparesis and left hemianopia [7]. If the posterior circulation is compromised, symptoms such as diplopia, bulbar palsies, dysphagia, unilateral dysmetria and incoordination and reduced levels of consciousness might appear. In a cerebellar infarction, the patients present with ataxia, dysarthria, nausea, vomiting, and vertigo [5].

Noncontrast computed tomography (CT) is the standard method to determine the cause of the stroke, whether it is ischaemic or haemorrhagic. CT angiography is recommended to identify the occlusion of intracranial vessel and assessment of the extracranial vessels. Magnetic resonance imaging might be used for a minor stroke, where deficits are mild [5, 7].

'Time is brain', so fast diagnosis followed by effective acute treatment that restores an adequate blood flow is the key to success. Intravenous thrombolytic therapy (rt-PA) should be administrated in the first 4.5 hours or 9h in selected patients, excluding

contraindications. After thrombolytic treatment, the patient should be evaluated with the possibility of endovascular therapy within 6h or 24h in selected patients, with the help of the CT angiography. In the case of atrial fibrillation, anticoagulation is imperative. The recovery is prolonged, sometimes with the need for physical therapy and occupational therapy, allying with the prevention of risk factors, i.e. screening and monitoring for hypertension, diabetes, and hyperlipidaemia, smoking cessation, and weight loss. Secondary prevention is a combination of standard and target strategies depending on stroke aetiology. This prevention includes antiplatelet and anticoagulation, high-dose/high-potency statins and carotid endarterectomy for severe symptomatic carotid artery stenosis, without excluding the management of lifestyle risk factors [9]. The recurrence of a new stroke is high, and complications are common and include pain, falls, urinary tract infection, pneumonia, and bedsores. Furthermore, many patients remain disabled or have partial neurological deficits that affect their private and professional life [5, 16, 17].

To this date, the treatment of stroke is still minimal, and stroke continues to have high morbidity and mortality. Since the thrombolytic treatments have a narrow therapeutic time window and the endovascular therapy are only suitable for certain situations, not all patients with acute stroke meet the criteria to receive it. As a result, the available treatments continue to be ineffective to improve quality of life in the subacute to chronic phases of a stroke [14].

Argonaute-2

Argonaute-2 (Ago2) is a protein with a crucial role in gene-silencing pathways guided by small RNA [18]. The protein forms an RNA-induced silencing complex (RISC) with a small RNA that targets a complementary RNA and causes its cleavage, a process named RNA interference (RNAi). Argonaute proteins (Ago) mediate gene silencing pathways in a transcriptional and post-transcriptional level [15].

Ago can be classified into two groups: The Ago and Piwi subfamilies [19]. The Ago subfamily is ubiquitously expressed, whereas the Piwi subfamily is restricted to germ cells. Humans express eight family members, with Ago2 being one of the most important given its catalytic activity. Ago proteins are localised in the cytoplasm of somatic cells and cytoplasmic processing bodies (P-bodies). Ago2 was also found in the nucleus and stress granules (SG) - structures induced by cellular stress containing mRNA-protein complexes [18]. Evidence shows a re-localisation of Ago2 to SG after treatment of cells with substances that induce oxidative stress. This accumulation did not occur in P-bodies. Induction of cell stress translocated Ago2 from an active site (Ago2 is in an active

form) to an inactive site (Ago2 in an inactive form) which includes SG, increasing metabolic stability and decreasing RNAi [20]. These suggest that upon induction of cellular stress, a process also seen on ischaemia, there is a decrease of RNAi-induced gene silencing activity.

Ago2 has a molecular weight of about 100 kDa and is composed of the following structural features: the PAZ domain, the MID domain, and the PIWI domain. The PAZ Domain forms a specific binding component for the characteristic 2-nucleotide(nt) 3′ overhangs produced by RNase III-type enzymes such as Dicer. This domain is required for Ago2 to form the RISC complex. The MID domain has a highly basic pocket, which links the characteristic 5′ phosphate of small RNA attaching, in this way, the small RNA onto Ago2. The PIWI domain folds similar to RNase H (endogenous enzyme that cleaves the RNA strand of an RNA–DNA duplex) and gives the protein the ability to cleave substrate RNA complementary to the bound small [18, 21].

Ago2 performs its functions by using its catalytic activity to initiate degradation of target mRNA and translation repression of target genes in four ways (i) competing with the translation initiating factor of target mRNA to prevent the attachment of ribosomes and the initiation of translation; (ii) blocking the formation of 80s ribosomes from 60s and 40s ribosomes; (iii) inducing deadenylation, uncapping and degradation of target mRNA (iv) combining with other AGO proteins such as Ago1 or Ago3 to form a complex that prevents peptide extension during translation. In heterochromatin, Ago2 inhibits target gene expression in a mechanism that is RNAi independent. In addition to all these functions, Ago2 can also act as RNA slicer in Dicer independent way and a regulator of miRNA maturation [22]. With all these functions, Ago2 was revealed to be necessary for embryonic development, cell differentiation, and stem cell maintenance as well as being involved in angiogenesis, and metastasis in different cancer types [21, 22].

Small RNA

Small RNA are essential for Ago2 functions and include Piwi-interacting RNA (piRNA), short interfering RNA (siRNA) and microRNA (miRNA) [18].

piRNA are the longest small non-coding RNA with 2′-O-methyl modification sites at the 3′ end and uridine at the 5′ end. piRNA are transcribed from piRNA clusters in a Dicer-independent process. piRNA precursors can be processed in two ways before being exported to the cytoplasm: cleavage by Zucchini, an endonuclease anchored to mitochondria and ping-pong processing. In the ping-pong process, piRNA can amplify the silencing signal. piRNA bound with Piwi proteins (Aubergine, Piwi and Ago3) to be

trimmed and methylated, resulting in a mature PIWI-piRNA-induced silencing complex that is transferred to the nucleus. These complexes target RNA through base pairing, and by the endonucleolytic slicer activity of the Piwi proteins, the complementary RNA is cleaved. piRNA are necessary to preserve normal gametogenesis and reproduction in animal germ cells since they silence transposons (mobile genetic elements) and prevent genetic elements from integrating into the host genome [23].

siRNA are a short double-stranded RNA (dsRNA) with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides. The longer double-stranded RNA is processed and cleaved into siRNA by Dicer, having two nucleotides overhangs on the 3′ end of each strand. siRNA are bound to RISC, and both strands are separated, integrating the more stable 5′-end to the active RISC complex. The guide strand then guides and aligns the RISC complex on the target mRNA, and by the catalytic action of Ago2, the messenger RNA (mRNA) is cleaved. Through transfection, siRNA can be incorporated into cells using liposomes [24]. Synthetic siRNA can be a vital drug targeting, to target dominant oncogenes as well as viral oncogenes that are involved in carcinogenesis, or even map functionally regulated oncogenes. Also, siRNA can silence essential molecules for tumour-host interactions and tumour resistance to chemo and radiotherapy [24].

miRNA have 20 to 25 nucleotides and perform crucial roles in the normal and neuropathological brain. miRNA genes are transcribed by RNA polymerase I and II and processed by RNase III enzyme Drosha to a miRNA precursor (pre-miRNA). Drosha leaves two nt 3′ overhangs and 5′ phosphate groups. Pre-miRNA are further transported to the cytoplasm via the export-receptor Exportin-5. Both long dsRNA and secondary fold-back structures such as hairpin are recognised and processed by the cytoplasmic Dicer. Dicer binds and cleaves dsRNA preferentially from the ends. Both strands of such dsRNA are separated, and one strand is incorporated into RISC and binds to Ago2. Ago2 uses the miRNA as a guide to identify complementary target mRNA leading to its silencing by degradation or sequestration [21]. Pro-inflammatory, anti-inflammatory, and mixed immunomodulatory miRNA regulate neuroinflammation not only in ischaemic stroke but also in several diseases like Alzheimer's disease, multiple sclerosis, and spinal cord injury. In ischaemic stroke progression, besides microglial activation, miRNA target BBB disruption, oxidative stress, excitotoxicity, and caspase-mediated cell death signalling [25].

Aims

Ago2 is the only member of AGO family with catalytic activity capable of directly initiating the degradation of target mRNA and is a crucial element of the RISC complex. Thus, Ago2 can initiate target mRNA degradation, stop target gene translation, and regulate miRNA quantity, function, maturation and stability [22]. When Ago2 is reduced/increased, a decline/rise of overall mature miRNA expression levels is seen, leading to disruptions in cellular function [26].

Since Ago2 participates in essential steps in gene silencing and modulates the miRNA-associated inflammatory response, it becomes necessary to understand its role in microglia, cells critical in an inflammatory response in ischaemia conditions. There is evidence that stroke changes the profiles of Ago2-bound miRNA in neural progenitor cells and that genes targeted by miRNA are essential for neurogenesis [27]. Additionally, studies revealed an over-expression of Ago2 in carcinomas that influence tumour cell growth and the overall survival of patients [22]. For this reason, Ago2 can emerge as a potential new therapeutic target for ischaemic stroke due to his role in gene-silencing pathways and confirmed influence in other diseases.

Considering the previous findings, our goal is to measure Ago2 expression in microglial cells and assess the impact of its silencing on the inflammatory response in an experimental ischaemic model.

Chapter 2 – Materials and methodology

All experiments were performed following protocols approved by national ethical requirements for animal research, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU) for the care and use of laboratory animals.

Primary microglia culture

3-5 days old C57BL/6 mice were euthanised by decapitation and brains were removed in 0.15M phosphate-buffered saline (PBS) The cortical hemispheres were dissected, and meninges and cerebellum were removed. The tissue was chopped and transferred to a test tube with 5 ml of medium. Each test tube contained three brains. The medium was composed by Dulbecco's Modified Eagle's medium-high glucose (DMEM-HG), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The tissue was mechanically dissociated by repeated trituration through a graduated pipette first and then a micropipette. The mixture was filtered and then centrifuged at 230 g for 10 min. The supernatant was discarded, and 5 ml of the new medium was added. The content was transferred to a culture flask previously coated with 0.01 % poly-L-lysine, and final medium volume was 15 ml in flasks. The seeded flasks were kept at 37°C in a 95% atmospheric air and 5% CO_2 humidified atmosphere. The media were replaced every two days, for nine days. To separate microglia cells from astrocytes, the cultured flasks were gently shaken (7h at 37°C) and the microglia harvested from the bottom-attached astrocytes. The medium was transferred to a tube test and centrifuged at 250 g for 10 minutes at room temperature. The microglial cells were plated onto 6-well coated with 0.01% poly-L-lysine.

Cell Treatment with siAgo2

The siAgo2 was added to microglial cells in order to silence the expression of protein Ago2. We performed a screening of the siRNA and exposed the cells to several time conditions in a 6-well with 2 ml each. Media (DMEM-HG) and 2 µg Lipofectamine 2000 (Invitrogen, Life Technologies, USA) was added to every well with 0.05 µM siAgo2 and cells were exposed for different periods, i.e. 6h, 12h, and 24h. In parallel, cells were treated with 2 µg lipofectamine 2000 and 0.05 µM scramble siRNA. The samples were mixed for 5 minutes in the vortex and added to the microglial cells after 30 minutes. Six hours was the period of exposure that showed a more considerable reduction in Ago2 levels, so, for this reason, this time point was selected for subsequent studies.

Cell treatment with lipopolysaccharide (LPS)

As a positive control, microglia cells were treated with 100 ng/ml of LPS for 24h and incubated at 37ºC. In cells subjected to silencing of Ago2's expression, the LPS was added 30 minutes before the treatment with 0.05 µM siAgo2 for 6h.

Oxygen and glucose deprivation (OGD)

To mimic the ischaemic environment, the samples were kept in a MIC-101 modular incubator chamber (Billups-Rothenberg Inc., Del Mar, California, USA) with a 5% CO_2 and 95% N_2 gas environment (0.1% O_2) for 1h and the medium was replaced with PBS. After that, the samples recovered for 24h with complete media (DMEM-HG).

Western Blotting (WB)

The samples were harvested with radioimmunoprecipitation (RIPA) lysis buffer (0.15MNaCl, 0.05MTris, 5mM ethylene glycol tetraacetic acid, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate, and 10mM dichlorodiphenyltrichloroethane) with proteinase inhibitors (Roche Diagnostics Ltd., Mannheim, Germany). The total protein from cell lysates was quantified using the BCA Protein Assay Kit (Thermo Scientific, USA). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Mini-PROTEAN® Tetra Handcast, Bio-Rad, CA, USA), at 120 V for 2h with a Tris-glycine running solution (pH 8.3; Acros Organics, Geel, Belgium) and then transferred to polyvinylidene fluoride membranes (GE Healthcare, Little Chalfont, UK) through semi-dry transfer (Trans-Blot® Turbo™ Blotting System, Bio-Rad) at 25 V for 30 min in a Towbin transfer buffer (25mM Tris, 192mM glycine, and 20% methanol; pH8.3). To block non-specific binding, membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST; Sigma-Aldrich) and 0.1% gelatine (Sigma-Aldrich) for 1h, at room temperature (RT). The membrane was incubated with primary antibodies for 1h at RT and overnight at 4ºC. The primary antibodies used were rabbit anti-Ago2 (1:1000; Cell Signalling, MA, USA), rabbit anti-mouse TRAF (1:1000; Thermo Scientific) and mouse anti-tubulin (1:5000; Sigma-Aldrich) as housekeeping control. The membrane was then incubated for 1 hour at RT with respective secondary antibodies. The secondary antibodies used were goat anti-rabbit (1:5000) and goat anti-mouse (1:10000). In between every change of solution, the membrane was washed with TBST. The membrane was revealed using a ChemidocMP imaging system (BioRad) and quantified using the software ImageJ software (NIH).

Enzyme-Linked Immunosorbent Assay (ELISA)

The samples were processed using a Human Protein Argonaute-2 (Ago2) ELISA Kit, as described in the manual. 50 µl Standard was added to corresponding standard wells and 50 µl Sample to every sample wells. Then 100 µl HRP-Conjugate Reagent was added to every well, and the plate was covered with a closure plate membrane and incubated for 60 minutes at 37°C. All the wells were washed four times with a 20×Wash Solution. 50 µl Chromogen Solution A was added to every well first and then 50 µl Chromogen Solution B to every well. The plate was mixed and incubated for 15 minutes at 37°C. 50 µl Stop Solution was added to every well, and the optical density was read at 450 nm using an ELISA reader within 15 minutes after adding the stop solution.

Griess assay

Media were processed using the Griess Reagent System (Promega, Madison, WI, USA) as described in the manual. A nitrite standard reference curve was prepared using 0.1M sodium nitrite with a concentration from 0–100µM in each well. Griess reagents were added to each well: 50 µl of 1% sulfanilamide in 5% phosphoric acid at room temperature for ten minutes, protected from light, and 50 µl 0.1% N-1-naphthyl ethylenediamine dihydrochloride at room temperature for ten minutes, protected from light. Nitric oxide (NO) production was determined by the formation and accumulation of nitrite (NO_2^-) by measuring the absorbance within 30 minutes in a plate reader with a filter between 520nm and 550nm. The total amount of protein was quantified using the bicinchoninic acid assay (Thermo Scientific).

Statistical analysis

Statistical significance was determined using the software GraphPad Prism 8 for Windows. The samples were independent and randomly chosen, and experimental conditions were performed in duplicate in three sets of independent experiments (n), unless otherwise specified. 1-way ANOVA followed by Turkey's Multiple Comparison Test was used to compare means with $p<0.05$ considered to represent statistical significance. All data are expressed as means ± standard error of mean (SEM) [28].

Chapter 3 – Results

Microglial cells were exposed to 0.05 μM siAgo2 for different periods of time (6h, 12h, and 24h) to downregulate Ago2. The expression of Ago2 was quantified and confirmed using WB.

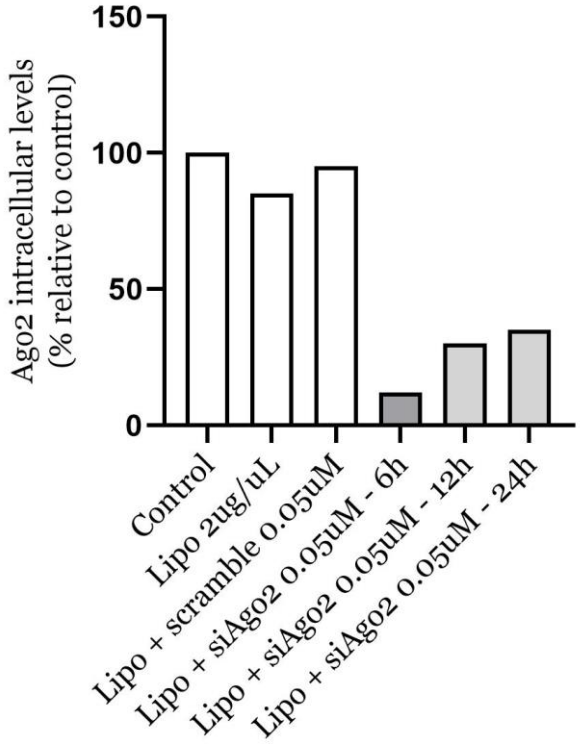

Figure 1- Screening of siAgo2 for 6h, 12h, and 24h. Microglial cells treated with 0.05 μM siAgo2 for 6h showed lower levels of Ago2 protein (n=1).

In both periods, a reduction of Ago2 protein levels was seen, suggesting an effective expression silencing of the protein (**Figure 1**). An evident decrease of Ago2 protein levels was observed at 6h. Therefore, this time duration was selected to be used in subsequent experiments.

Following the siAgo2 screening, microglial cells were exposed to specific stimuli, lipopolysaccharide (LPS, 100 ng/ml), or oxygen and glucose deprivation (OGD). LPS can activate the immune system, stimulating the release of inflammatory cytokines in various cell types, leading to an acute inflammatory response [29], while OGD mimics ischaemia conditions with a 24h recovery (OGD/R), where microglia are activated releasing both pro and anti-inflammatory mediators [11].

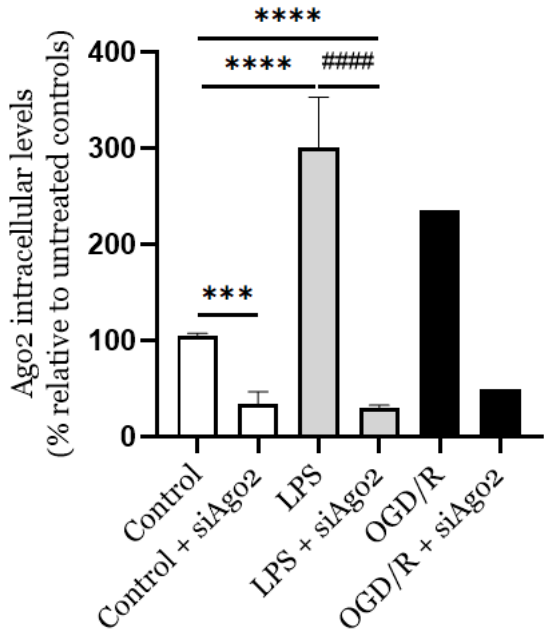

Figure 2- Quantification of Ago2 levels under control, OGD/R, and LPS conditions, with and without siAgo2 treatment (n=1-7). The expression of Ago2 was increased in ischaemic brain tissue (OGD/R) (n=1) and LPS-treated cells (n=5). The silencing of Ago2 caused a decrease of its intracellular levels in both conditions. ***p<0.001, ****p<0.0001, ####p<0.0001 using 1-way ANOVA followed by Turkey's Multiple Comparison Test.

The quantification of Ago2 protein levels was expressively superior in ischaemic cells and LPS-treated cells (LPS=300.13±53.13; ****p<0.0001 comparing to control (n=7; untreated cells) (**Figure 2**). In cells with siAgo2 treatment, both control cells (control+siAgo2=33.97±12.72; ***p=0,0002; n=3) and cells stimulated with LPS (LPS+siAgo2= 29.96±2.72; ####p<0.0001; ****p<0.0001; n=4), the Ago2 levels were significantly decreased in comparison with cells in the same conditions without treatment. In the end, the Ago2 protein levels in LPS+siAgo2 and control+siAgo2 was similar. These results indicate an effective silencing of Ago2 expression. In an ischaemic environment with siAgo2 (OGD/R+siAGo2), Ago2 experienced a considerable reduction in its intracellular levels, suggesting an effective silencing in this situation too.

To further characterise the effect of Ago2 in inflammatory conditions, we also evaluated the expression of inflammatory markers. When microglial cells are exposed to LPS, the neurotoxic form of microglia is activated and produces pro-inflammatory cytokines, such as IL-6, TNF-α, free radicals like NO and other metabolites [14]. Subsequently, we began by quantifying the levels of three pro-inflammatory markers (IL-6, TNF-α, NO) produced by microglial cells in a physiological and in an inflammatory context.

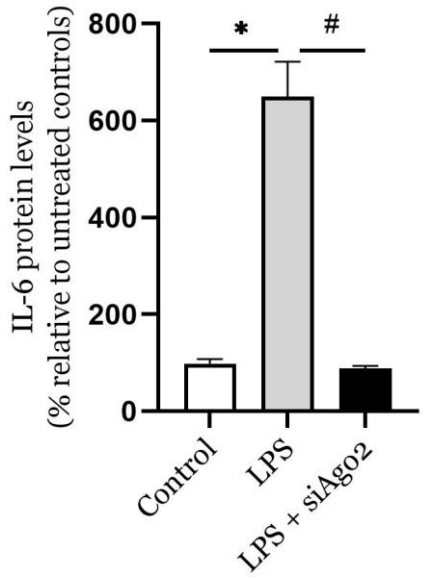

Figure 3 - Quantification of IL-6 after Ago2 silencing. The expression of IL-6 was significantly lower in cells with silencing of Ago2 exposed to an inflammatory condition (n=3). *p<0.05; #p<0.05 using 1-way ANOVA followed by Turkey's Multiple Comparison Test.

Quantification of IL-6 revealed that its levels, on Ago2 silenced cells exposed to an inflammatory stimulus, was significantly lower than in cells only exposed to LPS (Figure 3) (LPS + siAgo2= 88.39 ± 5.49; #p= 0.0306). IL-6 was considerably higher in LPS-stimulated cells comparing to control (LPS= 649.38 ± 72.58; *p = 0.0303).

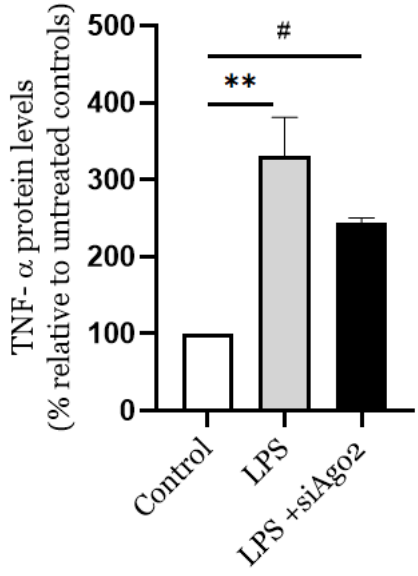

Figure 4 - Quantification of TNF-α after Ago2 silencing. A reduction of TNF-α was seen in siAgo2- treated cells in an inflammatory response (n=3). **p<0.01; #p<0.05 using 1-way ANOVA followed by Turkey's Multiple Comparison Test.

In an inflammatory context, quantification of TNF-α in cells with silencing of Ago2 had no statistically significant differences in comparison with the positive control (**Figure 4**). However, as expected, TNF- α levels were higher in LPS-stimulated cells (LPS= 330.20 ± 50.81; **p= 0,0045) than in LPS+siAgo2-stimulated cells and control. In comparison with control, LPS+siAgo2 showed a significant increase of the TNF-α (LPS+SiAgo2= 244.41 ± 5.86; #p= 0,0405), suggesting that the silencing of Ago2 might not have an impact in TNF- α.

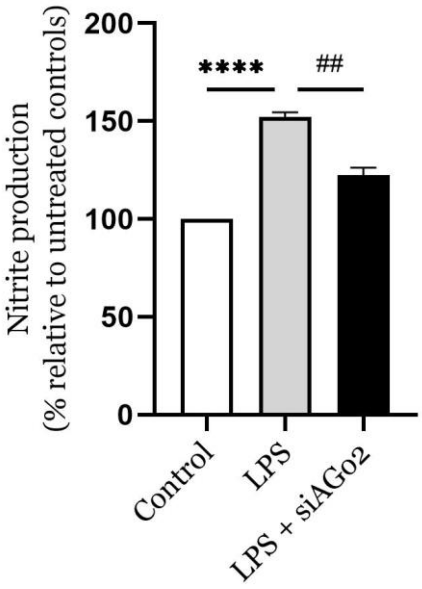

Figure 5 - Quantification of NO production after Ago2 silencing. The silencing of Ago2 decreased the production of NO after an inflammatory stimulus (n=5). ****p<0.0001; ##p<0.01 using 05 using 1-way ANOVA followed by Turkey's Multiple Comparison Test.

By measuring nitrite production, we demonstrated a significant reduction of NO in the cells were Ago2 was silenced comparing to the positive control (**Figure 5**) (LPS + siAgo2= 152.154 ± 3.92; ##p=0.0047), Additionally, LPS-stimulated cells produced significantly more NO than control (LPS= 122.40 ± 2.46; ****p<0.0001).

Chapter 4 – Discussion

Although, worldwide, stroke is a significant cause of death and disability, the available treatments are still inefficient, benefiting a small percentage of patients and producing severe side effects. Stroke activates inflammatory mechanisms trigged by several brain cells, being microglial cells an early intervenient. In this way, these cells can affect the pathophysiology of the disease and impact its outcome. Microglia cells are activated in defence of brain damages, acting as phagocytic cells that release inflammatory mediators like cytokines, chemokines, and reactive oxygen species, disrupting neural cells and the BBB. Those mediators can be pro- and anti-inflammatory, neuroprotective, and neurotoxic microglia, respectively. Briefly, neuroprotective microglia release anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13) and secrete remodelling factors, such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF), and matrix metalloproteinase-9 (MMP-9). These substances facilitate anti-inflammatory processes, axonal outgrowth, and angiogenesis after ischaemia that improve stroke outcome and promote brain repair. Neurotoxic microglia secrete pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) interleukin-12 (IL-12), interleukin-23 (IL-23), reactive oxygen and nitrogen species, and proteases, which exacerbate inflammation and tissue injury by disruption of neuronal function/synaptic transmission and neuronal oxidative stress/degeneration [14]. All these processes contribute to promote brain damage and poor outcome. During a stroke, the balance between neuroprotective and neurotoxic microglia is affected, and microglial activation phenotypes can transit between an initial beneficial neuroprotective phenotype, followed by a detrimental neurotoxic phenotype later on [30]. In this context, specific molecules that mediate mechanisms that modulate microglia response to adverse conditions like ischaemia will facilitate the development of new therapies capable of improving stroke outcome. Therefore, this present work tried to determine the role of Ago2 in microglia and its influence in an inflammatory response.

In this study, we measured the expression of Ago2 in microglial cells exposed to an inflammatory and ischaemic stimulus. Through ELISA and Griess Assay, we quantified the levels of critical pro-inflammatory markers in an inflammatory scenario and the impact of the silencing of Ago2 protein in these quantifications.

For the first time, an elevation of Ago2 on an ischaemic context in microglia cells was demonstrated, suggesting a crucial role of this protein in this situation. These results have only been performed once, and for that reason, we are unable to make any definitive conclusions. An overexpressed level of Ago2 indicates an effect in miRNA function and maturation during ischaemia, since Ago2 is a crucial regulator of this process. These findings are consistent with studies that demonstrated that during ischaemia/reperfusion, the expression of several miRNA is altered, and consequently alteration in mRNA related to cytokines, transcription factors, heat shock proteins, ion channels and neurotransmitter receptors in an ischaemic brain [31]. A single miRNA in combination with Ago2 can potentially target thousands of different mRNA, and multiple miRNA can target one mRNA. miRNA guide Ago2 to target complementary mRNA, and when this complementary is ideal, Ago2 uses its endonucleolytic cleavage capacity to interfere with translation or induce deadenylation of target mRNA [21]. miRNA was proved to be essential in microglial activation, expression of pro- or anti-inflammatory genes and other mediators, neuronal damage, and differentiation/polarisation of microglia. By manipulating specific miRNA, we can achieve beneficial results like inhibiting microglial activation that prevents neuronal damage, decrease pro-inflammatory mediators produced by microglia and induce anti-inflammatory genes, downregulate neurotoxic microglial phenotypic markers and upregulate neuroprotective microglial phenotypic markers, reduce brain oedema and cerebral infarction area [32]. It is possible that the process of gene silencing and modulation of miRNA-associated inflammatory response is intensified in an ischaemic situation. For this reason, it is crucial to understand if the augmentation or the reduction of Ago2 in ischaemic cells, can activate miRNA which function would enhance or worsen the stroke outcome. To improve stroke outcome, the regulation of Ago2 levels must lead to a decrease in inflammation and, posteriorly, a decline in brain damage and death cells. In this way, it could be useful to observe whenever cell death could be reduced when Ago2 levels are lowered too.

In the same way, an elevated level of Ago2 protein was observed for the first time in microglial cells exposed to an inflammatory stimulus. Inflammation is an underlying process in stroke, and miRNA control inflammation initiation and maintenance. Due to this elevated quantification of Ago2 in inflammation and ischaemic scenarios, we can also imply a protective role of Ago2. The overexpression of Ago2 could mean a late response from microglial cells as a control measure to decrease inflammation by activation of anti-inflammatory miRNA. Anti-inflammatory miRNA reduce the availability of mRNA responsible for coding inflammatory receptors, signalling

mediators, and cytokines., decreasing, in this way, inflammation by a negative feedback mechanism [25]. However, these results are insufficient to determine if Ago2 is associated preferably with a neuroprotective microglial phenotype or a neurotoxic microglial phenotype, since the Ago2 levels are similar in LPS and OGD/R condition. Although LPS stimuli is used mainly as an activator of microglial reactivity and pro-inflammatory responses, it also produces a mixed inflammatory outcome like in an ischaemia environment [29].

Noteworthy, when the Ago2 is silent in control, LPS and OGD/R condition, the microglial cells were still viable, so other feedback mechanisms must be present. A possible explanation could be that Ago1, Ago3 or Ago4 may assume Ago2 function, although they appear to lack its catalytic activity. However, some studies suggest that Ago1, Ago3 and Ago4 may be involved in RNA-mediated processes too [33].

Regarding the importance of inflammatory cytokines in the pathophysiology of stroke, we considered it necessary to study the variation in quantity of TNF-α, IL-6 and NO to approach the role of Ago2 in an inflammatory context. These molecules are pro-inflammatory markers that provoke and aggravate an inflammatory response after stroke.

The first marker, IL-6, was considerably reduced in cells treated with siAgo2 when exposed to an inflammatory stimulus. IL-6 is released by different brain cells, microglia included. As a mediator of the inflammatory process, it increases the migration of leukocytes and regulates the expression of chemokines and adhesion molecules, being a mediator in neurovascular dysfunction, neurodegeneration, and neuritis. This cytokine also has a role as a neurotrophic factor in the late phase of cerebral ischaemia by enhancing genes associated with post-stroke angiogenesis [34, 35]. This finding suggests that Ago2 is implicated in pathways responsible for exacerbating inflammation by recruitment of inflammatory cells and an increase in inflammatory mediators, that consequently contribute to early neurological deterioration. The significant reduction of IL-6 levels observed when Ago2 is silent might be useful to develop a potential therapeutic target.

The reduction of NO in cells where Ago2 was silent is a good indicator of the influence that this protein has in its expression. NO is a key radical produced by oxidative stress. L-arginine is converted by three different isoforms of nitric oxide synthetase (NOS) into NO. This marker has both beneficial and detrimental roles in cerebral ischaemia, i.e. restoring blood supply to the ischaemic area, reducing brain damage but also lipid

peroxidation, cellular toxicity and cell death by the rise of reactive oxygen and nitrogen species, increase of infarct volume, inhibition of DNA replication enzymes and upregulation of inflammatory mediators [34]. In pathological conditions like neuroinflammation, activated microglial cells expressed inducible NOS (iNOS) and the regulation of this isoform happens in a transcriptional level by NFκB [36]. Thus, Ago2 could be implicated in NFκB-mediated iNOS expression since some studies have reported that Ago2 also functions in the nucleus and could interfere in the transcriptional process [21].

Concerning TNF-α, its decrease on microglial cells with silencing of Ago2 in an inflammatory response compared to control was not significant, suggesting the Ago2 might not participate in the expression of this mediator. TNF-α is mainly produced by astrocytes and microglia. This cytokine can exacerbate stroke pathology by BBB disruption, oedema formation, accumulation of neurotransmitters and irons, alterations in infarct volume, release of neurotoxic mediators and enhance of apoptosis. It also has beneficial roles like inducing cerebral tolerance to hypoxia and ischaemia, modulates irons levels and neuronal plasticity, and stimulating cerebral microvasculature repair, anti-apoptotic factors, and anti-oxidant [34, 35]. TNF-α also stimulates the production of other inflammatory cytokines such as IL-6 via nuclear factor k-light-chain-enhancer of activated B cells (NFκB), a major regulatory transcription factor that induces genes involved in inflammation [37]. A significant reduction was observed in IL-6 in opposition to what was observed in TNF-α, and the NFκB pathway can explain this different effect. When cytokines like TNF-α stimulate cells, NFκB is activated and translocated into the nucleus to promote the expression of the gene encoding IL-6 [38]. In its turn, NFκB signalling pathway can be inhibited or activated by specific miRNA. In this manner, Ago2 associated with these miRNA can control the activation of this transcriptional factor [39]. Considering our results, the silencing of Ago2 could suppress the activity of NFκB-dependent regulation of the IL-6 pathway, resulting in the significant reduction of IL-6 levels but not TNF-α. Further investigation is needed to study the effect of Ago2 in the NFκB pathway.

Another important point should be discussed. One study demonstrated that some miRNA are predominantly associated with Ago2, that are originated from sense strands of the corresponding pre-miRNA: miRNA-451, miRNA-30, miRNA-21, miRNA-92, miRNA-99, miRNA- 183, miRNA- 27, miRNA-151, miRNA- 30, miRNA-182, miRNA-25 [40]. miRNA-21 and miRNA-27 are some of the most important miRNA in neuroinflammation. miRNA-27b is a pro-inflammatory miRNA that targets an anti-inflammatory transcriptional activator in human macrophages, PPAR-γ. This receptor is

disrupted in some neuroinflammatory diseases, as well as miRNA-27b is over-expressed in neuroinflammatory conditions. By interacting with PPAR-γ, miRNA-27b blocks the induction of an anti-inflammatory phenotype. In studies where miR-27b is inhibited, in an LPS condition, the production of inflammatory cytokines including IL-6 and TNF-α by macrophage, is reduced. Therefore, this miRNA might induce the production of these two cytokines. On the contrary, miRNA-21 is an anti-inflammatory miRNA, upregulated in activated immune cells, including macrophages. miRNA-21 inhibits PDCD4, responsible for increasing IL-6 and decreasing IL-10. In stroke, mRNA-21 is over-expressed and protects neurons from apoptosis, directly or by reducing microglial toxicity [25]. Although these miRNA are described in macrophages, we can assume that they induce the same effect in microglial cells, since microglia cells are considered the resident macrophage within the CNS and share similarities. Considering this information, in our research, we can speculate that miRNA-27b must be active and associated with Ago2 in an inflammatory stimulus, since in cells with silencing of Ago2 a significant reduction of IL-6 was seen, suggesting an inhibition of miRNA-27b.

To summarise, Ago2 is a limiting factor that conditions RISC activity, so an alteration like the silencing or overexpression of this protein compromises RISC activity and RNAi. In this way, RISC activity is increased in an ischaemic and inflammation context. Therefore, our research supports the relevant role of Ago2 in ischaemia and inflammation context, being implicated in the pathways that lead to the production of important inflammatory mediators.

Chapter 5 – Conclusion and Future Perspectives

In conclusion, the preliminary data collected appears to enable the possibility of an important role of Ago2 in microglia cells. An over-expression of this protein was seen in ischaemic cells, a situation where neuroinflammation is present. So neuroinflammation is related to elevated intracellular levels of Ago2. Also, a reduction of its intracellular levels led to a reduction of pro-inflammatory mediators in microglial cells exposed to an inflammatory stimulus. These last results were only obtained in inflammatory conditions and not in ischaemia conditions. Although we could predict that in an ischaemic scenario, the data will behave similarly, more studies need to be developed to portray the effect of Ago2 in pro-inflammatory mediators in this context to consider new therapeutic targets.

As future perspectives, specific topics could be further analysed in order to clarify some aspects that this study did not have the opportunity to elaborate on. Firstly, it would be essential to increase the sample size and understand if the descending of TNF-α is statistically significant. Secondly, assess the effect of the Ago2 in the level of anti-inflammatory mediators like Arg-1 and IL-4 and determine if this protein can preferably promote a neuroprotective or neurotoxic phenotype might be a pertinent point to study. Thirdly, the evaluation of silencing the Ago2 by immunocytochemical studies to quantify the expression of iNOS could be relevant to study the implication of Ago2 in NFκB-mediated iNOS expression and the implication of Ago2 in NFκB-associated regulation of IL-6. Moreover, miRNA implicated in ischaemia and inflammation in microglial cells could be further assessed and determine which of those are associated with Ago2.

To the best of our knowledge, this was the first time Ago2 was studied in microglial cells in these conditions. We can hypothesise that Ago2 has an impact in pro-inflammatory miRNA that reduce the availability of mRNA whose function is to inhibit activation of pro-inflammatory pathways. As a result, these miRNA amplify pro-inflammatory signalling cascades. Consequently, the reduced expression of Ago2 could be beneficial to control inflammation and induce cell regeneration. On the other hand, Ago2 could also be implicated in the anti-inflammatory mechanism to protect microglial cells exposed to danger.

Considering the promising results and the outcomes of stroke, further investigation in this topic would be an excellent contribution to the future.

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