The role of miRNA-9 and miRNA-29 and their specific strands (-5p and -3p) in Alzheimer's disease

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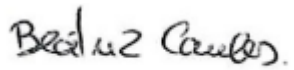

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Resumo

Os microRNAs (miRNAs) são pequenas moléculas de RNA de cadeia simples não codificantes que apresentam tipicamente 20 a 25 nucleótidos de comprimento. Estes desempenham um papel crucial na modulação de vários processos biológicos através da regulação da expressão genética a nível pós-transcricional. Devido ao seu papel biológico têm sido estudados no contexto de diagnóstico e tratamento de doenças neurodegenerativas, particularmente na doença de Alzheimer (DA) como biomarcadores ou potenciais agentes terapêuticos. A DA, classificada como a forma mais comum de demência, é uma doença neurodegenerativa irreversível com alta prevalência em idosos. Esta doença é caracterizada pela acumulação de peptídeos amiloides β (Aβ) e proteína Tau hiperfosforilada. Vários estudos têm demonstrado o efeito promissor dos miRNAs para silenciar proteínas desreguladas da via amiloide, responsáveis pela progressão da DA. Neste sentido, esta dissertação pretende estudar o efeito dos miRNA-9, e -29b, das suas cadeias específicas (-5p e -3p), bem como das suas formas precursoras (pre-miRNA-9-1 e pre-miRNA-29b-1), sobre as proteínas relacionadas com a DA, nomeadamente a APP, BACE1 e PS1. Para proceder a esta avaliação, as diferentes formas de miRNA foram encapsuladas em nanopartículas de quitosano e entregues na linha celular N2a695, um modelo *in vitro* da DA. Após a extração do RNA total das células, os níveis de mRNA das proteínas-alvo foram avaliados por reação em cadeia da polimerase em tempo real (qPCR). Este estudo demonstrou um potencial efeito de silenciamento por parte do miRNA-9 sobre os níveis de mRNA da PS1 e da BACE1, sendo o pre-miRNA-9-1, via miRNA-9-1-5p como sugerem os resultados, o mais promissor com quase 50% de silenciamento na expressão de mRNA da BACE1. Este trabalho demonstra que a compreensão do mecanismo pelo qual os miRNAs podem silenciar as proteínas envolvidas na via amiloide é crucial, devido ao potencial destes pequenos RNA como novas ferramentas terapêuticas para tratar ou atrasar a progressão da DA.

Palavras-chave

Doença de Alzheimer; Via Amiloidogénica; miRNA-9-1; miRNA-29b-1; Silenciamento Genético.

Abstract

MicroRNAs (miRNAs) are small non-coding, single-stranded RNA molecules, typically with 20-25 nucleotides in length. They play a crucial role in modulating several biological processes by regulating gene expression at the post-transcriptional level. Due to their biological role, their application is being evaluated in the context of diagnosis and treatment of neurodegenerative diseases, particularly Alzheimer's disease (AD), as biomarkers or potential therapeutic agents. AD, classified as the most common form of dementia, is an irreversible neurodegenerative disease with a high prevalence in elderly people. This disease is characterized by the accumulation of amyloid β (Aβ) peptides and hyperphosphorylated Tau protein. Several studies have demonstrated the promising effect of miRNAs for silencing the dysregulated proteins involved in the amyloid pathway, which are responsible for AD progression. With this in mind, this work aims to study the effect of miRNA-9, -29b, their specific strands (-5p and -3p), and as well their precursor forms (pre-miRNA-9-1 and pre-miRNA-29b-1) on the proteins related with AD, such as APP, BACE1 and PS1. To proceed with this evaluation, the different forms of miRNA were encapsulated in chitosan nanoparticles and delivered in the N2a695 cell line, an AD *in vitro* model. After extraction of the total RNA from the cells, the mRNA levels of the target proteins were evaluated by Real-Time Polymerase Chain Reaction (qPCR). This study demonstrated a potential silencing effect by miRNA-9 on the mRNA levels of PS1 and BACE1, with pre-miRNA-9-1 via miRNA-9-1-5p being the most promising, as it induced the silencing of BACE1 mRNA in almost 50%. In conclusion, understanding how miRNAs can silence proteins involved in the amyloid pathway is crucial because these small RNAs have the potential as new therapeutic tools for treating or controlling AD progression.

Keywords

Alzheimer's Disease; Amyloid Pathway; miRNA-9; miRNA-29b; Gene Silencing.

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List of Acronyms

3' UTR	3' untranslated region
AChEIs	Acetylcholinesterase Inhibitor
AD	Alzheimer's disease
AGO	Argonaut Protein
AICD	APP Intracellular Domain
APLP2	Amyloid Precursor-Like Protein 2
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
ASOs	Antisense Oligonucleotides
Aβ	Amyloid beta
BACE	β-Secretase
CDK5	Cyclin-Dependent Kinase 5
cDNA	complementary DNA
circRNAs	Circular RNA
CNS	Central Nervous System
CS	Chitosan
CSF	Cerebrospinal Fluid
CT	Threshold Cycle
CTFα	C-Terminal A Fragments
CTFβ	C-Terminal B Fragments
DEPC	Diethylpyrocarbonate
DGCR8	Digeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRNA	Double-Stranded RNA
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
Fw	Forward
GAPDH	Glyceraldehyde-3-phosphate
Glu	Glutamate
GSK-3	Glycogen Synthase Kinase-3
IL-6	Interleukin-6
Lipo 2000	Lipofectamine 2000
LMW CS	Low Molecular Weight Chitosan
MAP	Microtubule-Associated Protein
MCI	Mild Cognitive Impairment

miRISC	miRNA-Induced Silencing Complex
miRNA	microRNA
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MS	Multiple Sclerosis
N/P	Amines/Phosphates
ncRNA	Noncoding RNA
NFT	Neurofibrillary Tangles
NMDA	N-Methyl D-Aspartate
nt	Nucleotides
PACT	Protein kinase R-activating protein
PBS	phosphate-buffered saline
PD	Parkinson's Disease
PDEIs	Phosphodiesterase Inhibitors
PEI	Polyethyleneimine
PEN2	PS Enhancer
PET	Positron Emission Tomography
piRNAs	Piwi-Associated RNA
pre-miRNA	Precursor miRNA
pri-miRNAs	Primary Form
PS	Streptomycin-penicillin
PS1	γ-Secretase
p-tau	Tau protein
qPCR	real-time quantitative Polymerase Chain Reaction
RAN-GTP	GTPase Ras-related nuclear protein
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	Interference RNA
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT	Room temperature
Rv	Reverse
sAPPα	αAPP Fragments
sAPPβ	βAPP Fragments
siRNAs	Small-Interfering RNA
SIRT1	Sirtuin1
sncRNA	Small Noncoding RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
Th	T helper

TNF-α	Tumor Necrosis Factor-Alpha
TRBP	Transactivation-response RNA-binding protein
TRIzol	TripleXtractor
tRNAs	Transfer RNA
UV	Ultraviolet
XPO5	Exportin 5

Chapter I
Introduction

1. Introduction

1.1 Alzheimer's Disease

According to the World Health Organization, dementia is currently the seventh leading cause of death and disability, with more than 55 million people registered with it worldwide (Health Organization, 2021). Alzheimer's disease (AD), an irreversible neurodegenerative disease with a high prevalence in elderly people, is classified as the most common form of dementia, contributing to 50–70% of cases and is estimated to reach 74.7 million by 2030 (Santana *et al.*, 2015). The average prevalence of dementia, according to the Organization for Economic Co-operation and Development across its 35 member countries, was 14.8 cases per 1000 persons, in 2017. In Portugal, for the same period, it was estimated that the number of dementia cases was 19.9, exceeding the average. This number is projected to increase to 31.3 by 2037 (*Health at a Glance 2017*, 2017). These statistics demonstrate a tendency for a higher number of AD cases every year. Hence, the demand for innovative treatments and diagnosis methods is urgent and crucial for the well-being of those affected.

AD is characterized by cognitive impairment, such as the loss of neurons and synapses in the cerebral cortex, which consequently leads to memory loss and a huge impact on the patient's social life (Pan *et al.*, 2021). AD can occur in 2 forms known as early-onset familial, which can appear between 30 and 50 years old, and late-onset sporadic, which usually appears in people after 65 years old. AD can be classified into different stages: (1) preclinical, where it shows abnormal amyloid beta (Aβ) peptide levels and decreased glucose metabolism but no symptoms; (2) prodromal, where very mild cognitive impairment (MCI) appears accompanied by elevated Tau protein dysregulation and memory problems; (3) dementia, that can be further classified as mild dementia where assistance in some daily activities starts to be required, moderate dementia when the patient presents difficulties in communication and routine tasks, and severe dementia that shows biomarkers aggravation and symptoms interfere with everyday tasks; (4) and lastly, very severe dementia where dementia is fully installed, which can trigger body-wide inflammation, resulting in organ failure ("2020 Alzheimer's Disease Facts and Figures," 2020; Riscado *et al.*, 2021). Due to the complexity and severity of this disease, a more effective diagnosis method is required to detect early stages of AD and prevent the spread of the disease, as it leads to irreversible neurological damage. Additionally, a better understanding of pathological causes is needed, which can facilitate the establishment of more effective diagnosis methods.

From a biological point of view, the pathological hallmarks of AD are synaptic damage and loss, mitochondrial failure, oxidative stress, glial and astrocytic activation, and microRNA

(miRNA) deregulation. These are primarily caused by Aβ peptide depositions and hyperphosphorylation of tau protein (p-tau), a microtubule-associated protein (Pradeepkiran & Hemachandra Reddy, 2020). Therefore, there have been proposed many hypotheses for the etiology of AD. The cholinergic hypothesis is based on the cholinergic deficit by which the whole central neurotransmitter system is affected, suggesting a correlation between the central cholinergic deficit and the degree of cognitive disorder (Martorana *et al.*, 2010). The drugs developed to contradict this deficit, the acetylcholinesterase inhibitors, do not repair the damaged neurons, instead, they alleviate the symptoms without curing or even preventing the progression of the disease (Zhang *et al.*, 2023). On the other hand, the amyloid cascade hypothesis, one of the most accepted hypotheses to explain AD pathological changes such as neurotoxicity and inflammation, is found in the Aβ peptide accumulation that leads to neuronal damage and disease progression (Zhang *et al.*, 2023). Another well-accepted theory is the tau hypothesis, which is built on the pathological hyperphosphorylation of tau microtubule-associated protein (MAPs), a highly soluble protein found predominantly in neurons that promotes microtubule assembly and regulates dynamic instability (Biswas & Kalil, 2018). The phosphorylation of tau is regulated by multiple kinases and phosphatases such as glycogen synthase kinase-3 (GSK-3), cyclin-dependent kinase 5 (CDK5), and the MAP/microtubule affinity-regulating kinase (MARK), which are pathologically activated by Aβ oligomers and inhibited by the intracellular formation of neurofibrillary tangles (NFT) consequently leading to mitochondrial swelling and functional defects (Noble *et al.*, 2013). A more recently studied theory is the mitochondrial cascade hypothesis, which states that in late-onset sporadic AD, changes in mitochondrial function affect amyloid-beta precursor protein (APP) expression and Aβ accumulation in a manner that triggers the amyloid cascade (Abdallah, 2024). The oxidative stress hypothesis has also been recently in focus and is based on the increased levels of iron (Fe), aluminum (Al), and mercury (Hg), which can generate free radicals. This hypothesis is also supported by the increased lipid peroxidation and decreased polyunsaturated fatty acids. Furthermore, in the AD brain, there is also an increase in protein and DNA (deoxyribonucleic acid) oxidation, and it is believed that Aβ helps to generate free radicals, contributing also to oxidative stress (Markesbery, 1997). The excitotoxicity hypothesis focused on the overstimulation of N-methyl d-aspartate (NMDA) receptors by endogenous glutamate (Glu), which causes excitotoxic neuronal degeneration in the acute central nervous system and neural plasticity impairment, resulting in the synapse failure seen in AD (Zhang *et al.*, 2023). Lastly, the neuroinflammatory hypothesis is also one of the more accepted hypotheses for AD, where high levels of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are detected in AD brains and suggested to activated microglial cells, stimulating the release of proinflammatory mediators, leading to neurotoxicity, neuronal damage, and impairment of Aβ clearance (Pimplikar, 2014).

Over the years, researchers have struggled to find the correct hypothesis to explain the cause of AD and, consequently, continue to have difficulties in developing its prevention and treatment. Nevertheless, Aβ has been considered an essential target in AD pathological processes, and the "amyloid cascade hypothesis" has become a leading theory of AD pathogenesis (Zhang *et al.*, 2023). So, this work will focus on this theory, as well as its associated proteins to study a potential way to revert the amyloid cascade in AD.

1.1.1. Amyloid Pathway Hypothesis

AD pathophysiology is characterized by the upstream brain accumulation of Aβ species and plaques caused by the abnormal cleavage of APP, which precedes the spreading of p-tau and neuronal loss. These sequences of events are supported by the amyloid hypothesis (Hampel *et al.*, 2021).

APP is a type I transmembrane protein with a dynamic cellular localization, it has a large luminal domain in the C-terminal and a short cytoplasmic domain in the *N*-terminal. In humans, the *app* gene is located on chromosome 21, and its posttranscriptional alternative splicing results in six isoforms, where APP695 is the most dominant protein type in the normal brain, and APP751 is found at a higher level in the brain of AD patients (Zhao *et al.*, 2017). Overall, the function of APP is unclear but has been assumed to be related to cell adhesion and trophic support due to its interaction with laminin or collagen and, its co-localization with integrins. This glycoprotein is processed through amyloidogenic or non-amyloidogenic pathways. The non-amyloidogenic pathway is an innate way to prevent the generation of Aβ and it is the natural biological pathway that occurs in a healthy brain (Sabo *et al.*, 2003). APP is cleaved by α-secretase within the Aβ domain, generating soluble α-APP fragments (sAPPα) and two C-terminal α fragments, CTFα and C83. C83 is then cleaved by γ-secretase, producing non-toxic P3 and APP intracellular fragments (Figure 1). These last fragments play a pivotal role in gene transcription, apoptosis, development, and cytoskeletal dynamics (Müller *et al.*, 2008). On the other hand, the amyloidogenic pathway is the pathologic process of Aβ biogenesis, where APP is firstly cleaved by β-secretase (BACE1), producing soluble β-APP fragments (sAPPβ) and C-terminal β fragments (CTFβ, C99). The resulting C99 fragment from BACE1 cleavage is then cleaved by γ-secretase, generating the APP intracellular domain (AICD) and the Aβ peptide, which accumulates and creates the amyloid plaque (Wilkins & Swerdlow, 2017).

BACE1 is a 501-amino acid type 1 transmembrane protein belonging to the pepsin and retroviral aspartic protease family. Its activity is present in several cells and tissues of the body. However, the highest activity is found in neural tissue and neuronal cell lines. Therefore, it

was predicted that BACE1 should be expressed at higher levels in the neurons of the brain. Another aspartic protease called BACE2, which predominantly exists in astrocytes was later discovered also to cleave the β-amyloid sequence within APP (Sathya *et al.*, 2012). In this pathway, BACE1 controls the rate of Aβ-peptide formation, and high enzymatic activity levels of this secretase are found in human AD brain extracts, showing that in AD exist a tendency for higher levels of Aβ peptides to be formed (Hampel *et al.*, 2021; Li *et al.*, 2023).

The γ-secretase is also an aspartic protease composed of four subunits including a catalytic core (PS1 or PS2), nicastrin as a substrate receptor, anterior pharynx defective as a stabilizer, and PS enhancer (PEN2) as a regulator/enhancer of activity (Wilkins & Swerdlow, 2017). PS1 plays a critical role in maintaining cellular homeostasis and function by modulating membrane protein degradation, intracellular vesicle/protein trafficking, lysosomal activity, and autophagy (Oikawa & Walter, 2019).

The resulting Aβ peptides of the amyloidogenic pathway are small molecules composed of 39–43 amino acids with a variety of biophysical states, in which Aβ1-40 and Aβ1-42 are the most common isoforms, corresponding to the cleavage at the 40 and 42 positions, respectively. Aβ is found in different intermediate aggregation states, including dimers and trimers, soluble oligomers, and protofibrils until it forms fibrils that accumulate in plaques, which is believed to be one of the AD neuropathological hallmarks (Sun *et al.*, 2015). Although the exact pathogenic role of Aβ is unknown, the pathogenicity of Aβ is amplified when Aβ monomers become Aβ oligomers. The Aβ oligomers were found to be primarily responsible for the cognitive impairment because they have a greater surface area to interact with the synapses of cells in the brain, becoming more pathogenic compared to Aβ plaques (Mucke & Selkoe, 2012). Amyloid plaques can lead to neurotoxic processes such as loss of mitochondrial function, generation of reactive oxygen species (ROS) by increasing the oxidative stress, disruption of calcium homeostasis, activation of microglia causing neuroinflammation and synaptic distortions in cortical regions closer to the Aβ plaques. Furthermore, as mentioned before, Aβ peptides alter the kinase/phosphatase activity, leading to Tau protein hyperphosphorylation, which causes the formation of neurofibrillary tangles (NFTs), and eventual synaptic and neuronal dysfunction (Riscado *et al.*, 2021). Based on this hypothesis, multiple ongoing clinical trials focusing on therapeutic approaches aim to inhibit the secretases to prevent amyloid plaque formation and incorrect cleavage.

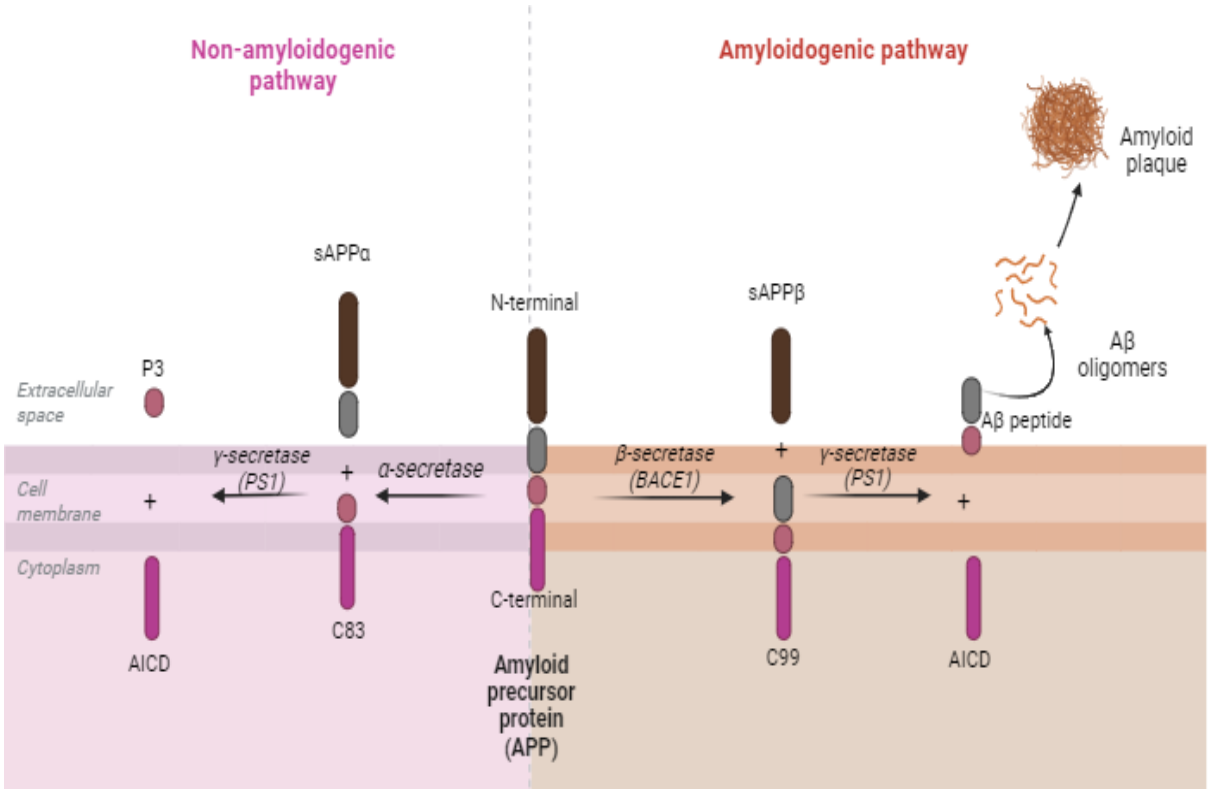

Figure 1. Schematic representation of the different processing pathways of APP.

1.1.2. Current diagnostic and therapies

To diagnose AD, there is a previous interview about the person's overall health, and cognitive tests can also be performed along with blood and urine tests. Brain scans may be used to detect dementia, including computed tomography, magnetic resonance imaging (MRI), and positron emission tomography (PET) scans (Turk *et al.*, 2021). There have been many ways to detect biomarkers of AD, such as by cerebrospinal fluid (CSF) analysis of $A\beta_{42}$, tau protein, and phosphorylated tau181. Low levels of CSF $A\beta_{42}$ are associated with cortical amyloid detected by PET and often depend on the presence of apolipoprotein E (ApoE), a major cholesterol carrier that regulates lipid transport and injury repair in the brain (Fagan & Perrin, 2012; Zafari *et al.*, 2015). *ApoE ε4* allele increases AD risk by 20–30% and is overexpressed in the blood of patients affected by AD. This glycoprotein additionally participates in the regulation of glucose metabolism, neuronal signaling, and Tau-mediated neurodegeneration, becoming an important biomarker (Riscado *et al.*, 2021). Levels of p-tau are correlated with tissue damage, reflecting the neuronal degeneration and neocortical tangle. Also, p-tau181 predicts progression from MCI to AD, as proven by Maccioni and co-workers (Maccioni *et al.*, 2006). Another current diagnosis for AD is using PET or MRI to

detect Aβ deposits and p-tau presence, which are mostly used to detect preclinical and clinical stages (Zafari *et al.*, 2015). All of these procedures are time-consuming, costly, or invasive and require specialized skills for execution and interpretation (Martinez & Peplow, 2019). Currently, advances in AD research are mainly in the diagnosis area. This is due to the complexity of this multifactorial disease, which makes it difficult to understand which targets are the best for therapeutic research.

Nevertheless, there are also attempts to stop or delay the progression of AD, but despite those efforts, to date, no effective therapies are available. However, sequential proteolytic cleavages of full-length APP by BACE1 are considered a prime target for therapeutic inhibition of Aβ production in AD (Wilkins & Swerdlow, 2017). Moreover, as Aβ plays a strong role in AD pathogenesis, it is widely accepted that reducing Aβ production or enhancing Aβ clearance may be a way to inhibit the cascade of Aβ-induced pathological events (Hampel *et al.*, 2021). There are advances in anti-Alzheimer drugs approved for its treatment, such as:

(1) Cholinergic drugs like donepezil, a reversible acetylcholinesterase inhibitor (AChEI) indicated for symptomatic treatment of patients suffering from mild-to-moderate AD (Abdallah, 2024);

(2) Phosphodiesterase inhibitors (PDEIs) like vinpocetine that repair cognitive impairment, downregulate BACE1, and decrease oxidative stress, even with the clinical trials being often inconclusive (Ali *et al.*, 2019);

(3) Anti-Aβ drugs that, through immunotherapy, are designed to degrade the amyloid plaques by activating phagocytosis or microglia. In this context, Lecanemab (Leqembi®), a monoclonal antibody directed against both soluble and insoluble forms of Aβ peptides, received approval for the treatment of early-stage AD (Park *et al.*, 2023);

(4) BACE-1 inhibitors, like Umibecestat (CNP520), are designed to meet the requirements of prevention treatment, but "The Alzheimer's Prevention Initiative Generation Program" announced that CNP520 worsens cognitive functions (Abdallah, 2024);

(5) γ-secretase inhibitors, like Semagacesthat, which decrease CSF and plasma Aβ in humans, still, this drug did not improve cognitive functions (Doody *et al.*, 2013).

In total, the U.S. Food and Drug Administration (FDA) has approved five drugs for the treatment of AD, Rivastigmine (Exelon®), Galantamine (Razadyne®), Donepezil (Aricept®), Memantine (Namenda®), and memantine combined with donepezil (Namzaric®), which temporarily improve cognitive symptoms by increasing the neurotransmitters in the brain ("2020 Alzheimer's Disease Facts and Figures," 2020).

Nowadays, RNA-based strategies have attracted attention to develop a novel AD therapy. RNA offers a wide range of capabilities, such as regulating gene expression or altering messenger RNA (mRNA) splicing (Dowdy, 2017). Due to these capacities, recent approaches, including drugs in clinical trials, use RNAs as promising therapeutic tools in many neurodegenerative diseases, such as AD.

1.1.3. New therapeutic approaches

RNA-based therapeutic strategies, which can include the use of small-interfering RNAs (siRNAs), miRNAs, mRNAs, and antisense oligonucleotides (ASOs), have great potential to target and regulate a large part of the currently inaccessible genes, and this area of research has become increasingly popular and important for future treatments to prevent or stop several diseases (Dowdy, 2017).

The study of ASOs is gaining significant attraction as a novel therapeutic approach for AD. These short single-stranded synthetic oligonucleotides can bind to RNA through complementary, based on Watson-Crick base pairing, and can modulate the miRNA precursor form (pre-miRNA) splicing, resulting in the repair of defective RNA or elimination of disease-associated proteins. Several ASOs aimed at lowering the levels of toxic Aβ. The OL-1 is an example, which was designed to target APP mRNA region corresponding to the 17-30 amino acid fragment of Aβ, improving cognitive performance and reducing neuroinflammation (Farr *et al.*, 2014; Riscado *et al.*, 2021). Another ASO example is 2'-OMePS designed and synthesized by Chakravarthy and Veedu, which demonstrated the ability to induce a decrease of 90% of BACE1 transcript and BACE1 protein levels by 45%, however, there's still no validation *in vivo* (Chakravarthy & Veedu, 2019).

Therapeutic approaches using siRNA, small noncoding RNAs (sncRNAs), involve introducing synthetic siRNA into the target cells to elicit an RNA interference (RNAi) mechanism, thus inhibiting the expression of specific mRNA and causing gene silencing (Lam *et al.*, 2015). RNAi is a natural cellular process that silences gene expression by promoting the degradation of mRNA and was first described by Fire and Mello, where, according to their observations, long double-stranded RNA (dsRNA) mediated potent and specific silencing of homologous genes (Fire *et al.*, 1998). Since then, siRNAs have become valuable tools to inactivate target gene expression by interacting and activating the RNA-induced silencing complex (RISC) (Lam *et al.*, 2015). Hérard and colleagues were pioneers in showing that siRNA can suppress protein expression in *in vivo* synapses (Hérard *et al.*, 2006). They discovered that siRNA administration via intraocular leads to a significant decrease in the APP and amyloid precursor-like protein 2 (APLP2) production and axonal transport in the retinal termini of adult rat brains (Hérard *et al.*, 2006). Olufunmilayo and Holsinger showed that, in

APP-transfected SH-SY5Y cells, siRNAs targeting catalytic and adjacent regions of BACE could provoke an 18% reduction in BACE1 mRNA, resulting in an 83% decrease in secreted Aβ (Olufunmilayo & Holsinger, 2023). Also, Kao and coworkers designed four siRNAs, where two of the siRNAs reduced BACE1 mRNA levels by more than 90% and Aβ production by 36–41%. Moreover, in that work, it was noticed that the suppression of BACE1 expression may protect neurons from oxidative stress-induced cell death (Kao *et al.*, 2003). Many siRNAs have been evaluated in clinical trials, and in 2018, Patisiran (ONPATTRO®) became the first RNAi-based therapeutic approved by the FDA. It was developed to treat hereditary transthyretin-mediated amyloidosis, where the formed amyloid is molecularly characterized as an accumulation of misfolded transthyretin protein. Patisiran specifically targets the mRNA 3' untranslated region, reducing transthyretin protein levels (Adams *et al.*, 2018; Traber & Yu, 2023). Later, 4 more siRNA drugs were approved by the FDA, all developed by Alnylam Pharmaceuticals. Givosiran (GIVLAARI®), approved in 2019, targets aminolevulinate synthase 1 and is used to treat acute hepatic porphyria, next Lumasiran (OXLUMO®) in 2020, was designed to treat primary hyperoxaluria type 1, one year after FDA approved Inclisiran (LEQVIO®) for the treatment of hypercholesterolemia that targets PCSK9, a protein involved in cholesterol regulation, and recently Vutisiran (AMUTTRA®) approved in 2022 for treating polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (Narasipura *et al.*, 2023).

miRNAs, another type of sncRNAs, also have attracted considerable attention because of their involvement in gene regulation at a post-transcriptional level. Due to its essential role in several biological processes, such as synaptic functions, neurotransmission, and neuroinflammation, many researchers evaluate their therapeutic potential. For example, miRNA-106a-5p, - 363-3p, -106b-5p, and -25-3p are involved in brain development, while miRNA-124-3p and miRNA-9-5p are important in the neurogenesis, axonal growth, and neuronal migration. Also, miRNA-125-5p is involved in astrocyte differentiation (Olufunmilayo & Holsinger, 2023). A study by Parsi and colleagues has shown that miRNA-16 mimics, in mice, regulated the expression of APP, BACE1, and nicastrin (a component of the γ-secretase complex) (Parsi *et al.*, 2015). In addition, many miRNAs have also been evaluated in clinical trials, for example, a mimic of miRNA-34a, MRX34, was tested as a cancer therapeutic because of its antagonistic characteristics to cancer cell viability (Traber & Yu, 2023). Another example is CognimiR®, a panel of 24 brain-enriched and inflammation-associated miRNAs in blood, for early detection of MCI and asymptomatic AD patients, with 90% accuracy from controls, which is currently in phase I of clinical development, branded by DiamiR (Walgrave *et al.*, 2021). Gemfibrozil, an FDA-approved drug to decrease cholesterol

and lipids, has undergone a phase I trial to assess its ability to increase miRNA-107 levels for preventing AD in cognitively healthy and MCI individuals (Jicha *et al.*, 2019).

The therapeutic potential of siRNAs and miRNAs has been demonstrated in treating many different diseases, thus becoming interesting and potential targets for drug discovery and development (Lam *et al.*, 2015). Compared with conventional small therapeutic molecules, siRNAs and miRNAs offer the advantages of being highly potent and able to act on "non-druggable" targets (Hayes *et al.*, 2014). As mentioned above, several ongoing clinical trials are evaluating siRNA and miRNA-based therapeutics (Table 1), however, as far as we know, no such formulations have advanced to clinical trials for treating AD.

Table 1. Some examples of miRNA and siRNA drugs in clinical trials or approved by FDA.

RNA based therapeutics	**Disease**	**Company/Institutions**	**Status**
miRNA			
miR-MRX34	Primary Liver Cancer, SCLC, Lymphoma, Melanoma, Multiple Myeloma, Renal Cell Carcinoma, NSCLC	Cancer Prevention Research Institute of Texas	Terminated phase 1
miRNA-107	AD	Shanghai Mental Health Center	Recruitment
Gemfibrozil	Preclinical AD	University of Kentucky	Phase 1
miRNA-34a	Melanoma	Mirna Therapeutics, Inc	Withdraw Phase 1, Phase 2
CognimiR®	Detection of MCI	DiamiR Biosciences	Phase 1
siRNA			
Patisiran	Hereditary transthyretin-mediated amyloidosis	Alnylam Pharmaceuticals	Approved for marketing
TD101	Pachyonychia Congenita	Huntsman Cancer Institute United States	Completed Phase 1
Givosiran	Acute Hepatic Porphyria	Alnylam Pharmaceuticals	Approved for marketing

Lumasiran	Primary Hyperoxaluria type 1	Alnylam Pharmaceuticals	Approved for marketing
Inclisiran	Hypercholesterolemia	Alnylam Pharmaceuticals	Approved for marketing
Vutisiran	Hereditary Transthyretin-mediated Amyloidosis	Alnylam Pharmaceuticals	Approved for marketing

Since many diseases result from the expression of undesired or mutated genes, the discovery of siRNA and miRNA opened a whole new therapeutic approach for treating diseases by targeting the genes involved. It is believed that future treatments to prevent or stop the progression of AD and preserve brain function may involve the use of miRNAs. Altered levels of miRNAs have been linked to the progression of the disease, making them potential biomarkers and therapeutic tools.

1.2. MicroRNAs

Noncoding RNAs (ncRNA) are a diverse family of non-protein-coding transcripts, that modulate cell function by controlling gene expression. They bind to DNA, RNA, and proteins, being able to regulate various processes including gene transcription, RNA turnover, mRNA translation, and protein assembly (Idda *et al.*, 2018). They can be divided into housekeeping and regulatory ncRNAs. Housekeeping ncRNAs are expressed constitutively and ubiquitously, playing an essential role in routine cell maintenance, and are subdivided into: transfer RNAs (tRNAs), which are the most abundant one, with the function to be the link between the coding sequence of an mRNA molecule and the amino acid sequence of a polypeptide chain during the translation process (Baptista *et al.*, 2021); ribosomal RNAs (rRNAs), that are also involved in mRNA translation and form the structure of ribosomes (macromolecular structures essential for the translation); and there are also the small nuclear (snRNAs) and small nucleolar RNAs (snoRNAs), both involved in the processing or modification of specific RNAs and regulatory events. Then, the class of regulatory ncRNAs play an important role in the regulation of gene expression at a post-transcriptional level and are divided into circular RNA (circRNAs), which can form a covalently closed loop, without 5′ and 3′ polar regions; and linear RNA which is the most investigated type for being useful as biomarker and therapeutic tool in numerous pathologies. In its turn, linear RNA can be divided into long ncRNAs

(lncRNA) that can transcriptionally and post-transcriptionally regulate gene expression and present a length of ≥ 200 nucleotides (nt) or sncRNAs with 20-30 nt in length. This last class includes siRNAs that trigger degradation of mRNA and translation repression, piwi-associated RNA (piRNAs) that can suppress the transcription of transposable elements in reproductive cells, and lastly miRNAs that are also capable of inducing translation inhibition or mRNA degradation (Baptista *et al.*, 2021; Idda *et al.*, 2018).

As mentioned, miRNAs are non-coding single-stranded RNA molecules with 20- 25 nucleotides. They are involved in the regulation of various biological processes, such as cell proliferation, growth regulation, dendritic ridge development, apoptosis, and pathogenic mechanisms, such as oxidative stress (Li *et al.*, 2023; Radhakrishnan & Alwin Prem Anand, 2016). The first miRNA, lin-4, was discovered in 1993 in a study that examined the developmental regulatory genes in *C. elegans*, and it was quickly found to be a class of small RNA molecules that negatively regulate gene expression. This RNA class was found to be 22 nt in length. Nowadays, the shorter *lin-4* RNA is considered the first member of the large class of miRNAs (Lee *et al.*, 1993).

The presence of miRNAs in diverse biological fluids emphasizes their clinical relevance and potential as possible biomarkers for many diseases. miRNAs are involved in the control of post-transcriptional gene expression, as they form a double-stranded complex with mRNA affecting many processes by blocking/inhibiting gene expression (Li *et al.*, 2023). As shown in Figure 2, the miRNA gene is transcribed by RNA Polymerase II into its primary form (pri-miRNAs). In the nucleus, the pri-miRNA is processed by Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8)/Pasha microprocessor complex, giving origin to precursor miRNA (pre-miRNA). Drosha is a member of the ribonuclease (RNase) III family that selectively processes RNAs with prominent double-stranded features (Zeng & Cullen, 2005). The resulting pre-miRNA is exported into the cytoplasm through the nuclear membrane protein Exportin 5 and its co-factor GTPase Ras-related nuclear protein (XPO5/RAN-GTP), which recognize the 2–3 base pair overhang at the 3' end of the pre-miRNA stem-loop structure. Once in the cytoplasm, the pre-miRNA is processed by Dicer (ribonuclease III) into a miRNA duplex (around 22 nt long). This processing step is mediated by TRBP (transactivation-response RNA-binding protein) and PACT (protein kinase R-activating protein) (Pereira *et al.*, 2017; Saraiva *et al.*, 2017). The duplex is also named miRNA-miRNA*, where the miRNA is the antisense miRNA strand and, miRNA* is the sense miRNA strand. This mature double-stranded miRNA duplex is further incorporated into the induced silencing complex (RISC) with Argonaute (AGO) protein, where the sense strand is released and targeted to Ago2-catalyzed endonucleolytic cleavage, forming the miRNA-induced silencing complex (miRISC). The guide strand is selected based on the thermodynamic

stability of the two ends of the miRNA duplex, so the less stable strand at the 5' end is usually selected as the guide. However, it is still unclear what influences this selection process, and there is minimal research about the potential use of both strands. Nevertheless, it is now known that each strand can be incorporated into the RISC complex and can regulate different mRNA targets, leading to various biological functions. A relevant example of that is miRNA-21, extensively studied in cancer therapy. For example, a particular study demonstrated that miRNA-21-5p, in colorectal cancer, can downregulate the transforming growth factor beta, leading to pyroptosis. On the other hand, another study showed that miRNA-21-3p can work as an oncogene promoting cellular mobility through epithelial-mesenchymal transition (Jiang *et al.*, 2021; Jiao *et al.*, 2017). In a study using flies, it was observed that different strands were expressed in different organs. For example, miRNA-92a-3p, miRNA-988-3p, and miRNA-284-5p were found in ovaries, while miRNA-92a-5p, miRNA-988-5p, and miRNA-284-3p were in excess in fly heads (Medley *et al.*, 2021). These findings support the idea that each miRNA strand can act to regulate different processes and if so, it is crucial to study each strand of miRNA to fully understand their distinct roles in gene regulation and their potential impact on biological processes, to further develop new therapeutic tools.

The RISC complex induces translation inhibition by translation blocking or mRNA degradation, by binding the incorporated miRNA at the 3′UTR of the target mRNA based on sequence complementarity (Radhakrishnan & Alwin Prem Anand, 2016; Slota & Booth, 2019). This protein-miRNA complex then mediates the degradation, destabilization, or repression of the mRNA. The miRNA has a wide range of targets and one miRNA can target multiple mRNAs (Li *et al.*, 2023). The binding to the mRNA occurs mainly through the recognition of specific sites (called the 'seed sequence', 2–8 nt, based on Watson-Crick pairing recognition) located in the 5' end of the miRNA and the 3' untranslated region (3' UTR) of their target mRNA. When there is perfect complementarity, it results in direct cleavage and degradation of mRNA by the PIWI domain of Ago, often seen in plants. In the case of imperfect complementary, which happens with mammal miRNAs, the mRNA translation is blocked or deadenylation of mRNA is induced (Pereira *et al.*, 2017; Saraiva *et al.*, 2017).

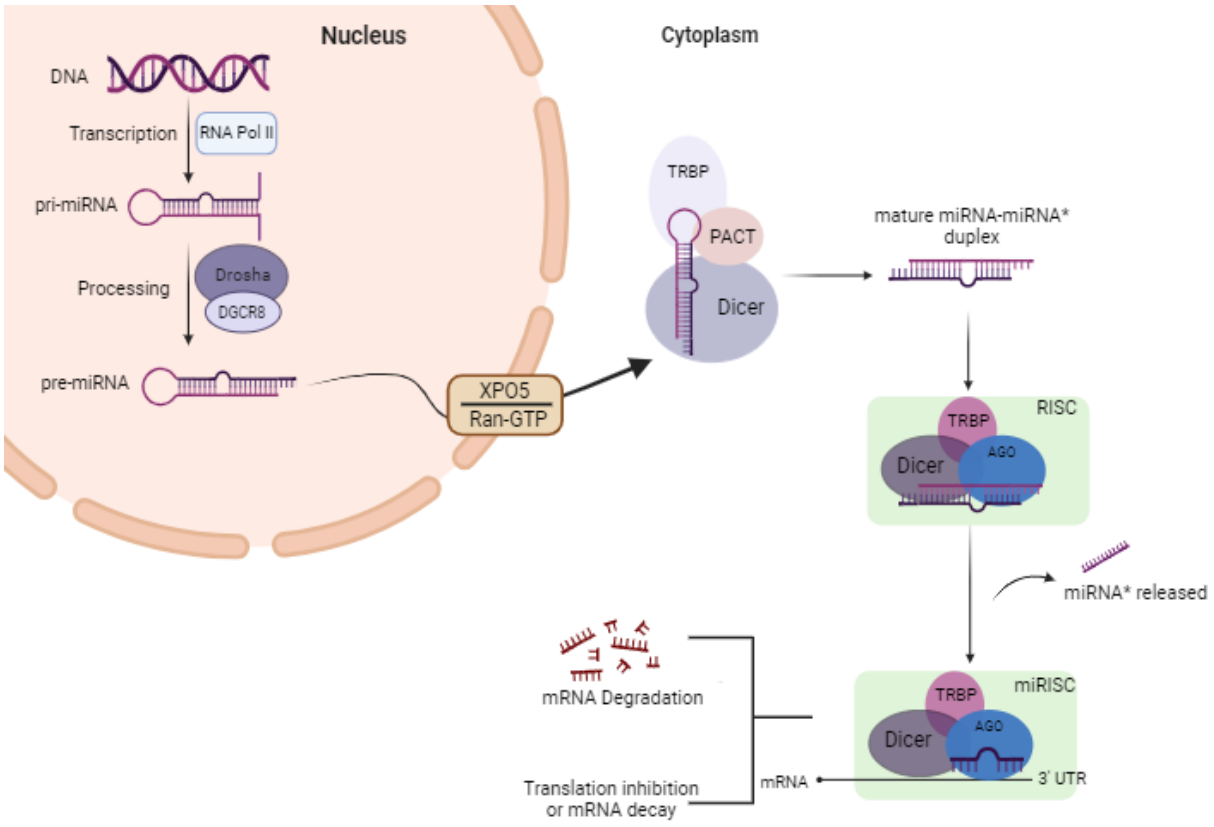

Figure 2. Schematic representation of the miRNA biogenesis (Adapted from Saraiva *et al.*, 2017).

Due to this mechanism of action, various studies have explored the application of miRNA in neurogenerative diseases. These small RNAs have been implicated in the pathogenesis of a variety of neurological diseases, like Multiple Sclerosis (MS), Parkinson's disease (PD), and AD. This is consistent with their recognized roles in neuroinflammation, and therefore, they could serve as potential biomarkers in neurodegenerative diseases and could be an ideal therapeutic target (Li *et al.*, 2023; Slota & Booth, 2019). The approved medications for PD treatment include Levodopa, but more recently miRNA-based approaches started to be investigated. A study performed with miRNA-29c-3p mimics inhibited microglia activation and suppressed NLRP3 inflammasome in *in vitro* PD mouse models by directly targeting the nuclear factor of activated T cells (Nhung Nguyen *et al.*, 2022). In the case of MS, recent studies demonstrated that miRNAs are an important tool for diagnosing MS working as biomarkers. For example, miRNA-145 was found to be overexpressed in peripheral blood, showing a relationship with patients with MS (Keller *et al.*, 2009). Another study, using miRNA-467b mimics showed downregulation of T helper (Th) 17 differentiation by targeting eukaryotic initiation factor 4 F, preventing infiltration of inflammatory cells into the central nervous system (CNS), being the main cause of neurocognitive deficits in MS (Wu *et al.*, 2021). With these findings, it is evident that miRNAs can play a crucial role in neurodegenerative diseases.

Furthermore, the use of the precursors has attracted attention within this field. A study made with recombinant pre-miRNA-29b encapsulated into polyplexes formulated with chitosan (CS) and polyethyleneimine (PEI), showed a decrease in the BACE1 mRNA expression levels in an AD cell model, with around 78% with CS/pre-miRNA-29b and 86% with PEI/pre-miRNA-29b, plus approximately 50% reduction for the Aβ42 levels. These findings suggest a post-transcriptional mechanism involving direct degradation/destabilization of BACE1 mRNA and inhibition of BACE1 protein expression. These results may prove that pre-miRNAs are more efficiently recognized and processed within the cell, than the mature form of miRNA (Pereira *et al.*, 2016). Therefore, this study will focus on the effect of pre-miRNA-9 and pre-miRNA-29b as therapeutic agents in AD, specifically their interactions with the amyloid pathway.

1.3. miRNAs role in Alzheimer's Disease

Many researchers have discovered that miRNAs are involved in the progression of neurodegenerative disorders. In AD, it is suggested that specific miRNAs can play an important role in the pathogenesis of the disease and, that their levels also appear to be dysregulated in the blood of AD patients. Neuroinflammation is among the many processes which are regulated by miRNA and this process is involved in the development of many neurodegenerative diseases, like AD. Some miRNAs, such as miRNA-124 and miRNA-21, have been discovered in exosomes released from neurons, where they regulate nearby cells such as microglia. This suggests that the miRNAs expressed in neurons may play a role in inflammatory signaling when they affect adjacent glial cells (Slota & Booth, 2019). So, trying to understand the miRNA regulation of the amyloid pathway is crucial for further comprehending its relationship with the inflammatory response. Some miRNAs, instead of downregulating pathological pathways can have the contrary effect, contributing to the pathogenic inflammatory signaling in AD. For example, Aβ42 induces the expression of miRNA-146a resulting in the attenuation of inflammatory signaling (Cui *et al.*, 2010). Nevertheless, other miRNAs can have a positive effect by downregulating pathological pathways. For example, miRNAs are involved in the regulation of APP expression, alternative splicing of APP, regulation of BACE and Tau, and even lipid metabolism (Riscado *et al.*, 2021; Slota & Booth, 2019). Some examples of miRNAs implicit in these processes are shown in Table 2.

Table 2. miRNAs involved in the regulation of target genes related to Alzheimer's disease.

miRNA	Target	miRNA expression pattern	References
miRNA-9	BACE1, CAMKK2, SIRT1, PS1	Upregulated/downregulated	(Idda *et al.*, 2018);(Bastien *et al.*, 2008)
miRNA-15a	ERK1	Downregulated	(Hébert *et al.*, 2010)
miRNA-16	APP, ERK1	Upregulated	(W. Liu *et al.*, 2012)
miRNA-20a	APP	Downregulated	(Delay *et al.*, 2011; Idda *et al.*, 2018)
miRNA-27a-3p	PS1	Downregulated	(Frigerio *et al.*, 2013)
miRNA-29b-1, -29a, -29c	APP, BACE1	Downregulated	(Bastien *et al.*, 2008; Yang *et al.*, 2015)
miRNA-34a	PS1/APP, Tau	Upregulated	(Jian *et al.*, 2017)
miRNA-101, miRNA-106a, -106b	APP	Downregulated	(Sonntag, 2010; Vilardo *et al.*, 2010)
miRNA-96-5p	$A\beta_{42}/A\beta_{40}$ ratios	Upregulated	(Li *et al.*, 2023)
miRNA-107	BACE1	Downregulated	(Idda *et al.*, 2018; Wang *et al.*, 2008)
miRNA-147	APP	Downregulated	(Delay *et al.*, 2011; Idda *et al.*, 2018)
miRNA-298/miRNA-328	BACE1	Downregulated	(Boissonneault *et al.*, 2009; Idda *et al.*, 2018)

As mentioned above, the cleavage of APP by BACE1 has been a research focus on the understanding of AD pathogenesis. Numerous studies have found that various miRNAs, such as miRNA-485-5p regulated BACE1 mRNA levels. In this case, miRNA-485-5p could inhibit BACE1 translation by binding to BACE1 exon 6, which reduces the protein level by 30% (Faghihi *et al.*, 2010). Another study shows that upregulation of miRNA-29a, -29b-1, and -29b-c levels could directly inhibit BACE1 by binding its mRNA 3'UTR (Zhao *et al.*, 2017). These findings demonstrated that miRNAs could alter BACE1 translation which could affect the metabolic processes of APP protein. So, the miRNA-29 family seems to be a good candidate as a key regulator for the downregulation of BACE1 in AD, enabling the therapeutic inhibition

of β-secretase to decrease the production of all forms of Aβ peptides, including the pathogenic Aβ42 (Sathya *et al.*, 2012). The miRNA-29 family will be focused on in this work.

miRNA-29 family is composed of miRNA-29a, miRNA-29b, and miRNA-29c. This family is one of the most interesting miRNA families because its dysregulation strongly impacts many diseases. miRNA-29a and miRNA-29b-1 are encoded on human chromosome 7q32.3 as miRNA-29a/b-1 cluster, and miRNA-29b-2 and -29c are found on chromosome 1q32.2 known as miRNA-29c/b-2 cluster. In this case, the miRNA-29-3p strand represents the most abundant and functional species (Kwon *et al.*, 2019). This family is known to regulate several biological processes in various tissues and organs. In the immune system, miRNA-29b controls the innate and adaptive immune response by targeting interferon-γ producer mRNA, and miRNA-29a/b regulates Th1 differentiation through their increased expression in CD4+ T memory cells. miRNA-29 also regulates cell proliferation and apoptosis where its expression is increased in the initial steps of embryonic stem cell differentiation. Also regulates the extracellular matrix through the production of collagen, fibrillin 1, elastin, and others. Lastly, it can be also present in the regulation of hematopoiesis, muscle function, and skeleton, being essential for the differentiation of osteoblasts (Alizadeh *et al.*, 2019; Kriegel *et al.*, 2012).

As mentioned, in AD, it is recognized that this miRNA family targets the BACE1 mRNA, and it is believed that it can regulate its expression and, consequently, Aβ peptide levels (Idda *et al.*, 2018; Pereira *et al.*, 2016). This study will explore its correlation with AD protein levels. miRNA-29 levels are inversely correlated with BACE1 and APP levels in neuronal cellular models, accompanied by increased amyloid production *in vitro*. So, the loss of miRNA-29 results in increased BACE1 expression and Aβ peptide levels (Femminella *et al.*, 2015). These findings support the idea that miRNA-29 can be a potential therapeutic tool for pharmacological intervention in AD. A study by Zong and coworkers showed that miRNA-29c is highly expressed in the APPswe/PSΔE9 mouse and can lower BACE1 protein *in vitro* (Zong *et al.*, 2011). Another study revealed that low miRNA-29c-3p levels and high BACE1 levels were detected in the brain tissue of AD patients, contributing as a biomarker of AD. They also showed that miRNA-29c-3p overexpression relieved the abnormal phosphorylation of tau protein and can reduce the cytotoxicity caused by Aβ (Cao *et al.*, 2021). Additionally, these small RNAs are under-expressed in AD, which means that the loss of miRNA-29a/b-1 increased Aβ levels, associated with dysregulation of BACE1, thus, they play a crucial role in the amyloid pathway. A study about miRNA-29b-5p showed that if its expression were decreased, it would result in the overexpression of BACE1 and Aß42 (Duan *et al.*, 2023). Due to the evidence using miRNA-29b, this work aims to explore its impact on the proteins involved in the amyloid pathway of AD.

Another miRNA of interest in AD is the miRNA-9. It is one of the highly expressed miRNAs in the fetal hippocampus in the developing vertebrate brain, a region of the brain associated with memory and learning. It is involved in several cellular functions and development like proliferation, maturation, and differentiation of neurons in the brain. It is a brain-enriched miRNA, and its guiding strand can be generated either from the 5' strand (miRNA-9-5p or miRNA-9) or the 3' strand (miRNA-9-3p or miRNA-9*) (Saraiva *et al.*, 2017). At the moment, various studies demonstrate that in AD, miRNA-9 is one of the most frequently altered miRNAs, encoded by three different genes. It is downregulated due to the presence of Aβ peptides in primary neuronal cell cultures and is also implicated in the formation of Tau hyperphosphorylated forms by interacting with sirtuin1 (SIRT1) (Femminella *et al.*, 2015; Krichevsky *et al.*, 2003). Additionally, a reduced level of miRNA-9 may result in elevated BACE1 expression and an over-production of Aβ (Chen *et al.*, 2021).The miRNA-9-5p is closely related to cell growth, metastasis, and differentiation, as well as modulates neuroinflammation in the central nervous system. However, its mechanism in AD is yet not clear. Liu and coworkers revealed that miRNA-9-5p expression was found downregulated in the frontal gyrus and the cortex of sporadic AD patients, and by its overexpression, inhibited oxidative stress, cell apoptosis, and mitochondrial dysfunction is induced by targeting GSK-3β (Liu *et al.*, 2020). This miRNA is known to have different levels of expression within the stages of AD, being described as the miRNA-9-5p having lower levels at the early stage of AD and higher levels at the late stage, in mice. These findings are important to prove the potential of miRNA-9 to be used as a tool for better distinguishing the early and late stages of AD (Chen *et al.*, 2021). On the other hand, miRNA-9-3p, a brain-enriched miRNA known to regulate neuronal development, has been implicated in several neurological disorders. It was suggested by Sim and team, that miRNA-9-3p mediated gene regulation plays important roles in synaptic plasticity and hippocampus-dependent memory. Inhibition of miRNA-9-3p impairs hippocampal long-term potentiation and results in memory deficits, showing its critical role in neuronal differentiation (Sim *et al.*, 2016). This miRNA can be a solid candidate as a biomarker for neurodegenerative diseases, and the studies mentioned above prove its potential as an innovative therapeutic tool for AD.

Considering the relevance of studying miRNAs involved in AD, the present work focuses on the study of miRNA-9 and miRNA-29-b, in mature (both -3p and -5p strands) and precursor forms, and their involvement in particular pathological signatures of AD, hoping to provide evidence on the potential of these RNAs as innovative targets and therapeutic agents for AD.

Chapter II
Aims

2. Aims

The therapeutic potential of miRNAs has garnered significant attention due to their role in the regulation of gene expression at a post-transcriptional level. Specific miRNAs have been implicated in AD pathology and understanding how these small RNA affect the amyloid pathway, holds promise for the development of novel treatment strategies.

Thus, this project aims to study the effect of specific miRNAs and precursor forms in the regulation of proteins expression present in the amyloid pathway of AD. So, the objectives of this study are to:

- Evaluate the effect of miRNAs-9-1 and miRNA-29b-1 on the mRNA levels of APP, BACE1, and PS1, proteins involved in the amyloid pathway.
- Compare the silencing effect of the miRNAs mature and precursor forms and try to understand which form can be the most efficient to be considered as biopharmaceutical.
- Determine which miRNA strand is more active and efficient in the silencing of the target proteins.
- Evaluate which miRNA strand can result from pre-miRNA processing and play the more relevant role at controlling the target proteins expression.

Chapter III
Materials and Methods

3.1 Materials

For *in vitro* assays, the mouse neuroblastoma cell line stably transfected with cDNA encoding human APP695 (N2695), was kindly provided by Professor Wenjie Luo (Weill Cornell Medical College). For cell culture, trypsin from Sigma-Aldrich (Missouri, USA) was used. The cell line was maintained in a T-flask of 25 cm^2 and seeded in 12-well plates from VWR International, LLC (Pennsylvania, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma-Aldrich (Missouri, USA), and OptiMEM obtained from Gibco Life Technologies (New York, USA). The medium was supplemented with Fetal Bovine Serum (FBS) from PAN-Biotech (Aidenbach, Bayern) and streptomycin-penicillin (PS) purchased from Grisp (Porto, Portugal). To transfect the cells, miRNA-29b-1-3p, miRNA-29b-1-5p, miRNA-9-1-3p, and miRNA-9-1-5p were used and purchased from STAB VIDA (Lisbon, Portugal). Additionally, pre-miRNA-29b and pre-miRNA-9-1 obtained from Integrated DNA Technologies (Iowa, USA) were also used to transfect the N2a695 cell line. The sequences of the RNAs used in the study are represented in Table 3. In the transfection assay was also used Lipofectamine 2000 (Lipo 2000) Reagent obtained from Thermo Fisher Scientific Inc. (Waltham, USA), and for the formulation of the nanoparticles were used the following reagents: low molecular weight chitosan (LMW CS) and acetate buffer composed of sodium acetate, glacial acetic acid, Milli-Q water, all obtained from Sigma-Aldrich (Missouri, USA).

Table 3. Synthetic RNA sequences used in the *in vitro* studies.

RNA	Sequences (5'-3')	Length (bp)
pre-miRNA-9-1	CGG GGU UGG UUG UUA UCU UUG GUU AUC UAG CUG UAU GAG UGG UGU GGA GUC UUC AUA AAG CUA GAU AAC CGA AAG UAA AAA UAA CCC CA	82
pre-miRNA-29b-1	CUU CUG GAA GCU GGU UUC ACA UGG UGG CUU AGA UUU UUC CAU CUU UGU AUC UAG CAC CAU UUG AAA UCA GUG UUU UAG GAG	81
miRNA-9-1-3p	AUAAAGCUAGAUAACCGAAAGU	22
miRNA-9-1-5p	UCU UUG GUU AUC UAG CUG UAU GA	23
miRNA-29b-1-3p	UAG CAC CAU UUG AAA UCA GUG UU	23
miRNA-29b-1-5p	GCUGGUUUCAUAUGGUGGUUUAGA	24

The reagents used to extract total RNA from N2a695 cell line were TripleXtractor (TRIzol) from Grisp (Porto, Portugal), 1x phosphate-buffered saline (PBS), and chloroform purchased from Fisher Scientific (UK). To precipitate and wash the RNA, isopropanol and ethanol 75% were prepared with Milli-Q (ultra-pure deionized water purified with Millipore Milli-Q system obtained from Billerica (MA, USA)) treated with 0.01% diethylpyrocarbonate (DEPC) purchased from Thermo Fisher Scientific Inc., (Waltham, USA). The RNA integrity was analyzed by agarose gel electrophoresis in which was used agarose and Green Safe (0.012 µL/mL) obtained from Grisp (Porto, Portugal). The gels were visualized under ultraviolet (UV) light exposure with a UV chamber (UVITEC Cambridge, Cambridge, United Kingdom). The quantity of RNA was measured by the Thermo Fisher Scientific NanoDrop Microvolume Spectrophotometer (Waltham, USA).

For the primers optimization, conventional PCR was performed using DreamTaq DNA Polymerase and DreamTaq Green Buffer from Thermo Fisher Scientific Inc. (Waltham, USA), deoxyribonucleotide triphosphate (dNTPs) from NZYTech Genes and Enzymes (Lisbon, Portugal), and Forward (Fw) and Reverse (Rv) primers from STABvida (Lisbon, Portugal).

To analyze the mRNA levels of the proteins under study after transfection, complementary DNA (cDNA) synthesis was performed using the Xpert cDNA synthesis Kit purchased from Grisp (Porto, Portugal) composed of Xpert Reverse Transcriptase (Xpert RTase with RNase inhibitor), 5x reaction Buffer, random hexaprimer, dNTP mix, and RNase free water. To execute the real-time quantitative polymerase chain reaction (qPCR) technique the Maxima® SYBR Green/Fluorescein qPCR Master Mix Kit from Thermo Fisher Scientific Inc. (Waltham, USA) was used together with Fw and Rv primers designed specifically for each mRNA under study, and obtained from Stabvida (Lisbon, Portugal). Table 4 shows the primers sequences used in the PCR and qPCR.

Table 4. Primers sequences used in the PCR and qPCR.

mRNA	Primers sequences (5'-3')	
BACE1 (*Mus musculus)*	Rv	CCGTGGATGACTGTGAGACA
	Fw	CCATCCTTCCTCAGCAATACCT
APP (*Homo sapiens*)	Rv	GCCGTAGTCATGCAAGTTGG
	Fw	GTCGCCAAAGAGACATGCAG
PS1 (*Mus musculus*)	Rv	GCATTCAGGATCGAGTGCAG
	Fw	GGAAGGACGGTCAGCTAATCTA
GAPDH (*Mus musculus*)	Rv	AGTGTAGCCCAAGATGCCCTTCAG
	Fw	TGACGTGCCGCCTGGAGAAA

3.2 Methods

3.2.1. Primer design and optimization

To design the primers necessary to evaluate the mRNA levels for the target proteins from *Mus musculus* species, the NCBI database (https://www.ncbi.nlm.nih.gov/gene) was used to identify the transcripts sequences of APP, BACE1, and PS1. Next, the Primer-BLAST designed tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) was used to design several primer pair options. These options were then analyzed in each mRNA sequence file and the primer pair that amplified the largest number of transcripts for each protein was chosen. Lastly, the Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was used to confirm the primer amplification sequence. Each primer was resuspended in free-nuclease water to a concentration of 100 µM. A conventional PCR was performed for the annealing temperature optimization. The primers sequences were inserted in the Thermo Fisher Tm calculator (https://www.thermofisher.com/pt/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html) to estimate the possible annealing temperature for each primer pair, using the DreamTaq DNA polymerase. Then, a range of temperatures was chosen to test the optimal annealing temperature. For each gene, it was prepared 12 µL of mix composed of 9.25 µL of nuclease-free water, 1.25 µL of DreamTaq Green Buffer (Thermo Fisher Scientific Inc.), 0.25 µL of dNTPs (10 µM) (NZYTech Genes and Enzymes), 0.25 µL of Fw primer (5 µM), 0.25 µL of Rv primer (5 µM) (Stabvida), 0.25 µL of DreamTaq polymerase (Thermo Fisher Scientific Inc.), and then 1 µL of cDNA. The PCR program was performed with an initial denaturation step of 95˚C for 10 min, followed by 40 cycles at 95 ˚C for 15 min, test temperature for 30 sec, and 72 ˚C for 30 sec. In the end, the samples were analyzed by 3% agarose gel electrophoresis for 40 min at 120 V.

3.2.2. Cell culture

The N2a695 cell line, an *in vitro model* of AD, was used to evaluate the biological activity of miRNA-9-1, miRNA-29b-1, and their respective pre-miRNAs. N2a695 cells were cultured at passages 10-23 in a mixed medium of 50% DMEM (Sigma-Aldrich, Missouri, USA) and 50% OptiMEM (Gibco Life Technologies, New York, USA), supplemented with 5%

(wt/vol) FBS (PAN-Biotech, Aidenbach, Bayern) and 1% (wt/vol) PS (Grisp, Porto, Portugal), in a humidified atmosphere of 5% CO_2 at 37 ˚C.

3.2.3. Transfection assays

To optimize the transfection assays, different cell numbers such as 2 x 10^4 cells/well, 4 x 10^4 cells/well, and 6 x 10^4 cells/well, were tested at different incubation times, namely 24 h, 48 h, and 72 h. The better medium for plating and transfecting was also chosen by testing medium with 1% or 5% FBS. The time needed for the cells to be stabilized before transfection was confirmed by testing periods of 16 h and 40 h. Two transfection times (4 h and 6 h) were also tested. Additionally, to select the best transfection vector LMW CS (Sigma-Aldrich, Missouri, USA) a ratio of 1:30 (Amines/Phosphates (N/P)) and Lipo 2000 (Thermo Fisher Scientific Inc., Waltham, USA), following the manufacturer's instructions, were used.

After transfection optimizations, the bioactivity of the RNAs under study was performed as follows. After reaching 70-80% of confluence, the N2a695 cells were seeded in 12-well plates at a density of 2 x 10^4 cells/well with a culture medium mix of 1:1 DMEM and OptiMEM supplemented with 1% FBS. For each plate, there were always duplicates for each condition in the study and duplicates of untreated wells (negative control). The counting of the cells was done with trypan blue at a 1:2 dilution in a Neubauer chamber. After 24 h, the cell medium was changed with the same medium, to obtain a better confluence and number of cells for the transfection. 16 h later, the cells were transfected with 20 nM of miRNA-9-1-3p, -5p, miRNA-29b-1-3p, -5p, and pre-miRNA-9-1 and pre-miRNA-29b-1 from STAB VIDA (Lisbon, Portugal), previously incorporated in LMW CS nanoparticles. The formulation of CS nanoparticles was accomplished by the dropwise method, where 100 µL of LWM CS solution was added, in a ratio of 1:30 (N/P), (LMW CS stock solution of 10 mg/mL in 0.1 M acetate buffer, pH=4.5) to 20 nM of RNA in 400 µL of 0.1 M acetate buffer, pH=4.5 under a vortex, for 1 min. Then, the complexes were incubated for 15 min at room temperature (RT) and were centrifuged at 15 000 RCF, for 20 min at 4 ˚C. In a sterile environment, the supernatant was discarded, and the pellet was resuspended in 200 µL of OptiMEM and added to the cells. In the negative control, only 200 µL of OptiMEM was added. After 6 h of transfection, the culture medium was replaced by 1:1 DMEM and OptiMEM medium supplemented with 5% FBS and 1% PS. The transfected cells were incubated for 48 h in a humidified atmosphere of 5% CO2 at 37 ˚C and were finally harvested for total RNA extraction, as shown in Figure 3.

3.2.4. Total RNA extraction from eukaryotic cells

For RNA extraction, transfected N2a695 cells were rinsed in PBS (1x), and 300 µL of TRIzol (Grisp, Porto, Portugal) reagent was added to each well, promoting cell lysis. Then, homogenization was performed with "up and down" pipetting, and the lysed cells were transferred to tubes of 1.5 mL and incubated for 5 min at RT. After that, 60 µL of chloroform (Fisher Scientific, UK) was added, vigorously mixed, and incubated for 10 min at RT. The tubes were centrifugated for 15 min, 4 ˚C, at 12000 RCF, leading to the formation of three phases: the organic phase containing phenol, chloroform, proteins, and lipids (bottom phase); interphase, containing DNA; and the aqueous phase containing the RNA (top phase). The top phase was transferred to a new tube and the RNA was precipitated with 200 µL of iced-cold isopropanol (Billerica, MA, USA), and the mixture was slowly homogenized and incubated in ice for 15 min. Next, the tubes were centrifugated at 12000 RCF, 4 ˚C for 10 min, the supernatant was discarded, and the pellet was slowly homogenized with 200 µL of 75% ethanol (Billerica, MA, USA) in 0.01% DEPC water (Fisher Scientific Inc., Waltham, USA). The resuspended pellet was centrifuged at 12000 RCF, 4 ˚C for 5 min. The supernatant was again discarded, and the final RNA pellet was left to dry for 20 min at RT inside the extraction cabinet, and then resuspended in 20 µL of 0.01% DEPC water. To quantify the RNA a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used, and their integrity and purity were evaluated by 1% agarose gel electrophoresis using Green Safe (0.012 µL/mL) (Grisp, Porto, Portugal) where the samples were prepared with 3 µL of RNA sample and 0.5 µL of loading buffer (glycerol, bromophenol blue, milli-Q water). The electrophoresis was run at 120 V for 30 min in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH=8.0). The gels were visualized under UV light exposure with a UV chamber (UVITEC Cambridge, Cambridge, United Kingdom). The samples were then stored at -80˚C.

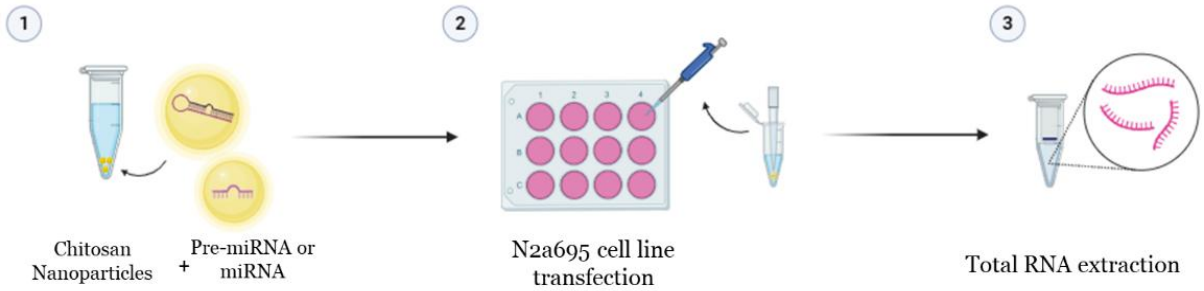

Figure 3. Schematic representation of N2a695 cells transfection.

3.2.5. cDNA synthesis

To proceed with cDNA synthesis, 1 µg sample of total RNA was used with the Xpert cDNA Synthesis Kit (Grisp, Porto, Portugal) according to the manufacturer's protocol. Each

reaction with a final volume of 20 µL was prepared as follows: 4 µL of 5x reaction Buffer, 1 µL random hexaprimer, 1 µL of dNTPs, 1 µg of total eucaryotic RNA, 1 µL of Xpert RTase with RNase inhibitor and the remaining volume was completed with nuclease-free water. The mix was incubated in a T100™ Thermal Cycler (BioRad) at 25 ˚C for 10 min, followed by 50 ˚C for 15 min, 85 ˚C for 5 min, and then incubated at 4 ˚C for 2 min. The samples were then stored at -20 ˚C.

3.2.6 Real-time qPCR

For quantitative analysis of the mRNA expression of BACE1, PS1, and APP, qPCR amplification of synthesized cDNA was performed with specific primers for each mRNA (Figure 4). qPCR reaction was prepared by adding: 1 µL of cDNA, 1.2 µL of Fw primer (5 µM), 1.2 µL of Rv primer (5 µM) from Stabvida (Lisbon, Portugal), 10 µL of Maxima® SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermo Fisher Scientific Inc., Waltham, USA), and 6.6 µl of nuclease-free water, to a final volume of 20 µL per reaction. In the negative control, cDNA was not included. The reaction conditions were 95˚C for 10 min for initial denaturation, followed by 40 cycles at 95 ˚C for 15min, 64 ˚C for 30 sec, and 72 ˚C for 30 sec. In the end, the samples were incubated at 65 ˚C for 5 sec with an increment of 0.5 ˚C until 95 ˚C for the melting curves. The primers were optimized by conventional PCR and the efficiency was optimized by qPCR, for an annealing temperature of 64ºC. All reactions were completed in triplicate and threshold cycle (CT) values were averaged from the triplicate.

Figure 4. Schematic representation of the target mRNA expression analysis assay.

3.2.7. Data analysis

qPCR data analysis was used to determine changes in the expression of the target mRNAs, where Glyceraldehyde-3-phosphate (GAPDH) was used to normalize expression and determine any amplification changes compared with the untreated cells. To calculate the expression, the comparative CT was used, where the amount of target was determined to be 2-

$^{\Delta\Delta Cq}$, where ΔΔCq = (Cq target gene - Cq housekeeping gene). Statistics were analyzed in GraphPad Prims 9 by ordinary one-way ANOVA, where a value of $p < 0.05$ was considered statistically significant. Data are shown as the mean ± SD of 3 separate experiments.

Chapter IV
Results and Discussion

4. Results and Discussion

4.1. Optimization assay

To achieve precise results in cell assays, optimizing key experimental parameters related to the cell culture is essential. This includes the number of cells, selecting an appropriate culture medium, determining the optimal growth and incubation conditions, and carefully selecting the most suitable transfection vector. All these factors were optimized and are described in this chapter. Additionally, the use of primers is crucial in guiding the amplification to the correct region of the DNA template, making their proper design essential for successful amplification. This ensures specificity and minimizes non-specific bindings, which is fundamental to the reliability of PCR-based techniques. Therefore, the annealing temperature was also optimized in this work to enhance application accuracy.

4.1.1. Transfection assay optimization

To perform the transfection assays with the RNAs under study, it was first optimized the number of N2a695 cells per well. The N2a695 cell line has a particular way of growth, as the cells grow on top of each other, making a "tower" of cells. As the cells do not occupy the entire well area, because of this characteristic, the number of cells and the medium used are crucial for successful and proper cell growth. A study made by Viallon and co-workers tested the viability of N2a695 cells by studying a range of 10000 to 100000 cells/well (Viallon *et al.*, 2020) So, in this work, the cells were cultured in a plate of 12 wells with 20000 cells/well, 40000 cells/well, and 60000 cells/well, in accordance with the range evaluated in the literature. These numbers were tested to ensure the correct confluence, but specifically to obtain a significant cell number on the transfection day and viable cells on the extraction day. Figure 5 shows the cell confluence in each well on the days of transfection and extraction. From the analysis of Figure 5, it can be seen that 20000 cells/well is the best condition because the cells have better conformation on the transfection day and presented a lower number of dead cells on the extraction day, with an incubation of 48 h. To ensure that this number of cells had enough RNA to proceed with the mRNA evaluation levels by qPCR, the total RNA was extracted from the cells and quantified by a NanoDrop spectrophotometer and, it was confirmed that these conditions allowed the recovery of the RNA for cDNA synthesis (data not shown).

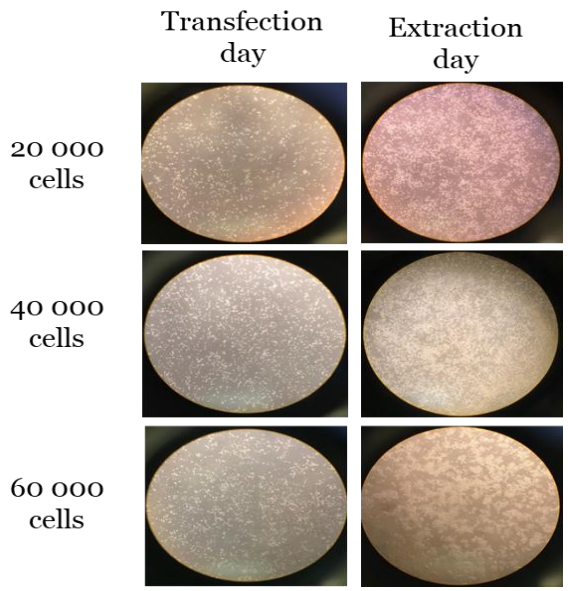

Figure 5. Representation of the cellular appearance on the transfection and extraction days, after initial cultivation with different cell numbers (20000, 40000, and 60000 cells/well). Cell culture images were acquired using a 50x magnification.

The composition of the medium used for cell plating and transfection was also tested, using 5% or 1% FBS with 0% PS. Following the handling information of N2a695 on the ATCC website (https://www.atcc.org/products/ccl-131), the culture medium must be supplemented with FBS to a final concentration of 10%. It is known that the percentage of FBS influences cell growth by strengthening cell adhesion. This influence was also studied by Maulik group, which compared the potential effects of varying FBS concentrations (0%, 5%, 10%) and concluded that those variations altered the cytotoxicity for cells, and could also induce an increase of the levels of APP when less percentage of FBS was applied (Maulik *et al.*, 2018). Having this in mind, testing how the different percentages of FBS could affect the transfection and extraction assays is essential. Figure 6 shows how the different percentages of FBS affect the cells' growth. With 5% FBS, cells grow too fast and do not present proper viability on the extraction day, as the majority lost their conformation on the day of transfection. So, the medium with 1% FBS and 0% PS was selected to maintain cell viability and ideal conformation for transfection and RNA extraction.

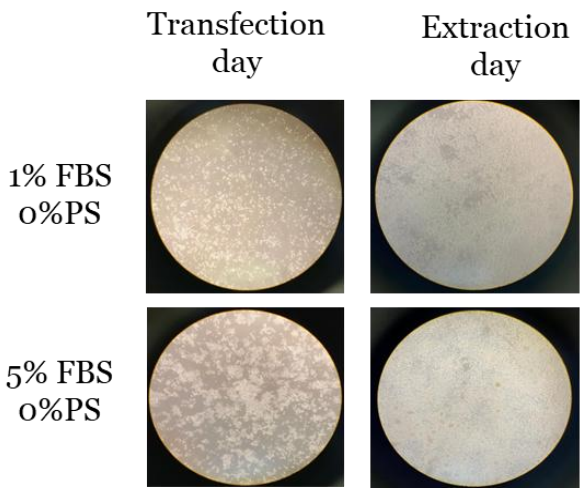

Figure 6. Cellular appearance in the presence of different FBS percentages on the days of transfection and extraction. Cell culture images were acquired using a 50x magnification.

After the ideal medium and cell number were chosen for this type of cell, it was important to try to increase the number of cells present on the transfection day because those could be insufficient for a successful transfection. Based on the guidelines from the SignaGen® website (https://signagen.com/), N2a695 cells should be plated 18 to 24 h prior to transfection. Additionally, in a previous study by our research group, the transfection was performed 12 h after cell seeding (Pereira *et al.*, 2020). Although this shorter time frame provided sufficient cell numbers for transfection in that case, a higher number of cells is required for the current experiment. So, it was evaluated the possibility of increasing the culture time for 24 h before the transfection to see if the cell confluence could improve. In this way, the appearance of the cells was compared for 16 h and 40 h of growth. As shown in Figure 7, the 40 h of growth was necessary for a better conformation on the transfection day and it is clear that the cells are less clustered. On the extraction day, it is possible to see that the experiment established with 16 h of growth presented agglomerates, which could be associated with lower cell viability when compared to 40 h of culture.

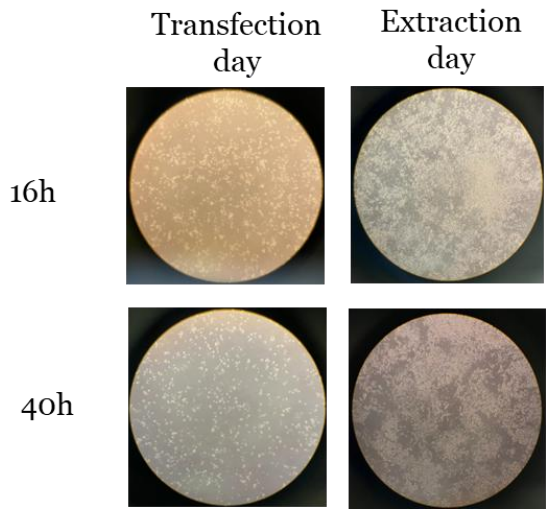

Figure 7. Cell appearance on the day of transfection and extraction, when evaluating 16 h or 40 h of culture before transfection. Cell culture images were acquired using a 50x magnification.

When this optimization was concluded, a transfection with CS and Lipo 2000 carrying the pre-miRNA-29b-1 was performed to select the best transfection vector. Comparing the silencing results in Figure 8, it was verified that the transfection with CS presented less variability. When the Lipo 2000 is used, no silencing is detected, which could indicate that the pre-miRNAs are not being well-transfected because, according to the literature, silencing should be seen at least in BACE1 mRNA levels. This interesting result with the CS-based systems, corroborates with a previous study made by Pereira and coworkers, in 2016, where it is shown that the polymeric nanoparticles, derived from natural materials, are preferred due to their biocompatibility, immunogenicity, and low cytotoxicity. In the previous study, it was confirmed that encapsulating the pre-miRNA-29b with CS resulted in efficient delivery and decreased levels of BACE1 expression. This transfection was carried out for 6 h (Pereira *et al.*, 2016). Figure 8 also shows inconclusive results regarding the mRNA silencing at 4 h of transfection, and it was thought that the cells could need more time to internalize the nanoparticles and to see the effect of the transfection with the miRNAs. Considering this, a comparison was performed for 4 h and 6 h of transfection. In this study, it was not verified a significant difference in the expression levels of the target proteins. Still, it was selected the condition of 6 h of transfection for further studies to ensure a more effective transfection, consistent with the protocol

performed by the study mentioned. So, in this work, the miRNAs were delivered to cells in CS nanoparticles, establishing a transfection time of 6 h with an incubation of 48 h.

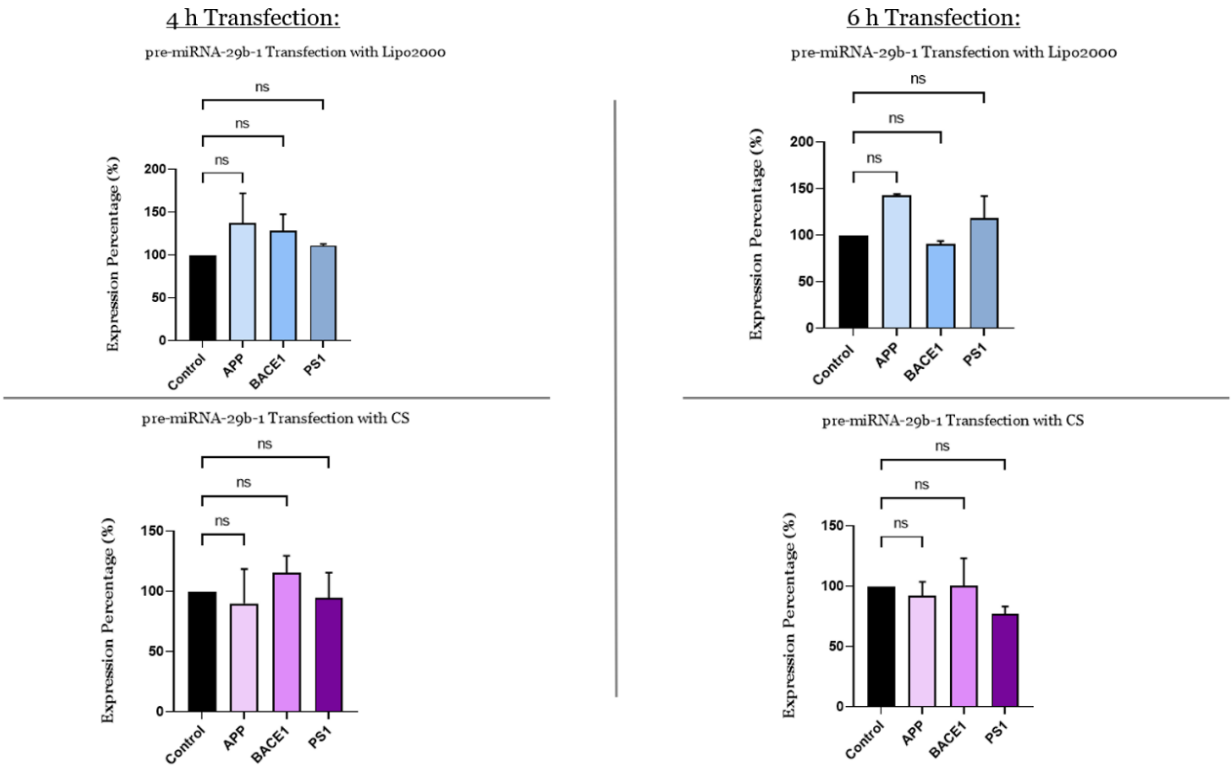

Figure 8. APP, BACE1, and PS1 proteins expression after transfection of N2a695 cells with pre-mir-29b-1 using CS and Lipo 2000 at 4 h and 6 h of transfection. The error bars represent ± SD of 3 separate experiments. The results are considered statistically significant when $p<0.05$. Where ns means non-significant.

4.1.2. Primers optimization

To amplify the specific DNA sequences corresponding to the mRNAs of the target proteins by qPCR, it was necessary to design specific primers and optimize the annealing temperature of each primer pair. After primer design, performed as described in the Methods section 3.2.1, the correct annealing temperature was optimized by conventional PCR, to obtain a specific amplification and avoid non-specificity or dimers formation. So, for that, several temperatures were tested until a single well-defined band was achieved for each gene. As illustrated in Figure 9, with the optimal annealing temperature of 64 °C, low or no dimers were shown in the negative control of each target. Moreover, each band amplified by the specific primer pair corresponded to the expected molecular weight of the fragments in amplification. For example, the primers designed for the amplification of the APP mRNA coding sequence should result in a 51 bp length fragment, and by the electrophoresis gel, it can be seen that the amplified band corresponds to this molecular weight. The same happened for the remaining

genes, as the BACE1 primers were designed to amplify a 93 bp fragment in the mRNA-encoded sequence, the PS1 primers an 85 bp fragment, and the GAPDH primers corresponded to the amplification of a 98 bp fragment. These results prove that the design of the primers was well performed and that if the qPCR is carried out at 64 ˚C with these primer pairs it is almost certain that the amplification for each target is going to be specific. Nevertheless, it was necessary to test these conditions with the qPCR equipment and reagents to make sure that the primer specificity was maintained. For that, primer efficiency in qPCR was calculated for each target DNA.

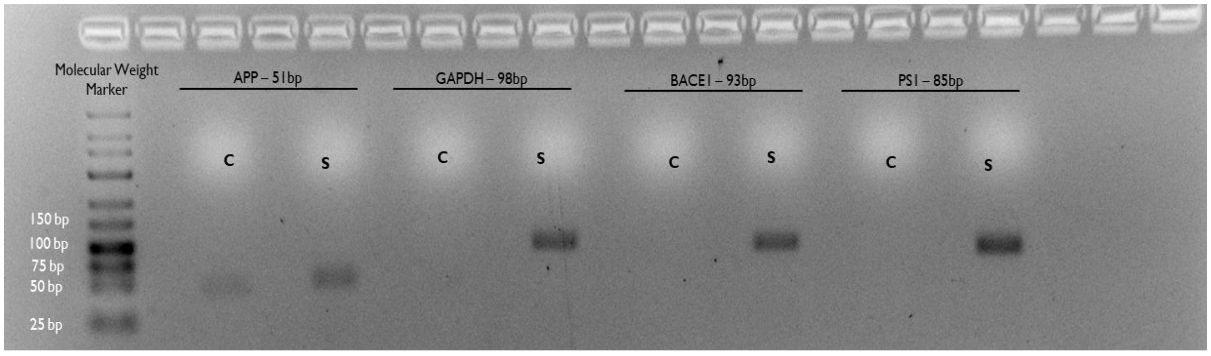

Figure 9. Agarose gel electrophoresis of the amplification for each target DNA after annealing temperature optimization. Where C is the negative control (only mix reagents without template) and S is the mix with DNA template.

The efficiency of the primers was determined using the equation:

$$\text{Efficiency (\%)} = \frac{10^{-\left(\frac{1}{\text{slope}}\right)} * 100}{2}$$

To calculate the slope value, 5 dilutions of cDNA (1:1, 1:3, 1:9, 1:27, 1:80) were prepared with control samples from the N2a695 cell line and run with the optimized conditions for the qPCR. The obtained Ct values were then plotted on a logarithmic scale along with their corresponding concentrations and a linear regression curve was generated ($R^2 \approx 0.99$). From the slope, the efficiency was calculated using the equation mentioned above. Figure 10 shows the primer efficiency obtained by qPCR for APP (108%), GAPDH (104%), BACE1 (93%), and PS1 (106%). The graphic analysis is crucial to determine how efficiently the primers amplify the target DNA, which is essential for ensuring accurate and reproducible results. An optimal amplification efficiency ranges from 90% to 110%, with any result above or below this range indicating a poor primer design, cross contaminations, or polymerase inhibition. So, based on Figure 10A, we can confirm that the primers are correctly designed and the conditions correctly optimized because all the primer pairs efficiency is in the ideal range. Analyzing the melting curves (Figure 10B), it can be confirmed that the primer is amplifying the desired

fragment because every sample only presents a main peak. If the curve had multiple peaks, it would indicate non-specific amplification (deviation to the right) or dimer formation (deviation to the left). In general, all genes were specifically amplified with no significant problems, except for the BACE1 gene as seen in Figure 10. Due to its low expression levels in the cell, detecting it in samples with lower concentrations can be challenging. However, once the sample corresponding to the 1:81 dilution was excluded, the amplification efficiency was within the expected range. It was important to consider the concentration range of the real samples. Upon analysis, the real samples exhibited Ct values that were higher than the removed ones from the calibration curve. This suggests that the range used for calculating efficiency is appropriate for studying BACE1 mRNA levels in the N2a695 cell line. Lastly, all samples amplified by qPCR were run in a 1% agarose gel electrophoresis to ensure correct amplification (data not shown) and the results were similar to Figure 9.

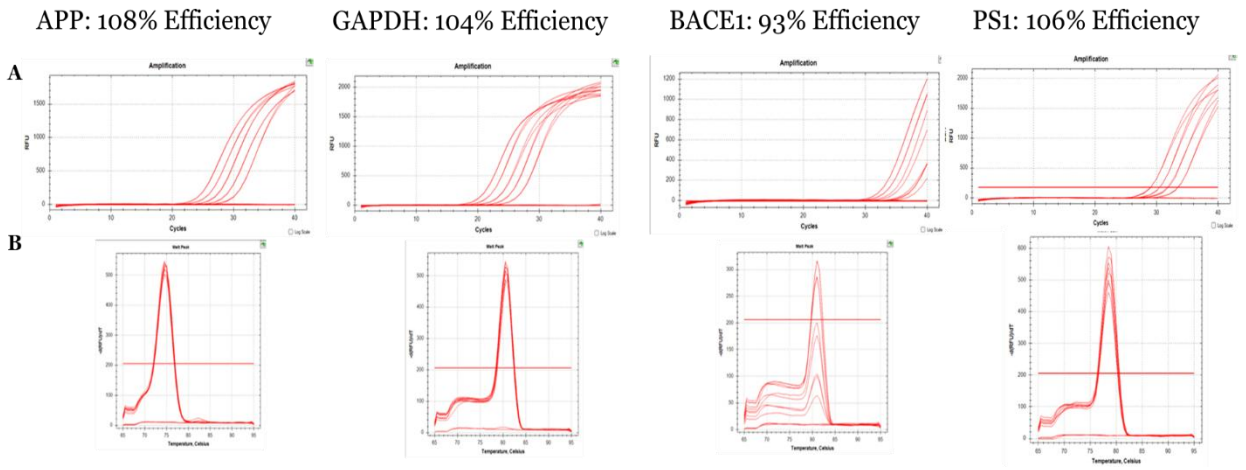

Figure 10. qPCR curves and efficiencies for APP, BACE1, PS1, and GAPDH primer pairs. A- Amplification curves; B- melting curves.

4.2. Analysis of the mRNA expression by qPCR

The N2a695 cell line was transfected with the miRNAs under study: miRNA-9-1-5p/-3p, miRNA-29b-1-5p/-3p, and its precursor forms pre-miRNA-9-1 and pre-miRNA-29b-1. After, the extraction of the total RNA of the cells using TRIzol was performed. The RNA samples were analyzed by electrophoresis in a 1% agarose gel to verify the integrity of the RNA and check for any DNA contamination. In the electrophoresis, presented in Figure 11, were visible three bands corresponding to ribosomal RNA (rRNA) 28s, rRNA 18s, and RNA of low molecular weight. To evaluate mRNA integrity, we focus on the rRNA bands because these are the most abundant in the eukaryotic cells with similar weight. So, observing the electrophoresis (Figure 11), it was confirmed the absence of DNA contamination, and the RNA extracted seems to be intact for qPCR evaluation.

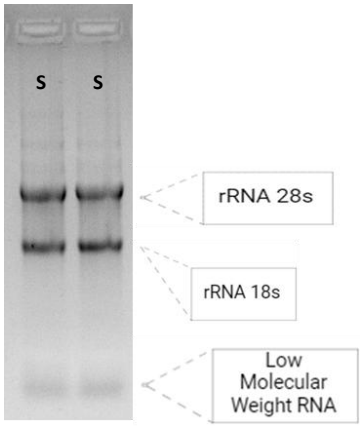

Figure 11. 1% agarose gel electrophoresis of the total RNA extracted from the transfected N2a695 cells. S-sample.

qPCR was used to study the effect of the target miRs and pre-miRs on the regulation of the expression of proteins associated with the AD pathological pathway (APP, BACE1, and PS1). With this study, we expected to determine the best way to interfere with altered protein expression. Figure 12 shows a comparison of the effect of each miRNA's specific strands (-3p and -5p) and its precursor form on the mRNA expression levels of APP, BACE1, and PS1.

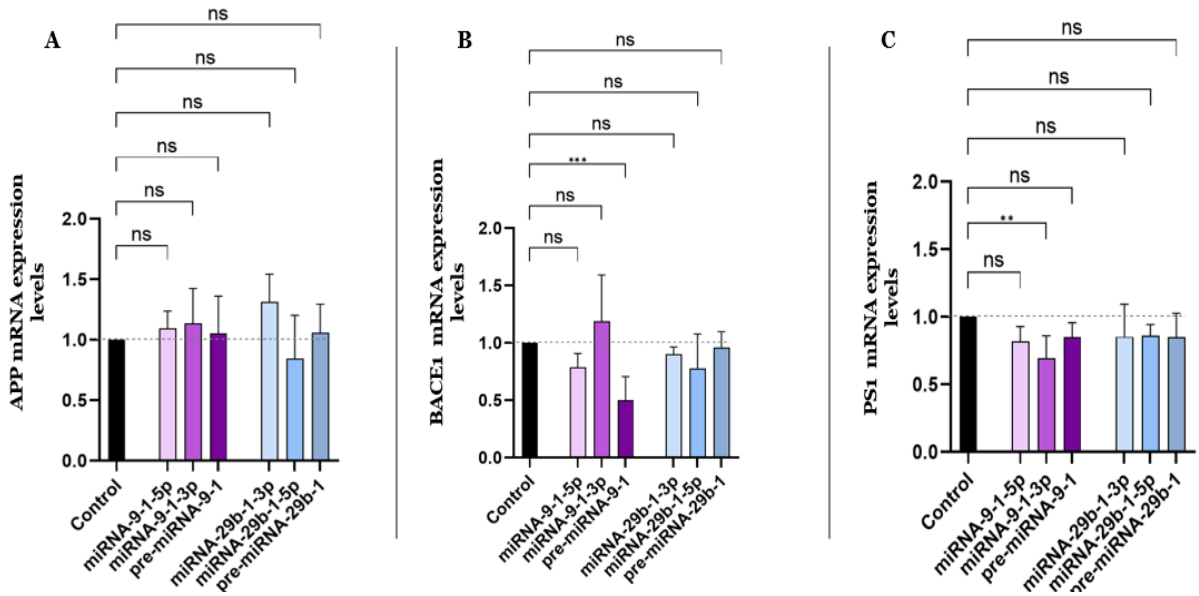

Figure 12. qPCR analysis of APP, BACE1, and PS1 mRNA expression levels, normalized to GAPDH mRNA, from N2a695 cells transfected with different types of miRNAs and pre-miRNAs. Control- non-transfected cells; ns- non-significant; A- Cells transfections performed with chitosan-nanoparticles, corresponding to the APP mRNA levels; B- Cells transfections performed with chitosan-nanoparticles, corresponding to the BACE1 mRNA levels; C- Cells transfections performed with chitosan-nanoparticles, corresponding to the PS1 mRNA levels. The error bars represent ± SD of 3 separate experiments. The results are considered statistically significant when $p<0.05$. Where ns means non-significant, ** means $p<0.002$ and *** means $p<0.0002$.

As illustrated in Figure 12A, according to the mRNA levels of APP, it seems that none of the RNAs transfected induces significant changes in the mRNA levels. However, there are significantly high error bars, therefore, we can not make an accurate statement. If we look at the tendency of the results, in general, the mRNA levels of APP seem to be not affected, although miRNA-29b-1-5p tends to decrease its expression, while miRNA-29b-1-3p tends to increase the APP mRNA expression. A study by Bastien and colleagues revealed that miRNA-29a and miRNA-29b-1 were implicated in the downregulation of BACE1. This downregulation indirectly impacted the levels of APP and Aβ, suggesting a propensity for these miRNAs to contribute to decreased APP expression. With the significant findings primarily associated with BACE1 levels (Bastien *et al.*, 2008). Given these findings, this tendency can be explained by the impact of miRNA-29b-5p on BACE1 levels as well. Even though the APP levels are subtle, this indirect effect mentioned via BACE1 downregulation aligns with the tendency observed in this work for the same miRNA.

By analyzing the mRNA levels of BACE1 (Figure 12B), it can be seen that the pre-miRNA-9-1 provokes 50% of silencing and the miRNA-9-1-5p 21% of silencing, in contrast to miRNA-9-3p which tends to increase the BACE1 expression. This suggests that when the pre-miRNA-9 is processed inside the cell, the -5p strand can be the one used in the RISC to regulate mRNA expression, being responsible for the silencing effect. A study by Bastien and coworkers showed that miRNA-9 can regulate BACE1 expression *in vitro*, supporting the results of this work when looking at the miRNA-9-1-5p effect (Bastien *et al.*, 2008). Based on these results, it can be concluded that the precursor form is more effective than the mature form, as documented in the literature. Their hairpin structure facilitates a more efficient recognition and processing in the cell, accelerating the processing by cellular machinery (Tsutsumi *et al.*, 2010). Regarding the effect of miRNA-29b-1 in the BACE1 mRNA levels, it seems that no significant effect is noticed, but it is not possible to accurately conclude about the influence on gene expression regulation, due to the significantly high error bars. However, the miRNA-29b-1-5p seems to induce some reduction in the mRNA levels, which is in line with other studies using miRNA-29b-5p, showing that if it is decreased, it would result in the overexpression of BACE1 (Duan *et al.*, 2023). However, with these results, it was not possible to conclude these findings, and it is suggested that if the cells had more time of incubation, maybe 72 h, it could result in more stable and better silencing effects.

In contrast with APP and BACE1, PS1 mRNA expression levels show a tendency to decrease with all miRs and pre-miRs tested, as demonstrated in Figure 12C. Comparing the effect of the different miRNA-9 forms, it seems that the 3p strand is a more promising one, inducing 30% silencing. An early study confirmed the downregulation of miRNA-9 and its

relationship with PS1 mutation, so its potential role in decreasing the mRNA levels of PS1, shown in this work, can be corroborated (Hemachandra *et al.*, 2017).

In general, although we tried to confirm the regulation of APP, BACE1, and PS1 by the miRNAs and pre-miRNAs under study, the results need confirmation due to the low silencing percentage and high error bars. As mentioned before, despite previous optimizations, the transfection protocol still needs optimization related to the RNA concentration and the incubation time needed for the cell to process these RNAs into an effective silencing mechanism. Reviewing the protocols from the previously mentioned studies, the N2a695 cell line was incubated for 72 h, resulting in a significant silencing effect on these proteins (Pereira *et al.*, 2016). This suggests that extending the incubation time in the current protocol could improve the silencing efficiency. In the same previous studies, the RNA concentration differed from that used in this work, ranging from 3.84 to 9.9 nM (Pereira *et al.*, 2016) and 10nM (Pereira *et al.*, 2020). However, other studies have used 20 nM of RNA (Sim *et al.*, 2016). This points out the importance of optimizing the protocol to ensure reliability and replicability in the result.

Chapter V
Conclusion and Future Perspectives

5. Conclusion and Future Perspectives

A new treatment for AD, or a way to stop its progression, has been highly investigated and has gained considerable interest over the years. Although there is still no cure, miRNAs have been an excellent tool for understanding the protein expression dysregulation in this disease, namely related to differences in the expression of APP, BACE, and PS1. Through the study of miRNAs, it was possible to discover their importance in regulating the expression of the proteins involved in the pathological pathway of AD. A wide range of miRNAs has been identified as potential tools in preventing the progression of AD and serve as biomarkers for this disease, as well as other neurogenerative diseases. In this study, we evaluated the effect of miRNA-9 and miRNA-29b, including their strands -5p and -3p, as well as the pre-miRNA-9 and pre-miRNA-29b, on the expression of proteins involved in the amyloid pathway, to try to determine which strand and which form can yield the best results as a innovate therapeutic agent.

By analyzing the qPCR results for APP and PS1, it was not possible yet to conclude with certainty if any miRNA could affect the mRNA expression. However, the miRNA-9-5p and its precursor form, pre-miRNA-9-1, have shown the best results in this work by silencing BACE1 mRNA levels. Also, this result allowed us to speculate that maybe the strand -5p of the mature form is the one that induces silencing when the pre-miRNA-9-1 is delivered and processed by the cell, also supporting the idea that the precursor form can be more efficient than the mature form, as various studies have proven. There are still a few studies about the difference between strands of the mature form and how they are selected for the RISC complex, so this study could help complement the information about miRNA's regulation of the amyloid pathway.

The silencing of these proteins is the key to suppressing or treating AD because of their role in the amyloid pathway. Decreased BACE1 mRNA expression is one of the most silencing interests because it is the main protein in the pathological pathway and that goal was achieved in this work. The results for APP may be explained by the fact that, in literature, it is proven that these miRNAs are complementary to the BACE1 mRNA rather than APP mRNA. This could explain why no-silencing effects were observed in this work. Since the APP is a natural protein with physiological functions, not being affected by the miRNAs does not necessarily imply a negative outcome. Given that APP is involved in both amyloid pathways, reducing its levels could potentially interfere with normal body processes.

The results for PS1 are interesting because this protein is also present in both amyloidogenic and non-amyloidogenic pathways. The results showed a tendency to decrease the mRNA expression. However, further investigation is required to determine if this decrease

would impact the non-amyloidogenic pathway or if it could be a positive result for halting the progression of this disease, having a similar outcome as the results for APP.

In conclusion, this work allowed the comparison of the effect of the mature and precursor forms of miRNAs in the expression of each protein (APP, BACE1, and PS1), specifically trying to evaluate which strand could cause the silencing mechanism. Although the results showed a more pronounced tendency in some miRNAs and proteins, there is still a need to improve the transfection method. It is necessary to perform an assay with different times of incubation, maybe increasing it to 72 h, to conclude if there is a more expressive silencing effect, and if so, try to select the best miRNA to silence the expression of these proteins responsible for AD, mainly BACE1. Additionally, transfection optimization is also needed to reduce the error bars and have more reliable results. In the future, it is intended to evaluate the protein levels as well, through the Western Blot technique, to support these results and see if the mRNA silencing can consequently lead to protein expression reduction.

Chapter VI
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6. References

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