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Evaluation of the role of miR-9 and miR-29 in amyloid pathway of Alzheimer's disease

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Resumo Alargado

A doença de Alzheimer (DA) é uma doença neurodegenerativa e a forma mais comum de demência, ocupando o terceiro lugar das patologias que causam incapacidade e morte nos idosos. Apesar do seu impacto, as causas desta doença são, ainda hoje, pouco compreendidas. Entender as vias e mecanismos que conduzem a uma transição entre um estado de saúde até à demência pode ajudar a desenvolver e estabelecer novos tratamentos mais efetivos. A DA caracteriza-se pela expressão desregulada de diversas proteínas envolvidas na formação de péptidos ß-amiloide, nomeadamente a proteína precursora amiloide (PPA), a proteína ß-secretase (BACE1) e a proteína presenilina 1 (PS1), sendo detetados níveis elevados nesta condição. Como estas proteínas fazem parte da complexa via patológica da DA, têm vindo a ser intensamente estudadas como alvos terapêuticos. No entanto, as terapias que utilizam inibidores dessas proteínas têm mostrado efeitos adversos e são muitas vezes descontinuadas, o que torna difícil, mas urgente, a criação de novas estratégias terapêuticas para controlar a progressão da DA.

Os microRNAs (miRs ou miRNAs) são moléculas de RNA não codificante, de baixo peso molecular, que têm como principal função regular o processo de tradução, com ação sobre o RNA mensageiro, permitindo uma diminuição dos níveis de expressão da proteína codificada. Os trabalhos de investigação utilizando miRNAs como estratégias terapêuticas para a doença de Alzheimer (DA) têm vindo a aumentar ao longo dos últimos anos. A ideia de utilizar os miRNAs como uma nova abordagem terapêutica nesta doença foi motivada essencialmente por dois fatores. Em primeiro lugar, estes miRNAs podem regular os RNA mensageiro (mRNAs) pós-transcricionalmente, e, consequentemente, controlar a expressão das proteínas responsáveis pelo surgimento da DA e, em segundo lugar, porque já foi amplamente descrito que os níveis de determinados miRNAs se encontram também desregulados, ocorrendo tendencialmente uma diminuição da sua expressão, na DA. Deste modo, os estudos que preveem o estabelecimento de uma relação entre os miRNAs e os seus mRNAs alvo, começaram a surgir e mostraram que certos miRNAs podem de facto controlar as principais proteínas relacionadas com os biomarcadores da DA. Os principais biomarcadores são os depósitos extracelulares de péptidos ß-amiloide juntamente com a acumulação intracelular da forma hiperfosforilada da proteína Tau associada aos microtúbulos. Apesar de alguns estudos mostrarem a regulação de proteínas pelos miRNAs na DA, esta é uma área vasta onde existem muito alvos ainda por descobrir, devido às múltiplas vias e funções que um só miRNA pode desempenhar.

Assim, o objetivo deste trabalho é estudar a nível celular, o papel de miRNAs cuja expressão se encontra diminuída na DA, dos quais foram selecionados o miR-29 previamente utilizado no grupo de investigação e o miR-9 que foi também relacionado com a regulação de vários mRNAs que codificam proteínas envolvidas em diferentes vias patológicas da DA. O foco

foi neste caso, nos miRNAs que apresentam níveis diminuídos na DA, considerando a possibilidade de entrega destes miRNAs às células, reestabelecendo a sua função e consequentemente contrariando assim o ambiente patológico existente na doença. Para isso foram utilizados miRNAs produzidos por métodos diferentes (síntese química ou enzimática), e apresentando a sua forma madura ou a forma precursora (pre-miRNAs), de modo a verificar a existência de um efeito biológico distinto entre eles. Os pre-miRNAs foram produzidos em laboratório através de transcrição *in vitro* utilizando um DNA plasmídico (pDNA) como template. Adicionalmente, foi também estudada a vantagem ou possível influência na atividade biológica, da introdução de um processo de purificação dos pre-miRNAs produzidos. De forma a avaliar a sua atividade biológica, as células N2A695 (linha celular de neuroblastoma de rato que expressa APP mutada, de maneira a replicar a via patológica da DA) foram transfetadas com os pre-miRNAs produzidos ou os miRNAs sintéticos, recorrendo à utilização de dois agentes de transfeção, como a Lipofectamina e nanopartículas de quitosano, que permitem igualmente a comparação destes sistemas. Após extração do RNA das células foi feita a avaliação dos níveis de expressão dos mRNAs que codificam as proteínas APP, BACE1 e PS1. Foram globalmente verificados efeitos de silenciamento para a APP pelo pre-miR-9 e para a BACE pelo miR-29b e pelo pre-mir-29b. A PS1 foi a proteína para a qual se verificou um efeito mais predominante, em termos de silenciamento, uma vez que todos os tipos de miRNAs/pre-miRNAs utilizados conduziram ao seu silenciamento.

Em suma, um melhor entendimento de como os miRNAs atuam e quais os seus principais alvos, poderá ter um grande impacto na indústria farmacêutica, pois em patologias onde estes são reguladores das vias patológicas, como é o caso da DA, poderão ser utilizados como uma nova abordagem terapêutica, de forma a regular a expressão de proteínas alteradas nesse ambiente patológico.

Palavras-Chaves

Doença de Alzheimer, miR-9, miR-29, Via Amiloide, Silenciamento Genético

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder which occupies the 3rd place of the diseases that cause disability and death in the elderly, but their causes are yet in need of a better understanding. Also, the search of the etiopathological path leading from a healthy state to full-blown dementia could help with the establishment of more effective treatments. MicroRNAs (miRs or miRNAs) are small regulatory non-coding RNAs (sncRNA), which are involved in post-transcriptional gene expression regulation. Diverse studies have shown that the expression levels of several miRNAs are decreased in AD patients and, there is also evidence that certain miRNAs can control the pathologic hallmarks related to the biomarkers of AD. The main biomarkers of AD are extracellular deposits of amyloid-β peptide (Aβ) along with an accumulation of intraneuronal hyperphosphorylated form of the microtubule-associated protein Tau.

The aim of this dissertation is to study the role of two under-expressed miRNAs in AD, miR-29 and miR-9, that are related to the regulation of messenger RNA (mRNAs) that encode proteins involved in various pathological pathways of AD. The selection of miRNAs with decreased levels was based on the possibility of performing its delivery into the cells, in order to contradict the pathological environment of AD. To accomplish this issue, synthetic miRNAs and precursors of miRNAs (pre-miRNAs) produced by two different methods (chemical and enzymatic synthesis, respectively) were used, in order to evaluate if the preparation method had influence in the biological activity. Thus, the pre-miRNAs were produced in the laboratory through *in vitro* transcription using a plasmid DNA (pDNA) as a template and were further subjected to a purification step. To evaluate the biological activity of the miRNAs, N2a695 cells (mutated APP expressing neuroblastoma mouse cell line that mimics the pathological pathway of AD) were used as *in vitro* model. Two different methods were also used for cells transfection, Lipofectamine and chitosan-nanoparticles for RNA encapsulation and delivery. After RNA extraction, the mRNA expression levels were evaluated for the APP, BACE1 and PS1 proteins. The findings demonstrated a silencing effect on the expression of APP mRNA by pre-miR-9 and of the BACE mRNA by miR-29b and pre-miR-29b. In turn, PS1 mRNA levels were decreased after cells transfection with all types of miRNAs/pre-miRNAs used in this study, being the most prominent result obtained by RT-qPCR. Western blot assays were also performed, in order to quantify the expression levels of the proteins in study.

In conclusion, a better understanding of how miRNAs act and what are their specific targets may have a major impact on the pharmaceutical industry, because being regulators in the pathologic pathways in certain diseases, such as AD, they can be used as new therapeutic approaches to regulate altered proteins expression in this pathological environments.

Keywords

Alzheimer's Disease, miR-9, miR-29, Amyloid Pathway, Gene Silencing

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List of Acronyms

15-LOX	Lipoxygenases
ABCA1	ATP-binding cassette transporter
AGO	Argonaut protein
AKT	Protein kinase B
ApoE4	Apolipoprotein E4
APP	Amyloid precursor protein
Aß	ß-amyloid peptide
BACE	ß-secretase
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CD2AP	CD2-associated protein
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
cDNA	Complementary DNA
CFH	Complement factor H
CNS	Central nervous system
CSF	Cerebrospinal fluid
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagles's medium
DNA	Deoxyribonucleic acid
DNAi	DNA Interference
dNTPs	Deoxyribonucleotide triphosphate
dsRNA	Double stranded RNA
E. coli	*Escherichia coli*
EGTA	Ethylene glycol tetra acetic acid
EPHA1	Ephrin type-A receptor 1 protein
Fw	Forward
GAPDH	Glyceraldehyde-3-phosphate
GSK3B	Glycogen synthase kinase 3 ß
GTP	Guanosine triphosphate
IRAK	Interleukin-1 receptor-associated kinase
LB	Luria broth
Lin-14	Lineage protein 14
Lin-4	Lineage-4
Lipo	Lipofectamine 2000
lncRNAs	Long non-coding RNAs
LPA	Logopenic aphasia
MAP	Microtubule associated protein

MAPT	Microtubule-associated protein tau
Min	Minutes
miR/miRNA	microRNA
miRISC	microRNA-induced Silencing Complex
MRE	microRNA response elements
mRNA	Messenger RNA
N2a695	Human mutated APP695 cellular line
ncRNAs	Non-coding RNAs
NFH	Neurofilament heavy chain
NFT	Neurofibrillary tangles
NMDA	N-methyl D-aspartate
NR2E1	Nuclear receptor subfamily 2 group E member 1
Nt	Nucleotides
PACT	Protein kinase R-activating protein
P-bodies	Processing bodies
PCA	Posterior cortical atrophy
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PEI	Poly(ethylenimine)
PET	Positron emission tomography
PHFs	Paired helical filaments
PI3K	Phosphoinositide 3-kinase
piRNAs	PIWI-interacting RNA
PLL	Poly(L-lysine)
PMSF	Phenylmethylsulfonyl fluoride
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PS	Presenilin
PSA	Ammonium persulphate
PSEN1	Presenilin1 gene
PSEN2	Presenilin2 gene
PVDF	Polyvinylidene difluoride membrane
qPCR	Quantitative polymerase chain reaction
RBPs	RNA-binding proteins
RNA	Ribonucleic acid
RNAi	RNA Interference
RNPs	Ribonucleoprotein
Rpm	Rotation per minute
rRNA	Ribosomal RNA
RT	Room temperature

RT-qPCR	Real-time quantitative PCR
Rv	Reverse
sAPPα	Secreted APPα
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
Sec	Seconds
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT1	NAD-dependent deacetylase sirtuin-1
sncRNA	Small non-coding RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SPT	Serine palmitoyltransferase
SYN-2	Synapsin II
TB	Terrific broth
TEMED	Tetramethylethylenediamine
TLR-7	Toll-like receptor 7
TRBP	TAR RNA binding protein
tRNA	Transfer RNA
TSPAN12	Tetraspanin-12

List of scientific communications

Poster Communications related with this Thesis

1. **Micaela Riscado**, Bruno Baptista, João A. Queiroz, Patrícia Pereira, Fani Sousa. The role of miR-9 and miR-29 biopharmaceuticals in Alzheimer's disease. III International Congress in Health Sciences Research: Towards Innovation and Entrepreneurship - Trends in Aging and Cancer. 14-16 november 2019, Covilhã, Portugal.

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Chapter I

Introduction

1.1 Ribonucleic Acid

Ribonucleic acid (RNA) is a polyanionic macromolecule, chemically and structurally similar to deoxyribonucleic acid (DNA), however, its single chain of four different nucleotides (nt), make it a simple source of genetic information, comparing to the double chain of the DNA (Sharp, 2009). Up until 1950, RNA was seen as a simple intermediary in the information flux between DNA and proteins (Alberts *et al.*, 2002). The transcription process depends on a gene (a short sequence of DNA that encodes for a protein), that is recognized and used as a model sequence by the RNA polymerase to produce a complementary RNA strand, called messenger RNA (mRNA). Following the rules of the genetic code, mRNA suffers translation into a protein, where the nucleotide triplets, also known as codons, found in the mRNA correspond to an amino acid in proteins. The genetic code is described as redundant because some amino acids are encoded by more than one triplet. This feature explains how 64 combinations of three nucleotides can be translated into only 20 different amino acids. For this connection between codons in mRNA and amino acids to exist, it is needed an adaptor molecule, the transfer RNA (tRNA) that is composed by 3 loops (Alberts *et al.*, 2002; Nahalka, 2019). One of the loops of the tRNA contains the anticodon - a complementary sequence of three nucleotides to the mRNA's codon – and, the 3' end has the complementary amino acid. The protein synthesis takes place in the cytoplasm, in a complex catalytic element (called ribosome), consisting of more than 50 different proteins and several RNA molecules, known as ribosomal RNAs (rRNAs). The ribosomal complex is composed by two subunits: the small subunit providing an accurate match between tRNA and mRNA, while the large subunit catalyzes the peptide bond between the carboxyl group at the end of a growing polypeptide and the amino group of the new amino acid to be incorporated on the sequence (Alberts *et al.*, 2002; Nahalka, 2019).

In 1990, Andrew Fire and Craig Mello discovered the RNA interference (RNAi) (Fire *et al.*, 1998), and showed that the RNA is more than a simple intermediate molecule in the genetic information transfer from DNA to proteins. Thus, RNA is currently recognized as a fundamental molecule for the regulation of gene expression (Sharp, 2009).

1.1.1 RNA Interference

RNAi promotes the post-transcriptional regulation of target genes by inducing translation blockage or degradation of the corresponding mRNA. Several studies have shown that RNAi is a well-conserved, endogenous mechanism in different species. In 1993, Ambros and colleagues identified the first RNAi in the nematode *Caenorhabditis elegans*, characterizing it as a non-protein-coding transcript named lineage-4 (lin-4). Lin-4 was shown

to negatively regulate lineage protein 14 (lin-14) by imperfect complementary binding to its 3' UTR mRNA (Lee *et al.*, 1993; Wightman *et al.*, 1993). In this species, the downregulation of lin-14 mRNA by the lin-4 helps to progress the embryo from the first to the second larval stage of development (Amakiri *et al.*, 2019; Mockly and Seitz, 2019). In the beginning, it was thought that the double-stranded RNA (dsRNA) had to unwind, so the antisense strand be able to bind to mRNA, however, the complete strand was never detected. In turn, Hamilton and Baulcombe discovered strands of antisense RNA with approximately 25 nt (short forms), which led the researchers to think that these short forms were needed for RNAi final function (Hamilton and Baulcombe, 1999). Posteriorly, several studies demonstrated the same result, suggesting that dsRNA was always cleaved in small intermediates, to which was given the name of small interfering RNAs (siRNAs). siRNAs have a complementary sequence for target mRNA transcripts and they had already been discovered in plants and mammals (Sen and Blau, 2006). Hence, the regulation of the expression of genes by RNAi occurs at a post-transcriptional level, through non-coding RNA molecules (ncRNAs) by blocking the translation or inducing the degradation of their target mRNA via sequence-specific (Ramachandran and Ignacimuthu, 2013).

There is a large group of ncRNAs-regulated gene sequences, some of which playing important roles in a variety of diseases, leading to a line of thinking that the RNAi can be considered for therapeutic proposes. The ongoing research on RNAi-based technology demonstrates that it possesses attractive characteristics such as high specificity, high efficiency, ability to induce a robust silencing of the targeted genes and the possibility to promote long-lasting therapies. In addition, some recent studies suggest that the dosage required for ncRNA therapeutics can be low, which can reduce the occurrence of undesirable adverse effects in the patients, one of the biggest problems encountered in the development of therapeutics (Burnett and Rossi, 2012; Deng *et al.*, 2014).

Although ncRNAs are not protein-coding genes, this does not mean that they are less important. On the contrary, they are important regulatory molecules in several processes, and cellular mechanisms related with development (heterochromatin formation and differentiation), viability (cell cycle regulation, stem cell self-renewal and epigenetic control) and integrity of the cell. For example, in neurodegenerative disorders, ncRNAs can affect the pathology by epigenetic and translational regulation, alternative splicing, mRNA stability change, and molecular decoys (Nahalka, 2019; Svoboda, 2014). ncRNAs can be classified into two main groups according to their length. So, if they have above 200 nt are called long non-coding RNAs (lncRNAs), and if they have bellow 200 nt are named small non-coding RNAs (sncRNAs). Both types of ncRNAs are particularly abundant in the central nervous system (Dogini *et al.*, 2014; Nahalka, 2019). LncRNAs appear later than sncRNAs, however, they maintain features common to protein-coding genes including promoter regions, intron-exon and alternative splicing. Compared to the sncRNAs, the lncRNAs are mainly localized in the

nucleus, less polyadenylated, more tissue-specific, less conserved and expressed at lower levels. To date, few lncRNAs have been discovered with specific biological functions and biochemical mechanisms, but it is already known that about 40% of lncRNAs are associated with chromatin-modifying complexes, leading transcription factors to activate or repress the gene expression (Nahalka, 2019). In turn, sncRNA has crucial roles in neuronal development and differentiation, synaptic plasticity and memory formation (Nahalka, 2019). For a classification and characterization, sncRNAs can be subdivided via biogenesis and mode of action in infrastructural RNAs [rRNA, tRNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA)] and regulatory RNAs [microRNA (miRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA) and PiWI-interacting RNAs (piRNAs)] (Li *et al.*, 2017).

1.1.2 MicroRNA

MicroRNAs are a class of endogenous sncRNAs of approximately 22 nt, with a role on the regulation of gene expression at post-transcriptional level, allowing them to shape the cell's proteome in a highly regulated manner, sensitive to the outside influence and internal feedback (Long *et al.*, 2019; Mockly, 2019; Nahalka, 2019). Distinct miRNAs are expressed in various cells and tissue types with different expression levels, for example, miR-122 has specificity in the liver, while miR-124 has specificity in the brain (Mott and Mohr, 2016). In turn, the genomic location of miRNA genes also varies, as they can be found in intra- or inter-genetic regions, and, therefore can be divided into two classes: miRNA associated with exons, forming the majority, or with introns (Amakiri *et al.*, 2019).

MiRNAs regulation can occur at several levels, including at the transcriptional level and at each step of its biogenesis. The first step of the biogenesis is the transcription of miRNA genes into a long primary transcript, known as primary-miRNA (pri-miRNA), with 1-4 kilobases by RNA polymerase II. These pri-miRNAs are capped at the 5′ end and polyadenylated at the 3′ end, forming double-stranded stem-loop structures. In the nucleus, the pri-miRNAs are recognized and cleaved by two proteins, namely, enzyme ribonuclease III Drosha and their regulatory subunit, the double-stranded RNA-binding protein called DiGeorge Syndrome Critical Region 8 (DGCR8), which dimerize to form a functional microprocessor complex (Amakiri *et al.*, 2019; Nilsen, 2007; Slota and Booth, 2019).

The cleavage of the pri-miRNAs produces a long stem-loop precursor of miRNA, called precursor miRNA (pre-miRNA), with a hairpin secondary structure of approximately 70 to 100 nucleotides, with two nucleotides overhang at its 3' end. After nuclear processing, the pre-miRNAs are transported to the cytoplasm by a nuclear transport receptor complex, Exportin-5/Ran-GTP (3' end-recognize). Once in the cytoplasm, pre-miRNAs are digested by a complex that contains the ribonuclease III Dicer, its co-factors TAR RNA binding protein (TRBP) and

Protein kinase R-activating protein (PACT) that removes the terminal loop. As a consequence, pre-miRNAs are converted into mature double-stranded miRNAs duplex (miRNA-miRNA* duplexes) with approximately 19-25 nt. After Dicer cleavage, mature miRNAs are associated to Argonaute (Ago) proteins, which are core components of RNA-induced silencing complex (RISC) (Amakiri *et al.*, 2019; Nilsen, 2007; Slota and Booth, 2019). The guide strand is selected based on the thermodynamic stability of the two ends of miRNA duplexes. The strand more stable at the 5' end (or sense strand, also referred to as miRNA*) is usually degraded by Argonaute-2 protein (Ago2), while the strand less stable at the 5' end (or antisense strand, also referred to as miRNA) is incorporated into the RISC, forming miRNA-induced Silencing Complex (miRISC), as represented in figure 1. In some cases, both strands can serve as mature functional miRNAs, depending on the cell type (Amakiri *et al.*, 2019; Nilsen, 2007; Slota and Booth, 2019).

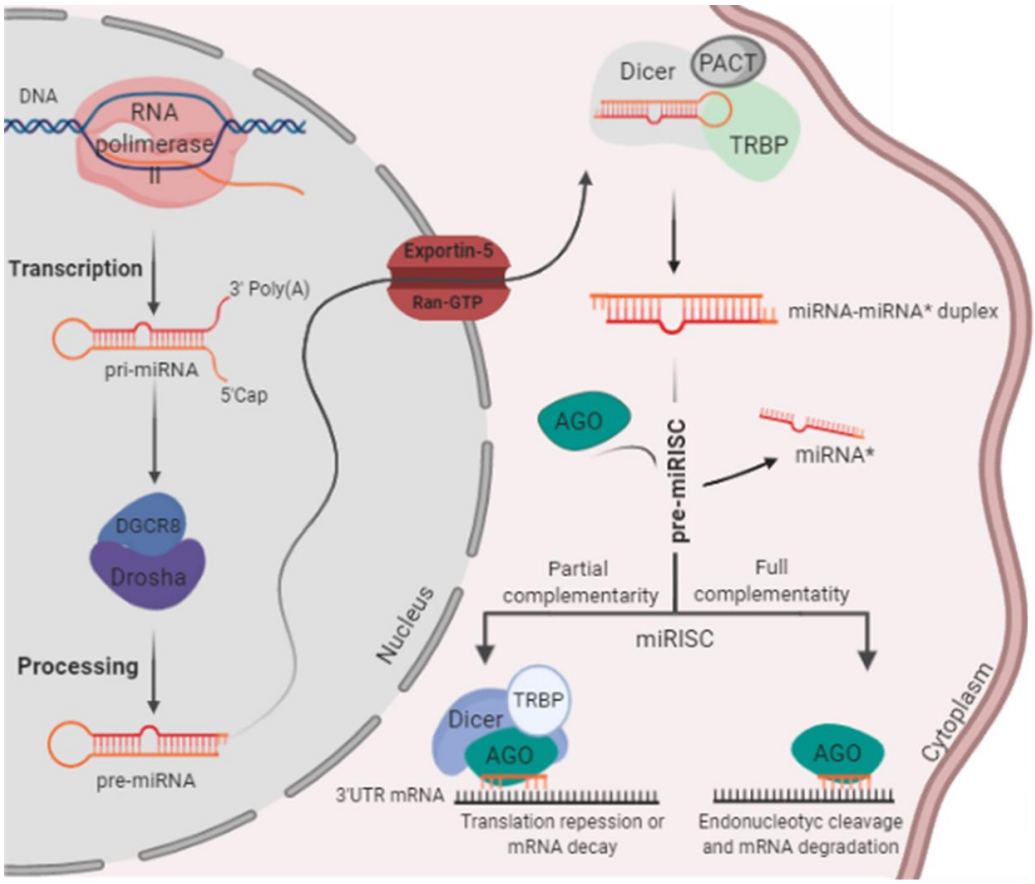


Figure 1. Schematic representation of the miRNA biogenesis (Adapted from Saraiva *et al.*, 2017).

For the formation of cytoplasmic ribonucleoprotein (RNPs) complexes, including miRISC, a direct interaction between two families of RNA-binding proteins (RBPs), AGO and GW182, is required. The RBPs are involved in the regulation of gene expression, and GW182 proteins, known for their glycine and tryptophan repeat motifs, are markers of processing bodies (P-bodies), which are dynamic cytoplasmic structures that contain untranslated

mRNAs. P-bodies are involved in the cellular stress response and contain proteins involved in diverse post-transcriptional processes (mRNA degradation, nonsense-mediated mRNA decay, translational repression and RNA-mediated gene silencing). In addition of being bound to P-bodies, the GW182 proteins bind to RNA through one or more globular RNA-binding domains and alter their fate or function. Regarding the AGOs proteins, these use single-stranded small nucleic acid molecules for RNA or DNA silencing (Nahalka, 2019; Perconti *et al.*, 2019). For example, prokaryotic AGOs participate in host defense by DNA interference (DNAi), while eukaryotic AGOs control a wide range of processes through the RNAI mechanism. Several studies in eukaryotes have indicated that, among the AGO proteins, AGO2 is catalytically active and involved in the mRNA cleavage process, whereas AGO1, 3 and 4 are catalytically inactive and are mainly involved in the translational repression. If AGOs and GW182 are in the nucleus, they are involved in the transcription and splicing control. However, if in the cytoplasm, they have a role in miRNA-mediated repression (Nahalka, 2019; Perconti *et al.*, 2019).

Thus, the miRISC complex composed by the AGO proteins and miRNA has endonucleolytic activity (PIWI domain), repressing target mRNAs by base-pairing (at least 7 to 8 nt of complementarity) with the "seed sequence" miRNA (corresponding to 2–8 nt localized at the 5' end of the antisense strand miRNA). However, miRNAs usually bind with partial complementarity to the 3'UTR of the target mRNAs, resulting in their cleavage or inhibition of protein synthesis (Long *et al.*, 2019; Mockly, 2019; Slota and Booth, 2019). Actually, depending on the degree of complementarity between miRNA and the target mRNA different outputs can occur. The mRNA undergoes endonucleolytic cleavage, when the complementarity is perfect, but, if the complementarity is not total, what is the most common in mammalian, the target mRNA is translationally repressed, through interference with the translational machinery (directed to polyribosomes in subcellular compartments, resulting in truncated protein) or by sequestering in cytoplasmic P-bodies (where mRNAs are degraded through exonuclease enzymes or de-adenylated by recruitment of de-adenylating enzymes, poly(A) nucleases). Because of the many ends that the miRNA can give to mRNA in eukaryotes this is a process still in study to better comprehension (Long *et al.*, 2019; Mockly 2019; Nilsen, 2007; Slota and Booth, 2019).

Nowadays, it is known that the main mode of action of mature miRNA is based on the recognition of specific sites [known as microRNA response elements (MRE)], typically present in the 3'UTR of their target mRNAs, however, some miRNAs can bind specific sites in the 5'UTR of the target mRNA with less frequency and efficiency of binding (Amakiri *et al.*, 2019; Long *et al.*, 2019; Mockly, 2019). At present, miRbase lists 1917 precursors and 2654 mature *Homo sapiens* miRNAs that recognize the 3'UTR of many genes. These data indicate that over 60% of human protein-coding genes show predicted miRNA target sites, indicating that miRNAs can control the expression of more than half of all proteome. Thus, each miRNA can

regulate many, even hundreds of different mRNA molecules, and multiple miRNAs can regulate the same mRNA that is likely to act synergistically (Long *et al.*, 2019; Nahalka, 2019; Selbach *et al.*, 2008; Slota and Booth, 2019). Overall, the miRNAs can regulate a variety of physiological and biological processes including: differentiation, developmental, cell cycle, proliferation, adhesion, apoptosis, stress response, metabolism, stem cell self-renewal, hematopoiesis, embryogenesis, neurogenesis and cellular communication (miRNAs are also released from cells in small extracellular vesicles that can be internalized by other cells). As a result of its versatility in several biologic processes, coupled with its efficacy and potential to inhibit the expression of a specific mRNA, miRNAs can be explored as therapeutic agents in certain pathologies that have genetic origins (Selbach *et al.*, 2008; Slota and Booth, 2019). Indeed, miRNAs-based therapeutics have been proposed in many disorders such as cancer, heart disease, diabetes and neuroinflammation (Nowek *et al.*, 2018; Sethupathy, 2016). Hence, the ability to control the expression of *in vivo* miRNA will serve as the basis for the development of treatments. Some strategies for controlling these levels have already been developed. For example, in order to raise miRNA levels, it is possible to use miRNAs mimics, small synthetic double-stranded molecules processed into functional miRNA, miRNA expression vectors to induce expression of a miRNA in cells and the delivery of the miRNA itself. In turn, to decrease miRNA levels, antagomirs and miRNA sponges, both synthetic sequences complementary to the miRNAs can be applied, blocking the binding to endogenous targets mRNA (Simonson and Das, 2015; Slota and Booth, 2019). Some of the therapeutic treatments with miRNAs, which are presently used in clinical trials are described in Table 1.

Table 1. Potential therapeutic applications of miRNA drugs currently in clinical trials (Updated as of 19-10-2019 a www.clinicaltrials.gov).

Company/ Institutions	miRNAs/Therapy	Disease	Status /Development
Chang Gung Memorial Hospital, Taiwan	miR-29a precursor	Shoulder Stiffness	Recruiting Clinical Trial
Cancer Prevention Research Institute of Texas	miR-RX34	Primary Liver Cancer, SCLC, Lymphoma, Melanoma, Multiple Myeloma, Renal Cell, Carcinoma, NSCLC	Terminated Clinical Trial Phase 1
Mirna Therapeutics, Inc.	miR-34	Melanoma	Withdraw Clinical Trial Phase 1, Phase 2

1.2 Dementia

According to *The World Health Organization*, dementia is the 5th largest cause of death in the world (World Health Organization, 2018). Dementia describes a group of symptoms affecting memory, thinking, social abilities, severely enough to interfere with daily life (Lane, 2018). Age is indicated as the biggest risk factor for the development of many forms of dementia. Actually, it is estimated that dementia affects approximately 47 million people worldwide and that by 2050, this number can reach about 131 million people (World Alzheimer Report, 2015). The increased prevalence of these diseases results from the growing aging population due to increased life expectancy, which causes more people living long enough to be affected. This growing number of people affected and the high impact that these diseases have on the quality of life, not only of patients but also of their families, are clearly arguments to make the dementia treatment an attractive opportunity for pharmaceutical companies (Lane, 2018; Reynolds, 2019). *The World Health Organization* has recognized Alzheimer's disease (AD) as the most common and devastating form of dementia of our time, where two-thirds (50-75%) of the people affected can face death in approximately 8.5 years after the onset of symptoms (Lane, 2018; Reynolds, 2019).

1.2.1 Alzheimer's Disease

AD is a multifactorial, progressive, chronic neurodegenerative disorder that occupies the 3rd place in the diseases that causes disability and death for the elderly, after cardiovascular/cerebrovascular diseases and malignant tumors. The death rate of AD is higher than the rate obtained for breast cancer and prostate cancer. It was estimated that there are about 47 million people with AD worldwide, and due to advances in healthcare, the average life expectancy of the human species has increased, which can contribute to the expected increased number of AD cases in the next few years. As a result, AD is associated with higher healthcare costs, leading to the estimation that the overall cost of health and social care reached 1 trillion dollars in 2018, which is expected to rise to 2 trillion dollars by 2030 (Du *et al.*, 2018; Martinez and Peplow, 2019; Nahalka, 2019; Wong *et al.*, 2019; World Alzheimer Report, 2015).

Alzheimer's disease was first described by Dr. Alois Alzheimer in his patient known only as "Auguste D.", in the early 20th century. The patient experienced memory loss, paranoia, and psychological changes. Dr. Alzheimer noted in the autopsy that there was shrinkage in and around nerve cells in the patient's brain (Alzheimer *et al.*, 1991). The pathological hallmarks of AD were first described in 1906 as being extracellular plaques, intercellular tangles and widespread neurodegeneration in the brain. Decades later, the ß-

amyloid peptide (Aß) and Tau were identified as the main constituents of these tangles and plaques (Glenner *et al.*, 1984). AD is commonly classified into two types: sporadic AD that can appear at any time in life, but usually appears after 65 and, familial AD that appears early-onset (between 30 and 50). The first form of AD – sporadic - is the most abundant and poorly understood, although it is thought that results from a combination of genetic (70%) – ABCA7, APOE and BIN1 -, and environmental (30%) factors (inflammatory, cholesterol metabolism and endosomal vesicle recycling pathways). In this case, when inflammatory pathways occur, microglia activation is initiated in response to amyloid peptide deposition, which is now recognized as a key factor in the pathogenesis of AD. The extremely uncommon form representing 1 to 2% of AD cases is the inherited autosomal, dominant (familial) form with clinical symptoms similar to sporadic AD, namely disease progression, biochemical and neuropathological changes (abnormal overproduction of amyloid-ß). This form is caused by mutations in three genes coding for amyloid precursor protein (APP), presenilin1 (PSEN1), presenilin 2 (PSEN2) proteins, which are linked to Aβ processing by γ-secretase complexes (Amakiri *et al.*, 2019; Lane, 2018; Long *et al.*, 2019; Martinez and Peplow, 2019; Reynolds, 2019). Generally, the risk factors for AD can also be divided into two types: modifiable and non-modifiable. The modifiable factors include poorly controlled type 2 diabetes, cardiovascular diseases (like stroke, hypertension), depression, traumatic brain injury, lifestyle and environmental factors (such as stress, alcohol, smoking, high blood pressure, high cholesterol, obesity, lack of exercise). In turn, the non-modifiable factors include genetic mutations, age, sex or genetic polymorphisms. An important example is a genetic mutation in apolipoprotein ε4 allele (APOE4), present in familial and sporadic AD. APOE isoforms have been difficult to determine, but it is known that it can promote Aß aggregation and impair Aß clearance in the brain, also participating in the regulation of glucose metabolism, neuronal signaling, and Tau-mediated neurodegeneration. Some examples of polymorphisms can be sortilin-related receptor 1, clusterin, complement component receptor 1, CD2-associated protein (CD2AP), Ephrin Type-A receptor 1 protein (EPHA1) and membrane-spanning 4-domains subfamily A (MS4A6A/MS4A4E) (Amakiri *et al.*, 2019; Reynolds, 2019; Wong *et al.*, 2019).

There are seven stages associated with Alzheimer's disease: preclinical (positive biomarkers but no cognitive impairment), prodromal (mild cognitive impairment), mild dementia, moderate dementia, severe dementia and, finally, death. The biological events leading from the first state to dementia are described in figure 2. Nowadays, AD is diagnosed using a combination of several tools, such as clinical examination by magnetic resonance imaging and positron emission tomography (PET) to detect Aß deposits; Cerebrospinal fluid (CSF) assays to detect biomarkers and neuropsychological tests which include cognitive performance or postmortem gross specimen analysis and histology of brain sections (Martinez and Peplow, 2019; Reynolds, 2019).

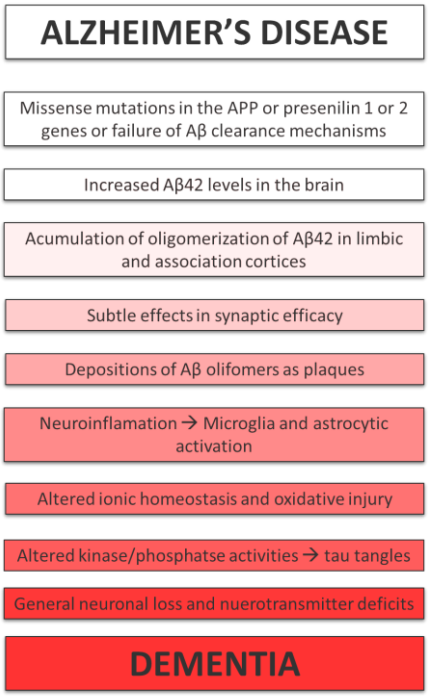


Figure 2. Overview of the main events leading from healthy state to full-blown dementia (Adapted from Lane, 2018).

Studies of biomarkers and PET scans suggest that signs associated with AD may be found in the patient brain 20 years or less before the first symptoms appear because the initial changes in the brain can be compensated. On the other hand, when the changes are no longer reversible, symptoms can gradually become apparent, thus, the first cognitive decline is visible when disease behavioral changes, impaired mobility, hallucinations and seizures occur. Then, it is visible the memory loss and in more serious cases basic daily functions are affected, leading to the inability of independently living. In the end, other clinical syndromes also emerge like posterior cortical atrophy (PCA), logopenic aphasia (LPA) and the frontal variant of AD, leading to dead (Amakiri *et al.*, 2019; Lane, 2018; Martinez and Peplow, 2019; Reynolds, 2019; Wong *et al.*, 2019). Histopathological and morphological examination of AD postmortem brains in combination with studies on transgenic mouse models with AD show multiple cellular changes, such as: cholinergic neuron damage, mitochondrial fragmentation, mitochondrial DNA damage, oxidative stress, inflammation, dystrophic neurites, astrogliosis, microglial activation, cerebral amyloid angiopathy, hormonal imbalance, altered neurotransmitter levels (mainly acetylcholine), neurofibrillary tangle and senile plaques. Downstream consequences of these processes include neurodegeneration with synaptic and neuronal loss, leading to macroscopic atrophy. These alterations are primarily observed in the learning and memory regions of the brain, including the entorhinal cortex and spread regions of the hippocampus, temporal cortex, frontoparietal cortex and subcortical nuclei (Amakiri *et al.*, 2019; Lane, 2018; Martinez and Peplow, 2019; Reynolds, 2019). Therefore, the biomarkers that are commonly used to document the underlying pathologic processes of AD are mainly three: amyloid deposition, pathologic Tau (microtubule-associated protein) and

neurodegeneration. Although the clinical characteristics and severity are better correlated with neurofibrillary tangles (NFT), data suggest that Aß pathology develops many years before clinical symptoms appear and, precedes Tau changes. Once the presence of the pathology of Tau can be related to the normal healthy aging process. It is still unclear how Aß and Tau are mechanistically linked, but some studies have suggested that this interaction occurs in the immune system, since activated microglia co-localize with amyloid plaques and some AD-risk genes are involved in immune system pathways (Lane, 2018; Nahalka, 2019).

Due to the complexity of this disease, the study of multiple molecular targets, mechanisms and pathways is still necessary, but several hypotheses have been proposed for the better understanding of AD. The most popular hypotheses are amyloid and Tau, where significant evidence supports an Aß-centered view of AD (Long *et al.*, 2019; Reynolds, 2019; Wong *et al.*, 2019).

1.2.2 Amyloid Hypothesis

The hypothesis of the amyloid cascade was proposed by Hardy in the early 1990s, where Aß forms, particularly $Aß_{42}$, sequentially led to the formation of neurotoxic oligomers, insoluble amyloid fibrils and, finally, amyloid plaques (Hardy and Allsop, 1991). Previously, in 1984, the scientists George Glenner and Caine Wong identified Aß as peptides of approximately 40 amino acids, initially described as a "novel cerebrovascular amyloid protein" (Glenner *et al.*, 1984). An amyloid peptide is generated by cleavage of transmembrane APP. This protein has non-pathogenic functions and has a vital physiological role in regulating metals by having metal-associated redox activity and by stabilizing the plasma membrane for iron transport. In the amyloidogenic pathway, APP is cleaved by the ß-secretase (BACE), an aspartyl protease with two isoforms BACE1 and BACE2, leading to the formation of sAPPß, that is then cleaved by γ-secretase complex to produce Aß. γ-secretase complex is composed by 4 subunits, including presenilins (PS) 1 and 2, nicastrin, anterior pharynx defective 1, and presenilin enhancer 2. Presenilin comprises the catalytic domain of γ-secretase, and the PS1 dysfunction has been directly linked to AD. γ-secretase cleavage is inconsistent, resulting in differences at the C-terminal end. These differences contribute to the existence of a variety of Aß peptides isoforms: $Aß_{1-36}$, $Aß_{1-37}$, $Aß_{1-38}$, $Aß_{1-39}$, $Aß_{1-40}$, $Aß_{1-41}$, $Aß_{1-42}$, and $Aß_{1-43}$. $Aß_{1-40}$ and $Aß_{1-42}$ are the most common isoforms, corresponding to the cleavage at the 40 and 42 positions, respectively. Moreover, $Aß_{1-40}$ is the most abundant form and is composed of two protofilaments, while $Aß_{1-42}$ is composed of only one protofilament. $Aß_{1-42}$ is slightly longer and less abundant, but is more hydrophobic and fibrillogenic, making it the main species accumulated in the brain of AD patients (Amakiri *et al.*, 2019; Ham *et al.*, 2018; Nahalka, 2019; Reynolds, 2019). The schematic representation of the possible processing pathways of APP is illustrated in figure 3. Hence, AD is not created by APP protein

but by its processing, because the balance between α-secretase/ß-secretase is inverted. So far, it is well known that almost all APP cluster mutations occur around the ß-secretase and γ-secretase cleavage sites, one example is the autosomal dominant mutation, V717I in the APP-coding genes, as discovered in 1991 (Goate *et al.*, 1991). In the non-amyloidogenic pathway, APP is initially cleaved by α-secretase, generating a soluble APPα ectodomain (sAPPα), which is further processed by γ-secretase in the middle, producing non-amyloidogenic fragments (Long *et al.*, 2019; Reynolds, 2019; Wong *et al.*, 2019). Although the exact pathogenic role of Aß is unknown, it is well documented that Aß toxicity depends on size, state of aggregation, diffusion in subcellular compartments and neuronal terminals. The pathogenicity of Aß is amplified when monomers become oligomers, leading to plaques formation. Several lines of evidence suggest that Aß accumulates in multiple cellular compartments like small cytoplasmic granules in neurites, perikaryal, trans-Golgi network, Golgi-derived vesicles, early and late endosomes, lysosomes, synaptic vesicles, and mitochondria (Amakiri *et al.*, 2019; Nahalka, 2019; Wong *et al.*, 2019).

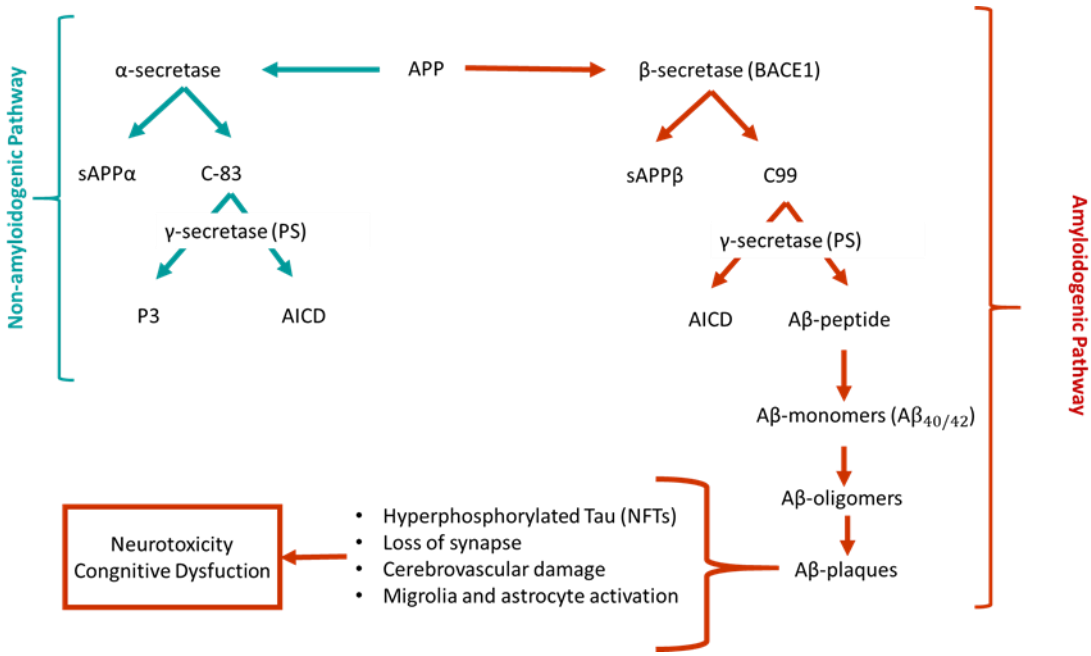


Figure 3. Schematic representation of the possible processing pathways of APP (Adapted from Kumar and Singh, 2015).

The intraneuronal accumulation of Aß peptides and amyloid plaques lead to a large number of neurotoxic processes such as loss of mitochondrial function, reaction with metal ions in the brain to produce reactive oxygen species (ROS) increasing oxidative stress, disruption of calcium homeostasis by Ca^{2+} influx to lipid vesicles or triggering of unregulated calcium flow through the plasma membrane, activation of microglia that release inflammatory mediators (inflammatory cytokines and chemokines) causing neuroinflammation

and neuritic alterations/synaptic distortions in cortical regions closer to the Aß plaques. Furthermore, these neurotoxic issues act as positive feedback, making it impossible to restore the original balance. In turn, several evidence suggest that Aß plays a role in inducing Tau hyperphosphorylation, leading to protein tangles, the second hallmark feature of AD (Amakiri *et al.*, 2019; Nahalka, 2019; Wong *et al.*, 2019).

1.2.3 Tau Hypothesis

Tau protein is a highly soluble microtubule-associated protein (MAP) encoded by the MAPT gene. Six Tau isoforms exist in human brain tissue and are mostly found in neurons. One of the Tau main functions is to modulate the stability of axonal microtubules, once the microtubules are naturally unstable, and require the interaction of Tau to maintain their structure. Tau is a phosphoprotein with 79 potential serine and threonine phosphorylation sites on the longest isoform, and its hyperphosphorylation results in disruption of microtubule organization (Guo *et al.*, 2017; Wong *et al.*, 2019). Under pathological conditions, the accumulation of hyperphosphorylated Tau in neurons leads to protein misfold and aggregation in intracellular NFTs, reducing their affinity for microtubules and influencing neuronal plasticity, causing neurodegeneration (Martinez and Peplow, 2019; Nahalka, 2019; Reynolds, 2019; Slota and Booth, 2019; Wong *et al.*, 2019).

1.2.4 Alzheimer's Disease Treatments

Treatments for AD have two main goals: 1) relieving cognitive symptoms, to improve the quality of life or maintaining cognitive and daily activity skills, and 2) slowing the progress of the disease. The problem is that the last goal is far from reality, because so far there are only six FDA-approved drugs to relieve AD symptoms (Amakiri *et al.*, 2019; Martinez and Peplow, 2019; Wong *et al.*, 2019). In 1970, the susceptibility of the cholinergic system was identified, leading to the emergence of the first effective drug for the treatment of cognitive symptoms of AD (Davies and Maloney, 1976; Francis *et al.*, 1999; Whitehouse *et al.*, 1982). Cholinesterase inhibitors are used in patients with mild to moderate AD, improving neurotransmission by acetylcholinesterase inhibition (hydrolysis of acetylcholine) in the synaptic cleft. Donepezil (Aricept®), Rivastigmine (Exelon®), Galantamine (Razadyne®) are currently approved drugs, with small but valuable clinical benefits. However, they cause adverse effects such as nausea, diarrhea and vomiting (Chu, 2012; Muir *et al.*, 1994; Reynolds, 2019; Sahakian and Coull, 1993; Weller and Budson, 2018). Another drug available to treat the cognitive problems of Alzheimer's disease is Memantine (Namenda®), an N-methyl-D-aspartate (NMDA) receptor antagonist prescribed for moderate-to-severe AD. The NMDA receptor is abundant in areas involved in cognition, learning, and memory. Memantine,

its antagonist, has moderate affinity that allows the physiological action of glutamate (NMDA ligand) without excitotoxicity (Chu, 2012; Lipton, 2006; McShane *et al.*, 2006; Reynolds, 2019; Weller and Budson, 2018). An alternative treatment is the use of antioxidants like selegiline, alpha-tocopherol (vitamin E) and vitamin D, but recent studies do not show consistent benefits. However, nutraceutical huperzine A shows benefits in memory and daily activities (Chu, 2012; Weller and Budson, 2018). In addition, one of the drug classes prescribed for AD patients are antipsychotics due to changes in their behavior, however, haloperidol and other antipsychotics have severe side effects like sedation, leading to physical injuries (Chu, 2012).

Considering all these reasons, limitations and adverse effects, it becomes clear the need to establish new therapeutic strategies, focusing on the pathologic pathway and recognizing the fact that there is an imbalance in the production and clearance of Aß peptides in the brain of AD patients. Some of the strategies under evaluation involve the use of secretase modulators, immunotherapy, amyloid binders, metal-chelating agents, anti-inflammatory and neuroprotective agents. For example, estrogens, corticosteroids, naproxen, ibuprofen, indomethacin, rofecoxib, tarenflurbil, rosiglitazone, xaliproden, and dimebon are some of the drugs already tested in the treatment of AD which, in the end, had negative results. Nevertheless, immunotherapy, specifically passive immunization with monoclonal antibodies directed to Aß peptides or Tau protein, have shown good results in the clearance of these proteins. Bapineuzumab has shown cognitive benefit in AD patients, while other antibodies have shown efficacy in animal models and even stimulate positive immune response in patient's trials (Golde *et al.*, 2013; Cummings *et al.*, 2018). Another therapeutic approach is the inhibition of enzymes involved in Aß production. Drugs targeting BACE1, like verubecestat, showed acceptable safety at doses that strongly reduce Aß levels in the plasma and CSF, but showed no cognitive or functional benefit (Kennedy *et al.*, 2016). γ-Secretase was another obvious target for inhibition with semagacestat. Unfortunately, target toxicity is inevitable due to the approximately 40 cellular substrates of the γ-secretase, leading to the closure of the clinical trials (Chávez-Gutiérrez *et al.*, 2012; Doody *et al.*, 2013). Although, γ-secretase modulators (Bursavich *et al.*, 2016; Hall and Patel, 2014) and γ-secretase stabilizers (Szaruga *et al.*, 2017) are still being tested (Chu, 2012; Reynolds, 2019; Weller and Budson, 2018; Wong *et al.* 2019). On the other hand, there are non-drug treatments that may also be recommended for AD patients, like the Mediterranean diet, regular aerobic exercise, and recreational physical activity (Chu, 2012; Weller and Budson, 2018).

Failures in AD treatment research are inevitable, but all research and information obtained so far can help in the identification of drug targets and the development of therapeutic strategies for this yet incurable disorder. Recent approaches point towards an alternative therapeutic strategy in neurodegenerative diseases, which can pass by the use of miRNAs as the next major therapeutic agents.

1.2.5 MicroRNAs and Alzheimer's Disease

Most reported miRNAs are found in the human brain and appear in complex interactive regulatory networks that govern the normal function of the brain and can be related with sporadic CNS diseases. As previously mentioned, several studies have demonstrated that miRNAs might play important regulatory roles in the molecular control of neuronal development, synaptic functions, neurotransmission, neurite outgrowth and neuroinflammation. Its levels can be detected in blood (mononuclear cells and erythrocytes), CSF, saliva and urine. miRNAs are stable and abundant, but their levels in these fluids are correlated with aging, diets and pathologic states. All of these reasons contribute to their potential as diagnostic biomarkers (Amakiri *et al.*, 2019; Martinez and Peplow, 2019; Nahalka, 2019).

In neurodegenerative disorders, such as AD, brain miRNA profiles are altered, so that their dysfunction may be a cause or a consequence for the onset of the disease, which is not yet well understood. The miRNA pool is controlled by various mechanisms, such as direct modification of miRNA sequences or their precursors that differ in length/sequence, sequestration, miRNA transport modulation and miRNA turnover modulation. Aß is also a powerful regulator of miRNA levels (Amakiri *et al.*, 2019; Long *et al.*, 2019; Martinez and Peplow, 2019; Nahalka, 2019; Slota and Booth, 2019). It is already clear that miRNAs are involved in many aspects of AD progression, including direct cell senescence, APP expression regulation, APP alternative splicing, BACE and Tau regulation, lipid metabolism and neuroinflammatory processes regulation. However, the identification of specific targets has been difficult due to the several pathways in which miRNAs participate and the interconnections of these paths, making it even more difficult to know if the influence on this target is direct or indirect. Therefore, it is important not to exclude a target just because there are no studies to support it. To try to identify miRNA targets some properties are taken into account, like if the mRNA in study has there expression level repressed in a miRNA-dependent way, if the loss of the mRNA interaction with the miRNA leads to a macroscopic phenotype, if mRNA interact physically with Ago proteins, if exhibits sequence complementarity to the miRNA or if their interaction is phylogenetically conserved due to biologically benefit. These are some common characteristic of the genes that are proven to be regulated by miRNAs. Some of the miRNAs and their targets already involved in or associated to AD are summarized in Table 2 (Long *et al.*, 2019; Mockly, 2019; Nilsen, 2007; Slota and Booth, 2019).

Table 2. Dysregulated miRNAs in Alzheimer's disease.

miRNA	Expression Levels	Target Proteins	References
Let-7	Down	TLR-7, APP	Lehman *et al.*, 2012; Maes *et al.*, 2009
miR-9	Up/**Down**	**BACE1, PSEN1,** NFH, SIRT1, MAPT, SPT, CFH, NR2E1	Batistela *et al.*, 2017; Delay *et al.*, 2012; Idda *et al.*, 2018; Li and Wang, 2018; Maes *et al.*, 2009
miR-15b, -29a, **-29b,** -29c, -107, -124, -186, -195, -328, -485-5p	Down	**BACE1**	Boissonneault *et al.*, 2009; Faghihi *et al.*, 2010; Hébert *et al.*, 2009; Geekiyanage and Chan, 2011; Lang *et al.*, 2016; Li and Wang, 2018; Jiao *et al.*, 2016; Kim *et al.*, 2016; Yang *et al.*, 2015; Zhu *et al.*, 2012
miR-16	Up	APP	Parsi *et al.*, 2015
miR-17, -20a, -27a -3p, -101a-3p, -147, -153, -323-3P, -644, -655	Down	APP	Barbato *et al.*, 2014; Boissonneault *et al.*,2009; Delay *et al.*, 2011; Hébert *et al.*, 2009; Liang *et al.*, 2012; Lin *et al.*, 2018., 2018; Long *et al.*,2012; Zhang *et al.*, 2014;
miR-21	------	PI3K, AKT, GSK3β	Feng *et al.*, 2018
miR-34	Up	TAU, SIRT1	Inukai *et al.*, 2012; Mariño *et al.*, 2010
miR-106b	Down	ABCA1, APP	Noren *et al.*,2010; Qiu *et al.*,2015
miR-125b	Up	CDKN2A, SYN-2, 15-LOX	Sethi and Lukiw, 2009; Van den Hove *et al.*, 2014
miR-137	Down	MAPT, SPT	Geekiyanage and Chan, 2011; Smith *et al.*, 2011

miR-146a	Up	CFH, IRAK-1, TSPAN12	Sethi and Lukiw, 2009; Van den Hove *et al.*, 2014
miR-155	Up	APP, CFH	Hébert *et al.*, 2009
miR-181	Down	SPT	Geekiyanage and Chan, 2011; Rodriguez *et al.*,2014

This study is focused on miR-29b previously used in the research group and miR-9 which is related to the regulation of various mRNAs encoding proteins involved in pathological pathways of AD. MiR-29 is a highly conserved family in humans, mice and rats. The elements of this family are miR-29a and miR-29b-1 transcribed from chromosome 7 (7q32.3), miR-29b-2 with mature sequences identical to those of miR-29b-1 (both called miR-29b) and miR-29c transcribed from chromosome 1 (1q32.2), both clusters are transcribed together. All of these miRNAs share identical sequences at the 2-7 nt position at the 5' end and have the same "seed region". Although they have similar sequences, they are located in different cell compartments, miR-29a is located in the cytoplasm, while miR-29b and miR-29c are mainly in the nucleus, because of these differences, their targets may also vary, leading to different biologic effects (Kwon *et al.*, 2019; Slusarz and Pulakat, 2015). One of the causes of this distribution is the hexanucleotide segmented sequence unique to miR-29b, that makes this miRNA to be mainly present in the nucleus, but its nuclear function is still not well understood. Another difference is the presence of a Tri-uracil sequence that makes miR-29a more stable and with a longer half-life, while miR-29c has a very low expression, perhaps due to the high turnover rate (Kwon *et al.*, 2019; Slusarz and Pulakat, 2015; Zong *et al.*, 2015). The differences in miR-29 structures are illustrated in figure 4.

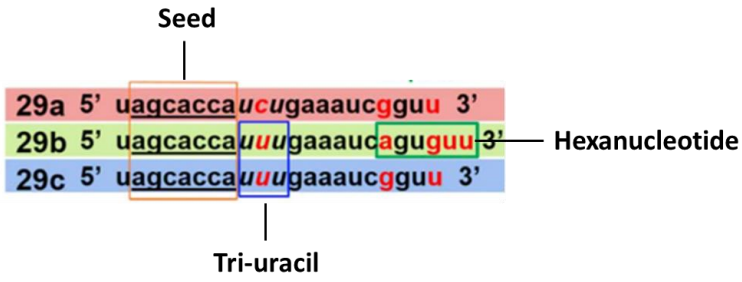


Figure 4. Schematic representation of the miR-29 family and their structural differences (Adapted from Kwon *et al.*, 2019).

This family has been shown an important role in several pathological disorders, like cardiovascular, AD and cancer (Roshan *et al.*, 2009). In turn, the dysregulation of this family has implications for neuronal differentiation, proliferation, communication and death. MiR-29b is downregulated in brains of AD patients and several studies have shown that it may be

involved in the regulation of APP and BACE1 expressions (Pereira *et al.*, 2016; Zong *et al.*, 2015). As mentioned above, the BACE1 protein levels are elevated in AD brains, suggesting that BACE1 dysregulation is directly implicated in AD pathogenesis through the formation of toxic Aβ peptides. Particularly, the work of Herbert and co-workers, in 2008, showed that the decreased levels of the miR-29a/29b-1 cluster were correlated with increased levels of BACE1 expression in patients with sporadic form of AD and that the introduction of synthetic miR-29 reduced the secretion of Aß peptides (Kwon *et al.*, 2019; Zong *et al.*, 2015).

MiR-9 has different origins from the 5' end of the precursor, named miR-9-5p (miR-9) or from the 3' end called miR-9-3p (miR-9*). However, the precursor and mature sequences are the same for both types. In mammals, the genes encoding miR-9 are located on chromosomes 1 (1q22) 5 (5q14.3) and 15 (15q26.1), highly conserved during evolution (Nowek *et al.*, 2018; Saraiva *et al.*, 2017). MiR-9 is highly expressed in the brain and its expression is often related to neurodegenerative disorders. Several studies have shown that miR-9 is downregulated in AD, more specifically in the hippocampus and cortex, but also in the blood serum and CSF (Nowek *et al.*, 2018; Saraiva *et al.*, 2017). In 2017, Riancho and collaborators demonstrated by microRNAome analysis that 14 miRNAs are differentially expressed in CSF fluid of AD patients, with miR-9 being one of them. When compared to the control group, miR-9 was detected in 50% of the control group, while in the AD group nothing was detected (Riancho *et al.*, 2017). Schonrock and co-workers also showed that the increase Aß levels leads to a considerable miRNA dysregulation, with miR-9 being one of the downregulated miRNAs (Schonrock *et al.*, 2012). In addition, some studies have suggested that miR-9 could regulate BACE1 (Hebert *et al.*, 2008) and PS1 (Krichevsky *et al.*, 2003; Reddy *et al.*, 2017). So, if this miRNA is able to negatively regulate targets that play a key role in AD, such as PS1 and BACE1, the same logical thinking given for miR-29 can be applied to miR-9, making this miRNA potential therapeutics for AD. The possible targets of miR-9 and miR-29b in AD pathologic pathway studied in this work are represented in figure 5.

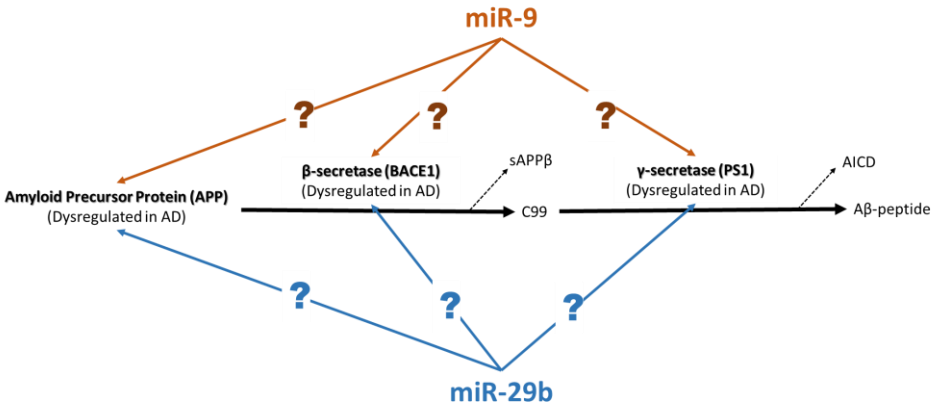


Figure 5. Schematic representation of the possible targets of miR-9 and miR-29b in AD pathologic pathway.

1.3 MicroRNA Manufacturing

For the preparation of a miRNA for pharmacological applications, certain characteristics have to be considered like the quantity required, the product quality, stability, safety and biological activity. There are currently three methods for the production of microRNAs, *in vivo* (recombinant), chemical and enzymatic synthesis. Being the last two approved by the Food and Drug Administration (FDA). Chemical synthesis is the most common method via phosphoramidite chemistry and has been automated for the production of oligonucleotides. This type of synthesis requires additional protection of the 2′-hydroxy group, adding t-butyldimethylsilyl (TBDMS) or tri-isopropylsilyloxymethyl (TOM), which can be posteriorly removed by a fluoride treatment. The success rate depends on the sequential deprotection-coupling and oxidation reaction (figure 6), but this method has not been consistently shown to produce high-quality RNA and the RNA produced must be subject to purification, usually by high-performance liquid chromatography (HPLC). In this production method, it is possible the addition of modifications (in the sugar or phosphate backbone) on RNA to improve its stability (resistance to nucleolytic degradation, leading to a longer half-life, increase the binding to plasma proteins for better renal clearance and increase metabolic stability) or selective fluorescence property, providing enhanced pharmacokinetic properties. Although biologic modifications are usually made in the nucleotides, their difference in location may alert the immune system to see RNA as an exogenous or pathogenic element. Moreover, these chemical modifications amplify *off-target* effects, change structure, which can lead to changes in functional properties. The process of synthesizing longer sequences is also more prone to errors and can lead to changes in functional properties, resulting in toxicity to the cell. Another limitation is the rapid increase in the cost of synthetic RNA parallel to the increase in length and number of modifications. The fact that the synthetic RNA is provided on a micromolar scale, which is sometimes not enough for animal and clinical investigations, also does not help (Ho and Yu, 2016; Pereira *et al.*, 2016).

Recombinant synthesis is done by using modified prokaryotic cells with the plasmid DNA (pDNA) coding for the target miRNAs sequences. This technique also needs a promoter insert in the pDNA, for example natural prokaryotic promoters, inducible lac promoter, lipoprotein gene promoter, rRNA promoter and the T7 transcription system promoter can be used. The cells grow in a fermentation medium where the prokaryotic cell express both pDNA and the target sequences, in this case the miRNA. Then the cells are disrupted, and the lysate needs to be purified for the recovery of pure miRNA, as seen in figure 6. The main challenges of these methods are the difficulty in purifying the samples and the fact that the RNA is highly susceptible to RNases activity in the medium of fermentation. Scaffolds RNA are used to try to increase RNA stability in the fermentation, exploiting the natural properties of

certain types of RNAs, like the four-leaf clover structure of tRNA that is resistant to heat-denaturation plus nucleolytic cleave and the widely expressed and stable structure of rRNA. The RNA sequence is included in the scaffold structure and processed by the cell, and in the end, it may be necessary the use of enzymes or just purification for obtaining the final product. This approach is more cost-effective than the chemical and enzymatic ones, but the big problem remains in the purification step, where more studies are needed to find a reliable technique (Ho and Yu, 2016; Pereira *et al.*, 2016; Ponchon and Dardel, 2011).

Enzymatic synthesis or *in vitro* transcription is widely used and is based on the use of bacteriophage systems (RNA polymerase) to produce RNA molecules from DNA sequences. In this production method it is necessary a DNA template for good efficiency and the action of the RNA polymerase highly specific for its 23-bp promoter sequence. The bacteriophage systems most frequently used are T3, T7, and SP6, where the T7 derives from the T7 phage of *E. coli*. The template most common is a linearized plasmid with the sequence of interest placed downstream from the promoter and the promoter placed upstream from a polylinker, yet PCR products and synthetic oligonucleotides with the promoter in the 5'end can also be used. Cloned templates are used for long transcripts, above 100 nt, while annealed oligomers are used for short transcripts but when large amounts of RNA are needed it is better to use cloned templates and techniques based on bacterial culture and plasmid extraction (figure 6). Some of the disadvantages of enzymatic production are the heterogeneity at 3' and 5' ends of the products, the decreased reliability of RNA polymerase as the transcript length increases and the absence of post-transcriptional modification machineries. The greater advantage of this method is the simplicity, existing many commercialized kits available. The versatility to generate RNA molecules of various lengths (from less 30 nt to over 104 nt) for different studies (*in vitro* translation, detection assays, structural or functional studies) in the microgram to milligram amounts is another great point. Preparative polyacrylamide gel electrophoresis and anion exchange fast protein liquid chromatography (FPLC) are then frequently used to further purify the RNA products. *In vitro* single-stranded RNAs may be annealed to give double-stranded RNAs that can be processed into siRNA by recombinant Dicer for silencing genes studies (Beckert and Masquida, 2011; Ho and Yu, 2016; P. Pereira *et al.*, 2016; Sherlin *et al.*, 2001).

In this work we focused on the two methods approved by FDA for the production of pre-miR-9 and pre-miR-29b. The selection of the precursor forms of miRNAs as molecules of interest is justified by the fact that it could be easier the bioprocessing and analysis, and the recognition and processing in the cell could be more efficient (Tsutsumi *et al.*, 2011).

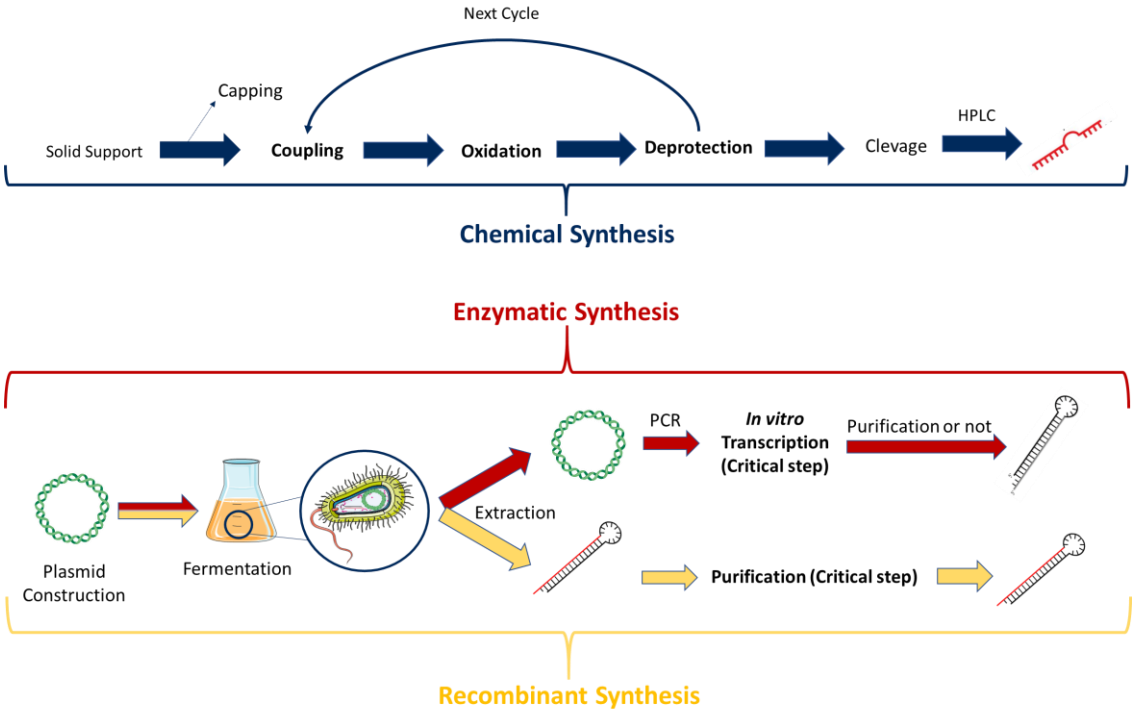


Figure 6. Schematic representation of the three methods currently used to produce microRNAs.

1.4 MicroRNA Delivery

One of the main obstacles of applying microRNA therapeutics in the treatment of brain disorders is the existence of the blood-brain barrier (BBB). The BBB is a specialized structural, physiological and biochemical barrier that regulates the movement of molecules from the blood to the brain. More than 98% of small drugs are denied to entry in the brain which makes it the most complicated microenvironment of the body. Of course, this can limit the development of efficient treatments, but before reaching the BBB it is also required and should be addressed the stable delivery of miRNAs and the entrance into the cells. To accomplish this, several options have been tried to encapsulate, protect and deliver miRNAs. Two main strategies have been exploited as delivery methods, including the non-viral methods, based on the use of lipid-based or polymeric nanoparticles, and viral vectors such adenovirus or adeno-associated virus vectors. These last, adeno-associated virus vectors are more powerful at transfection but have immunogenicity, potential toxicity, possibility of activation of oncogenes and difficulty in increasing production. On the other hand, the non-viral vector has an easy and reproducible method of production, presents higher biocompatibility, biodegradability, non-toxicity and non-immunogenicity. Giving these characteristics they are preferred over the viral vectors. The two main non-viral vectors found to be effective both *in vitro* and *in vivo* transfections are lipoplexes (Lipofectamine) and polymeric nanoparticles (Chitosan), also exploited in this work (Khurana *et al.*, 2013; Pereira *et al.*, 2017; Shende *et al.*, 2019).

Lipoplexes are formed by cationic liposomes that due to electrostatic interaction between the positively charged lipids and the negatively charged RNA, self-assemble in a positively charged system, allowing the binding to anionic molecules present in cells surface, facilitating that way the internalization of the encapsulated RNA by fusion with the cell membrane, also leading to endosomal release of RNA inside of the cell (figure 7). This system is stable, with reduced cytotoxicity and extremely efficient on cells transfection and RNA release, although the positive charge can induce inflammatory effects and unwanted interaction with negatively charged serum proteins, which can lead to opsonization and clearance of the lipoplex (Khurana *et al.*, 2013; Pereira *et al.*, 2017; Shende *et al.*, 2019).

Polymeric nanoparticles are more requested when derived from natural materials due to its higher stability in biological fluids and protection of the RNA against RNases degradation making them an efficient vector for RNAs delivery, having high drug-binding capacity (encapsulate large amounts of genetic material) and high delivery efficacy. Polymers like chitosan, Poly(L-lysine) (PLL) and Poly(ethylenimine) (PEI) can be modified with ligands or other polymers to enhance stability, transport properties, targeting and uptake, with relatively homogeneous sizes (up to 100nm) that facilitates the cellular uptake via endocytosis (figure 7). Chitosan (CS) nanoparticles and their derivatives have been extensively investigated for intranasal delivery of therapeutics to target the brain due to their mucoadhesive properties and had been successfully administered to silence target genes *in vitro* and *in vivo*, such as P-glycoprotein in brain endothelial cells (Khurana *et al.*, 2013; Pereira *et al.*, 2017; Shende *et al.*, 2019).

Several studies have reported the application of these non-viral vectors to DNA and siRNA delivery and, more recently, miRNAs as therapeutic agents. However, further studies are needed to ensure the best method for delivery awaiting a therapeutic application.

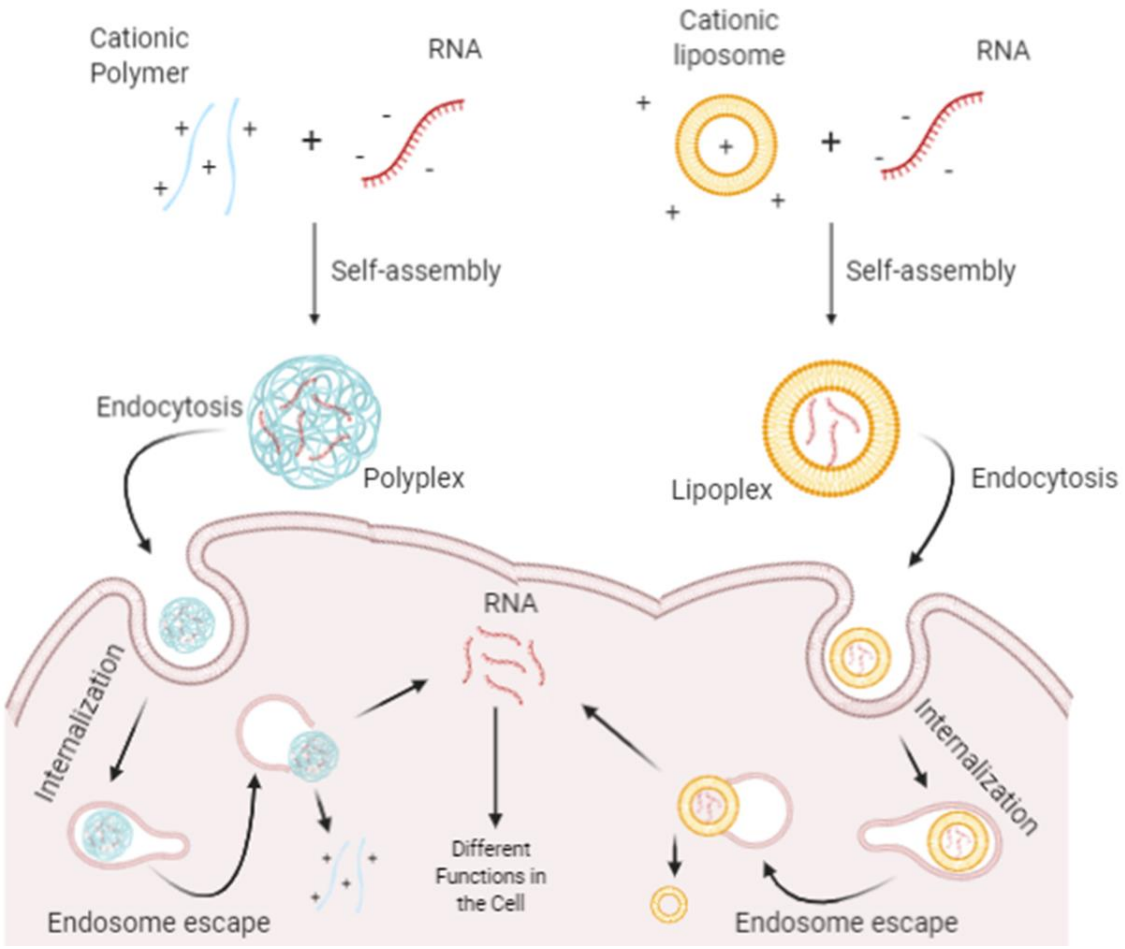


Figure 7. Schematic representation of the polyplex- and lipoplex-based non-viral strategies for delivering RNA into cell (Adapted from Wu *et al.*, 2018).

Chapter II

Aim of the work

2. Aim of the work

It is known that miRNAs can interfere with the gene expression regulation at post-transcriptional level, and in AD they are involved in amyloid pathway regulation. However, it is not specifically known what is affected by each specific microRNA. Thus, it is important to fill in these gaps for a better understanding of the AD and, consequently, to develop an efficient treatment. Actually, miRNAs can be promising biopharmaceuticals, but their effectiveness will be dependent on suitable and sustainable methods for producing and delivering these miRNAs. Thus, the specific objectives of this work are:

To study the effect of the pre-miR-9 and pre-miR-29b in inhibiting APP, BACE1, PS1 expression, proteins involved in the amyloid pathway;

To compare the biological activity of miRNAs and pre-miRNAs obtained by chemical and enzymatic methods, as well as, between purified and non-purified pre-miRNAs, in regard to the induced silencing;

To compare two different strategies of drug delivery, namely by using lipoplexes and polymeric nanoparticles, to assess their efficacy and influence on biological response.

Chapter III

Materials and Methods

3.1 Materials

For *Escherichia coli (E. coli) DH5a* bacteria growth was performed a solid culture medium - Luria Broth (LB) agar (Pronalab, Mérida, Mexico) - and a liquid culture medium composed by: tryptone and yeast extract (Bioakar, Beauvais, France), glycerol (Himedia), potassium hydrogen phosphate (K_2HPO_4) (Panreac, Barcelona, Spain) and potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, St. Louis, Missouri, EUA). Both culture media were supplemented with kanamycin antibiotic (Thermo Fisher Scientific Inc., Waltham, EUA). Posteriorly, the plasmid DNA extraction from *E. coli DH5a* culture was performed using NZYMiniprep kit (NZYTech Genes and Enzymes, Lisbon, Portugal).

For polymerase chain reactions (PCR) was used DreamTaq DNA Polymerase and DreamTaq Green Buffer from Thermo Fisher Scientific Inc. (Waltham, EUA), deoxyribonucleotide triphosphate (dNTPs) from NZYTech Genes and Enzymes (Lisbon, Portugal), Forward (Fw) and Reverse (Rv) primers from STABvida (Lisbon, Portugal). The primers sequences are represented in Table 3. After the amplification of the PCR products, its purification was carried out with NucleoSpin Gel and PCR Clear-up Kit (Machery-Nagel, Düren, Germany).

Table 3. Pre-miR-9 and pre-miR-29b primers sequences used in the PCR reaction.

miRNA	Primers sequences	
pre-miR-9	Fw	5'-GTT TTT TTT AAT ACG ACT CAC TAT AGG CGG GGT TGG TTG TTA TCT TTG G-3'
	Rv	5'-TGG GGT TAT TTT TAC TTT CGG TTA TC -3'
pre-miR-29b	Fw	5'-GTT TTT TTT AAT ACG ACT CAC TAT AGG CGG GGT TGG AAG CTG GTT TCA TAT GGT G-3'
	Rv	5'-CCC CCA AGA ACA CTG ATT TC -3'

In vitro transcriptions were performed with MEGAscript T7 Kit and, its transcripts were purified using MEGAclear T7 Kit (Thermo Fisher Scientific Inc., Waltham, EUA). To confirm the integrity and purity of the RNA samples, 1% agarose gel electrophoresis is used with GRS Agarose LE and Green-Safe (Grisp, Porto, Portugal).

Mouse neuroblastoma cell line (N2a695) harboring the wild type mutation for production of human mutated APP695, used in the *in vitro* assays, was kindly provided by Professor Wenjie Luo (Weill Cornell Medical College). N2a695 cells were cultured in the following medium: Dulbecco´s Modified Eagles´s Medium (DMEM) (Sigma-Aldrich, St. Louis,

Missouri, EUA), OptiMEM (Gibco, Life Technologies, EUA), 5% (wt/vol) heat-inactivated fetal Bovine Serum (FBS) (Biochrom, Berlin, Germany) and 1% (wt/vol) streptomycin-penicillin (Grisp, Porto, Portugal). In the transfection assays were used the following reagents: synthetic microRNA (miR-29b and miR-9) purchased from Stabvida (Lisbon, Portugal), *in vitro* pre-miRNAs (pre-miR-29b and pre-miR-9) produced in the lab, Lipofectamine 2000 Reagent (Thermo Fisher Scientific Inc., Waltham, EUA) and chitosan nanoparticles (CS) (Sigma-Aldrich, St. Louis, Missouri, EUA). The sequences of the microRNAs in study are represented in the Table 4.

Table 4. Sequences for miRNAs (miR-9, miR-29b, pre-miR-9 and pre-miR-29b) used in the *in vitro* assays.

microRNA	Sequences
miR-9 synthetic	5'- UCU UUG GUU AUC UAG CUG UAU GA -3'
miR-29b synthetic	5'- UAG CAC CAU UUG AAA UCA GUG UU -3'
pre-miR-9	5'-GGT TGT TAT CTT TGG TTA TCT AGC TGT ATG AGT GGT GTG GAG TCT TCA TAA AGC TAG ATA ACC GAA AGT AAA AAT AAC CCC A- 3'
pre-miR-29b	5'- CTT CTG GAA GCT GGT TTC ACA TGG TGG CTT AGA TTT TTC CAT CTT TGT ATC TAG CAC CAT TTG AAA TCA GTG TTT TAG GAG -3'

For total RNA extraction from N2a695 was used: TripleXtractor (TRIzol) (Grisp, Porto, Portugal), phosphate-buffered saline (PBS), chloroform (Fisher Scientific, UK), isopropanol (Thermo Fisher Scientific Inc., Waltham, EUA), ethanol 75% prepared with Milli-Q (ultra-pure deionized water purified with Milipore Milli-Q system (Billerica, MA, USA) and treated with 0.01% diethylpyrocarbonate (DEPC)) (Sigma-Aldrich, St. Louis, Missouri, EUA). Xpert cDNA synthesis Kit (Grisp, Porto, Portugal) was used for complementary DNA (cDNA) synthesis. In real-time quantitative PCR (RT-qPCR) experiments were used the following reagents: Maxima® SYBR Green/Fluorescein qPCR Master Mix Kit (Thermo Fisher Scientific Inc. Waltham, EUA), Fw and Rv primers for each mRNA in study (Stabvida, Lisbon, Portugal) (Table 5).

Table 5. Primers sequences used in the RT-qPCR experiments.

mRNA	Primers sequences	
bace1	Fw	5'- AGA CGC TCA ACA TCC TGG TG -3'
	Rv	5'- CCT GGG TGT AGG GCA CAT AC -3'
app	Fw	5'- AGA GGT CTA CCC TGA ACT GC -3'
	Rv	5'- ATC GCT TAC AAA CTC ACC AAC -3'
ps1	Fw	5'- GAG GAA GAC GAA GAG CTG ACA -3'
	Rv	5'- GAA GCT GAC TGA CTT GAT GGT -3'
gapdh	Fw	5'- TGA CGT GCC GCC TGG AGA AA -3'
	Rv	5'- AGT GTA GCC CAA GAT GCC CTT CAG -3'

For proteic extraction from eukaryotic cells were used the following reagents: PBS buffer, proteases inhibitors cocktail (Roche) and complete lyses buffer composed by Tris-HCl, EDTA, ethylene glycol tetra acetic acid (EGTA), Triton X-100, and phenylmethylsulfonyl fluoride (PMSF) from Sigma-Aldrich (St. Louis, Missouri, EUA). Then, the total protein levels were quantified by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc, California, EUA). For western blotting technique was used: reduction buffer (composed by glycerol, SDS, Tris-HCl and blue bromophenol), ß-mercaptoethanol (Merck, Whitehouse Station, EUA), 30% polyacrylamide (Grisp, Porto, Portugal), Tris-HCl buffer, ammonium persulphate (PSA) (Sigma-Aldrich, St. Louis, Missouri, EUA), tetramethylethylenediamine (TEMED) (Thermo Fisher Scientific, Geel, Belgium), TGS Buffer (composed by Tris-Base, glycine and SDS), 0.45 µm polyvinilidene difluoride membrane (PVDF) (GE Healthcare, Life Sciences, Germany), transfer buffer (composed by Tris-base, glycine and methanol), methanol (Fisher Scientific, England), filter paper Whatman, TBS buffer (composed by Tris-HCl and NaCl), TBS-T buffer (composed by Tris-HCl, NaCl and Tween-20), bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Missouri, EUA), powder milk and WesternBright ECL HRP substrate (Advansta Inc., San Jose, USA). Table 6 described the primary and secondary antibodies used for detection of the proteins in the study.

Table 6. Characterization of the antibodies used in the Western blot technique.

Proteins	Primary antibody	Secondary antibody
BAEC1	Anti-BACE1 antibody (ab10716): • Rabbit • Polyclonal • Dilution: 1:200 • Weight: ≈ 70 kDa (Abcam, Cambridge, England)	Goat Anti-Rabbit IgG Antibody, HRP-conjugate (12-348): • Anti-rabbit • Conjugated to horseradish peroxidase (HRP) • Detection by ECL • Dilution: 1:5 000 (Sigma-Aldrich, St. Louis, Missouri, EUA)
APP	Anti-Amyloid Precursor Protein antibody [Y188] (ab32136): • Rabbit • Monoclonal • Dilution: 1:1 000 • Weight: ≈ 95 kDa (Abcam, Cambridge, UK)	
PS1	Anti-Presenilin 1/PS-1 antibody [APS 18] (ab15458): • Mouse • Monoclonal • Dilution: 1:250 • Weight: ≈ 56 kDa (Abcam, Cambridge, UK)	m-IgGκ BP-HRP (sc-516102): • Anti-mouse • Conjugated to horseradish peroxidase (HRP) • Detection by ECL • Dilution: 1:1 000 (Santa Cruz Biotecnologia, Inc. Texas, EUA)
B-actin	Anti-β-Actin antibody (A1978): • Mouse; • Monoclonal • Dilution: 1:10 000 • Weight: ≈ 42 kDa (Sigma-Aldrich, St. Louis, Missouri, EUA)	

3.2 Methods

3.2.1 Pre-miRNAs synthesis

3.2.1.1 *Escherichia coli DH5a* growth

Escherichia coli DH5a strain modified with plasmids pHBSR1-RM containing the sequence of human pre-miR-29b and pre-miR-9 (pHBSR1-RM-pre-miR-29b or pHBSR1-RM-pre-miR-9, respectively) were used in this study for recombinant plasmid DNA production. First, *E. coli* stock stored at -80 ˚C was cultured on the Luria Broth medium supplemented with agar, overnight at 37 ˚C. Then, some of the cultured colonies were transferred with a loop into an Erlenmeyer of 500ml containing 125mL Terrific Broth (TB) medium (composed by 12 g/L tryptone, 24 g/L yeast extract, 5.5 x 10^{-5} glycerol, 23.14 g/L of KH_2PO_4 and K_2HPO_4), supplemented with 50 µg/mL Kanamycin, a medium/oxygen ratio of 1:4 for aerobic conditions. The pre-fermentation medium was incubated at 37 ˚C and 250 rotation per minute (rpm). The growth was monitored by measuring the optic density (OD) at 600nm, using a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer (Cambridge, UK). Once the pre-fermentation growth reached an OD_{600} ~2.6, a specific volume of culture was transferred into fermentation media in a 1L Erlenmeyer containing 250 mL of TB medium with antibiotic. The volume transferred from pre-fermentation to fermentation was calculated using the following equation: $V_{pre\text{-}fermentation} = (V_{fermentation\ x}\ OD_{fermentation})/(OD_{pre\text{-}fermentation} - OD_{fermentation})$, with the intent of starting the fermentation with an OD of approximately 0.2 (figure 8). The *E. coli* growth was maintained for 8 h, being then interrupted with an OD of approximately 6, corresponding to the final of the exponential phase (log phase). Then, the bacterial cells were recovered by centrifugation at 3900 g for 10 min at 4 ˚C and, the cellular pellets of 100 mL were stored at -20 ˚C.

3.2.1.2 Plasmid DNA extraction from *E. coli DH5a*

For the plasmid DNA extraction, the bacterial cells were lysed using NZYMiniprep kit, according to the manufacturer's indications with some modifications. Briefly, the bacterial pellet was resuspended in 25 mL of 0.8% NaCl, followed by the preparation of 2 mL fractions for centrifugation for 1 min, at room temperature (RT) and 12300 g. The supernatant was discarded, and the resulting pellet was resuspended in 250 µL of Buffer A1 and vortexed. The process of lysis was initiated with the addition of 250 µL of buffer A2 and homogenization. For neutralization step, 3 min later, 300 µL of buffer A3 were added and then centrifuged for 9 min at 123000 g, at RT. The supernatant was transferred to the NZYTech column was

centrifuged for 1 min, at 11000 g, at RT. This step promotes the binding of the DNA to the column, which posteriorly was washed with the addition of 700 µL of buffer A4. The column was again centrifuged during 1 min, at 11000 g, at RT. The plasmid DNA was carried out by addition of 50 µL buffer AE, and centrifugation for 1 min at RT, at 123000 g. The final pDNA sample was quantified using a NanoPhotometer (IMPLEN, United Kingdom). In turn, the integrity and purity of the sample was evaluated by horizontal electrophoresis in a 1% agarose gel (Hoefer, Holliston, MA, EUA) using Greensafe (0.012 µL/mL) as intercalating agent to enable the bands visualization. The samples were prepared with 5 µL of sample and 0.5 µL of loading buffer (glycerol, bromophenol blue, milli-Q water), ran at 120 volts, during 40 min in TAE buffer (pH=8.0, 40 mM Tris-Base, 20 mM acetic acid and 1 mM EDTA), then visualized in an ultraviolet chamber (UV) (UVitec Cambridge (Cambridge, UK). In the end, the samples were stored at -20˚C.

3.2.1.3 Conventional polymerase chain reaction

The sequences of the pre-miRNAs encoded in the plasmids pBHSR1-RM were amplified by PCR in a T100 Thermal Cycler (BioRad). In order to optimize the PCR reactions, 3 dilutions of the plasmid DNA were performed. In each reaction, a mix with a total volume of 25 µL was prepared with 1 µL of plasmid DNA for both pre-miRNAs and for the remaining components several volumes were tested to determine the best amplification conditions. For the negative control it was used the same mix but instead of 1 µL of plasmid DNA it was used nuclease-free water. The general program used for the amplification is described in table 7, with a variation in the melting temperature that had to be optimized for each pre-miRNA. For control of the PCR products, each sample was analyzed by 1 % agarose gel electrophoresis, for 30 min at 120 V.

Table 7. Experimental condition used in each step of the PCR for pre-miRNA amplification.

General PCR Program			
Step	Temperature (˚C)	Time	Cycle
Initiation of the reaction	95 ˚C	5 min	1x
Denaturation of DNA	95 ˚C	30 s	24x
Hybridization of the primers to DNA	Optimized for each pre-miRNA	30 s	
Extension	72 ˚C	30 s	
Final Elongation	72 ˚C	5 min	1x
	12 ˚C	∞	

3.2.1.4 Purification of PCR products

The PCR products were purified using the "NucleoSpin® Gel and PCR Clear-up" (Machery-Nagel, Düren, Germany) kit and following the manufacturer's recommendations. First, all the reactions were transferred to a tube of 1.5 mL and 20 µL of NTI buffer for each 10 µL of PCR products were added and homogenized. Then the mixture was transferred for the purification column. Next, the sample was centrifuged for 30 s, at 11000 g, at RT. Then, is added 700 µL of N3 buffer and centrifuged 1 min at the same velocity, new centrifugation was done for removing the remaining buffer. For recovering the DNA, it was added 30 µL of NE buffer to the column and the mixture is centrifuged at the same conditions. The amount of DNA was quantified by NanoPhotometer and the integrity and purity of the DNA was determined by 1% agarose gel electrophoresis.

3.2.1.5 *In vitro* transcription

In vitro transcription method was performed to obtention of the the pre-miR-9 and pre-miR-29b using MEGAscript T7 Kit. For a reaction mix of 20 µL of final volume it was added: 2 µL of 10X T7 Reaction Buffer, 2 µL of each rNTP, 0.4 µg of PCR products purified, 2

µL of T7 Enzyme Mix and the remaining volume was completed with nuclease-free water. Then, the reaction mix was incubated for 16 h at 37 ˚C. Posteriorly, 2 µL of DNase I was added and incubated again for about 20 min. Lastly, 2 µL of Stop solution (EDTA 0.5 M, pH 8) was added and incubated again during 15 min (figure 8). In the end the samples were stored at -80 ˚C. The RNA that was tested in a non-purified form it was immediately quantified by NanoPhotometer and analyzed in a 1% agarose gel electrophoresis for verifying their integrity and purity.

3.2.1.6 Purification and concentration of the RNA transcripts

Some of the samples obtained by *in vitro* transcription were purified with the MEGAclear T7 Kit, in accordance with the manufacturer's instructions. The first step consisted in the addition of 80 µL of elution solution to 20 µL of RNA followed by homogenization. After, the mixture was transferred to a new tube of 1.5 mL and was added 359 µL of binding solution concentrate. After homogenization, 250 µL of pure ethanol was added, this mixture was then transferred to the purification column, then centrifuged for 1 min at 12300 g, at RT. The column was washed through addition of 500 µL of wash solution and, centrifuged in the same conditions. An additional centrifugation is done to discard the remaining buffer of the column. For the recovery of RNA, to the column was added 50 µL of elution Solution, followed by incubation for 10 min in a heating block at 70 ˚C. Then, a new centrifugation was performed, and this step was repeated one more time with addition of more 50 µL of elution buffer, to improve the recovery of pure RNA. A step of concentration was applied with addition of 10 µL of 5M ammonium acetate plus 275 µL of pure ethanol onto the recovered RNA and, the mixture was incubated for 30 min at -20 ˚C. After centrifugation carried out at 4 ˚C, 15000 g for 15 min, it was added 500 µL of cold 70% ethanol prepared in DEPC water and centrifuged again. The resulting pellet was dried at RT for 10 min and resuspended with 30 µL of DEPC water. RNA concentrations were determined using a NanoPhotometer and analyzed in a 1% agarose gel electrophoresis. The purified RNA was stored at -80 ˚C up until use.

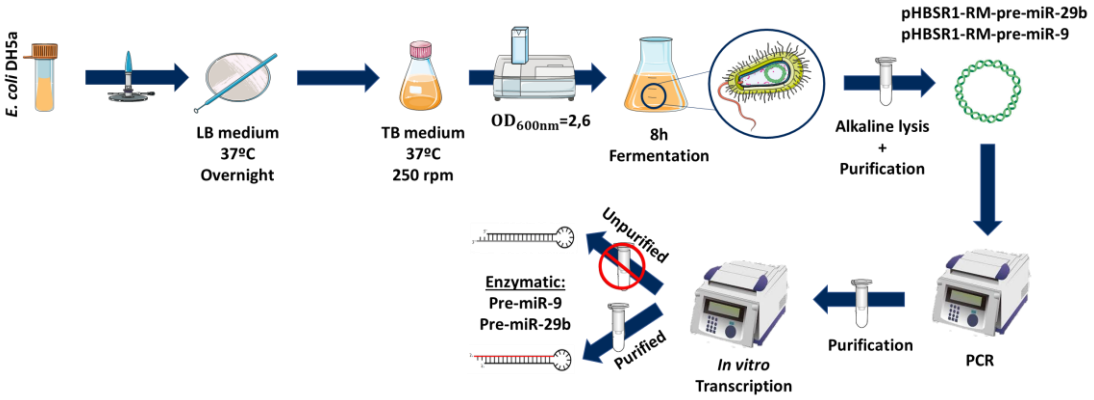


Figure 8. Schematic representation of the methods used to produce the *in vitro* pre-miRNAs.

3.2.2 Cell culture

3.2.2.1 N2a695 cells lines growth

To access the effects of the studied miRNAs *in vitro*, N2a695 cells were chosen because in these cells there is a constitutive endogenous human BACE1 expression, enabling greater sensitivity for detecting microRNAs induced changes in the human BACE1 expression at post-transcriptional level. First, N2a695 at passages 3-23 were cultured in the following culture medium: 1:1 mixture of DMEM and OptiMEM, supplemented with 5% (wt/vol) heat-inactivated FBS and 1% (wt/vol) penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 ˚C.

3.2.2.2 N2a695 cells transfection assays

After reaching 70-80% of confluence, the N2a695 cells were seeded in 6-well plates (at a density of $2.5x10^5$ cells/well) or 12-well plates (at a density of $2x10^5$ cells/well), for western blot technique or RT q-PCR, respectively. The counting of the cells was done with trypan blue at a 1:2 dilution in a Neubauer chamber. When 50 to 60% confluence was achieved, the medium was replaced by serum- and antibiotic-free culture medium. After 15 h, cells were transfected with 8 µg/µL of synthetic miRNAs (miR-29b and miR-9) and *in vitro* pre-miRNAs (pre-miR-29b/-9 purified and pre-miR-29b/-9 non-purified), as illustrated in figure 9. The non-transfected cells were used as negative control. After 6 h of transfection, the culture medium was then replaced by fresh medium supplemented with 1% FBS and 1% antibiotic, to allow the cells to remain metabolically active. The cells were then harvested 72

h after transfection for total RNA isolation and protein extraction. The transfection experiments were achieved with two different methods. In the first method was utilized Lipofectamine 2000 (Lipo), as transfection reagent, according to the manufacturer's protocol. Briefly, Lipo was incubated in optiMEM medium (5 µL to 250 µL for 6 wells plates and 2 µL for 100 µL for 12 wells plates) for 5 minutes, while miRNAs were diluted in the same proportions in optiMEN medium. Then, the optiMEM/Lipo mixture was added to the opiteMEM/miRNA mixture and incubated for 20 min. At the end, the resulting mixture was added to the cells (500 µL for each well in the 6 wells plates and 200 µL for each well in the 12 wells plates). For the second method were developed CS nanoparticles, using the method of simple complexation through electrostatic interactions that occur between positive charges present in the protonated amine groups of chitosan, and the negative charges of the phosphate groups of miRNA backbone. Briefly, the miRNA-loaded CS nanoparticles were obtained by dropwise addition of 100 µL of chitosan solution to the 8 µg of miRNA in 400 µL of 0.1M acetate buffer (pH 4.5, sodium acetate, glacial acetic acid, Milli-Q water) under stirring for 1 minute. After 15 min of incubation at RT, formulated nanoparticles were centrifuged at 15 000 RCF, 20 min, 4 ˚C. The supernatant was discarded, and the pellet was resuspended in 400 µL of optiMEM medium. At the end, the miRNA-loaded nanoparticles were added to the cells (400 µL for each well in the 6 wells plates and 200 µL for each well in the 12 wells plates).

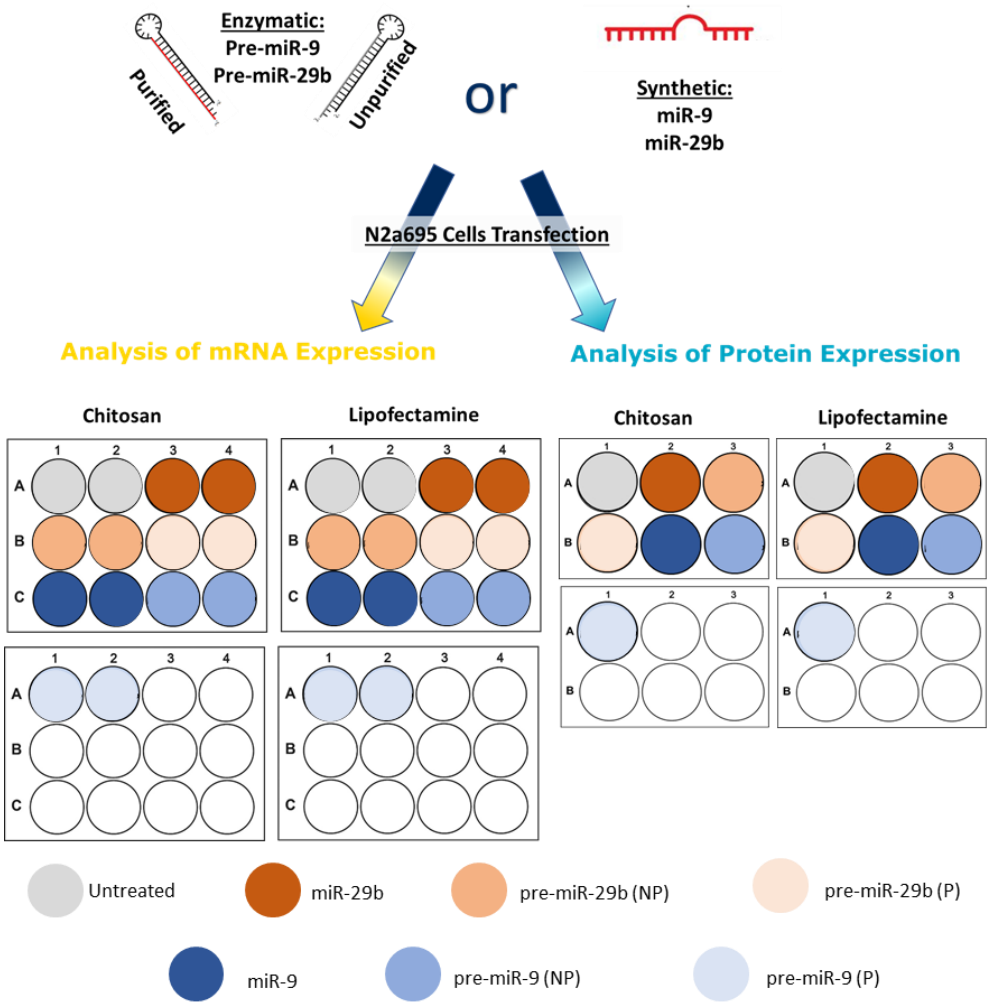


Figure 9. Schematic representation of N2a695 cells transfection. Where NP means non-purified transcript and P means purified transcript.

3.2.3 mRNA expression analysis

3.2.3.1 Total RNA extraction from eukaryotic cells

After transfection, N2a695 cells were rinsed in ice-cold PBS (pH=7.5, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4), and 300 µL of TRIzol reagent were added to each well of the 12-wells plate, promoting cellular lysis. Then, the lysed cells were transferred to tubes of 1.5 mL and incubated 5 min at RT. After incubation, 60 µL of chloroform was added and vigorously mixed. After 10 min at RT, a centrifugation was done for 15 min, 4 ˚C, at 12000 g. This step leads 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), which is the fraction of interest. This fraction was transferred to a new tube and, the RNA was precipitated with 180 µL of isopropanol at 20 ˚C. The mixture was, well homogenized and incubated in ice for 15 min. New centrifugation with the same parameters was done but only for 10 min. Then, the resulting pellet was

washed with 200 µL of 75% of ethanol in DEPC water and centrifuged with the same conditions for 5. The RNA pellet was left to dry for 20 min at RT and then resuspended in 20 µL of DEPC water. The RNA quantify was determined using a NanoDrop spectrophotometer and their integrity and purity were evaluated by 1% agarose gel electrophoresis.

3.2.3.2 Complementary DNA synthesis

To cDNA synthesis a 1 µg of total RNA of sample was reverse transcribed into cDNA using the Xpert cDNA synthesis Kit, according to the manufacturer's protocol. For each reaction of a final volume of 20 µL was added: 1 µL of Random primes, 1 µL of dNTPs, the volume needed for 1 µg of total RNA and the remaining volume was completed with nuclease-free water. The mix was incubated in a T100™ Thermal Cycler (BioRad) for 5 min at 65 ˚C and then incubated at 4 ˚C for 2 min. To the previous mixture the following reagents were added: 4 µL of reaction buffer, 0.5 µL of ribolock RNase inhibitor and 1 µL of xpertRTase were added. The mixture was incubated at 25 ˚C for 10 min followed by incubation at 50 ˚C for 15 min. The reaction was stopped by incubation at a 85 ˚C for 5 min. The samples were then stored at -20 ˚C.

3.2.3.3 Real-time quantitative-polymerase chain reaction

For quantitative analysis of the expression of BACE1, PS1, APP and GAPDH mRNA, RT-qPCR amplification of synthesized cDNA was performed in 96-well optical plates, using the Maxima® SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermo Fisher Scientific Inc.) in a CFX Connect™ Real-Time PCR Detection System (BioRad). RT-qPCR reaction was prepared containing: 1 µL of reverse transcription product - cDNA, 1.6 µL of forward primer, 1.6 µL of reverse primer, 10 µL of Maxima® SYBR Green/Fluorescein qPCR Master Mix (2X) and 6.6 µl of nuclease-free water, to a final volume of 20 µL per reaction. In the negative control was not added 1 µL of cDNA. The reaction conditions were 95 ˚C for 3 min for initial denaturation, followed by 40 cycles of 95 ˚C, 62 ˚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. All reactions were completed in triplicate and threshold cycle (CT) values were averaged from the triplicate (figure 10).

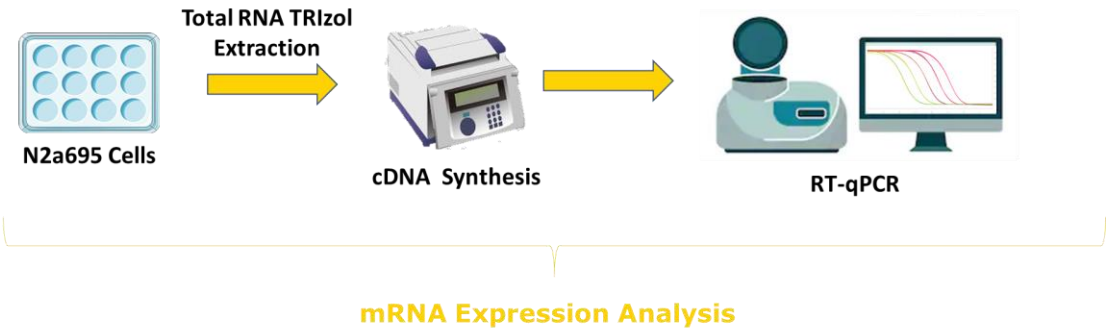


Figure 10. Schematic representation of the methods used for the target mRNA expression analysis.

3.2.4 Protein expression analysis

3.2.4.1 Protein extraction from eukaryotic cells

N2a695 cells were recovered in ice-cold PBS, and then centrifuged at 11000 g, for 7 min. The pellet was homogenized in 50 µL of cell lysis buffer: 25 mM Tris-HCl buffer (pH 7.5), 2.5 mM EDTA, 1% Triton X-100, 2.5 mM EGTA, 25 mM phenylmethylsulfonyl fluoride and complete EDTA Free protease inhibitor cocktail (Roche). Once homogenized, cell extracts were centrifuged at 11000 rpm for 7 min at 4 ˚C, for removal of cell debris. The supernatant was then quantified and stored at -80 ˚C.

3.2.4.2 Protein quantification

Total protein concentration in the supernatant was determined using 96-well plates and Bradford Protein Assay. Each sample was quantified in triplicate using: 1 µL of sample, 159 µL of Milli-Q water and 40 µL of Bio-Rad Protein Assay Dye Reagent Concentrate. For the reference it was used the same triplicate but with 1 µL of cell lysis buffer instead of 1 µL of sample. Then, the plate was read in a reader at 595 nm absorbance and the amount of protein was calculated using the following standard straight ($y = 0.1124x + 0.0993$), constructed from different known BSA concentrations (0, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/µL).

3.2.4.3 Western blotting

A mixture containing 15 µL of Milli-Q water and 50 µg of total protein was heated for 10 min at 95 ˚C with 3 µL of reducing buffer (composed by glycerol, 10% SDS, 1.25M Tris-HCl

with pH=6.8 and blue bromophenol) containing ß-mercaptoethanol. Then fractionated by molecular weight in a polyacrylamide gel electrophoresis on sodium dodecyl sulfate (SDS-PAGE) - 4 % stacking gel (3.463 mL of Milli-Q water, 1mL of 0.625M of Tri-HCl with pH 6.8, 500 µL of polyacrylamide, 50 µL of 10% SDS, 100 µL of 10% PSA, 20 µL of TEMED) and 12.5% running gel (6.7 mL of Milli-Q water, 4 mL of 1.5 M of Tri-HCl with pH 8.8, 5 mL of polyacrylamide, 160 µL of 10% SDS, 160 µL of 10% PSA, 16 µL of TEMED). 4 µL of a molecular weight marker was used and the electrophoresis was run at 180 V for 1 h in TGS buffer 1x. After, the proteins were transferred from the running gel to PVDF membranes using a system Trans-Blot Cell system (BioRad). First, the PVDF membranes was activated with pure methanol for 2 min, washed 3 times with Milli-Q water, for 5 min and, then incubated 15 min in transfer buffer (Tris-Buffered Saline: 20 mM Tris-HCl, pH=7.6 and 137 mM NaCl). The transfer sandwich was performed, where all components were soaked in transfer buffer (1 sponge, 4 filters, the gel, the membrane, 4 filters and 1 sponge) with the help of a roll, ensure that there no bubbles between each layer. The transfer was done for 90 min, at 0.75 A. After, the membrane containing the proteins was blocked in TBS supplemented with 3% BSA more 2% poured milk. Following, membranes were probed at 4 ˚C overnight with the following primary antibodies: anti-rabbit monoclonal APP, anti-rabbit polyclonal BACE1, anti-mouse PS1 monoclonal and anti-mouse polyclonal ß-actin, depending on the protein under analysis. After three washes with TBS-T (Tris-Buffered Saline–Tween: 20 mM Tris-HCl, pH=7.6; 137 mM NaCl and Tween-20), the membranes were incubated with HRP-labeled anti-rabbit/mouse IgG secondary antibody. The blots were visualized using ECL substrate according to manufacturer's instructions and the figures were acquired using the ChemiDoc system and analyzed with Image Lab software (figure 11).

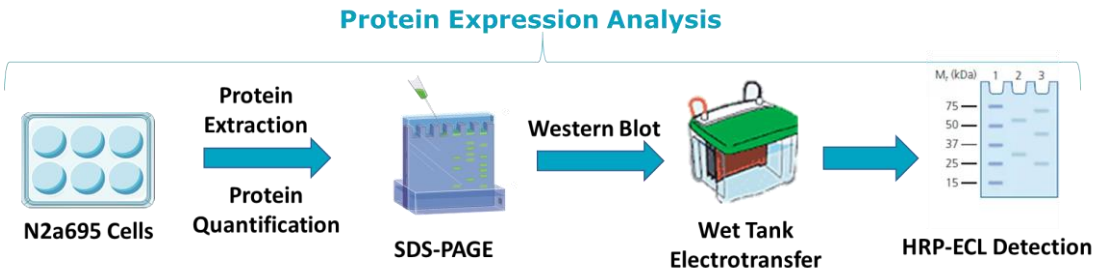


Figure 11. Schematic representation of the methods used for the target proteins expression analysis.

3.2.5 Data analysis

For RT-qPCR data analysis, a relative quantification method was used, in order to determine changes in the target mRNAs expression. Glyceraldehyde-3-phosphate (*gapdh*) was

used to normalize expression and determine any changes in the amplification in comparison with untreated cells. Fold changes in the expression were calculated for each sample using the comparative threshold cycle (CT), where the amount of target was determined to be $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = (Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}})$.

Chapter IV

Results and Discussion

4.1 *E. coli DH5a* growth and plasmid DNA amplification

In this study, the recombinant production of each plasmids encoding for the pre-miRNAs (pBHSR1-RM-pre-miR-9 or pBHSR1-RM-pre-miR-29b) was performed in a genetically modified organism, *E. coli* DH5α. As expected, *E. coli* was able to grow at 37˚C and 250 rpm. So, after transferring the transformed *E. coli* DH5α from the pre-fermentation to the 8 h fermentation medium, the OD was measured at 600 nm at each hour to follow the growth profile and verify that no contaminations were present. Figure 12 represents the typical cell growth obtained under these conditions, and it was very similar, independently of the pre-miRNA encoded.

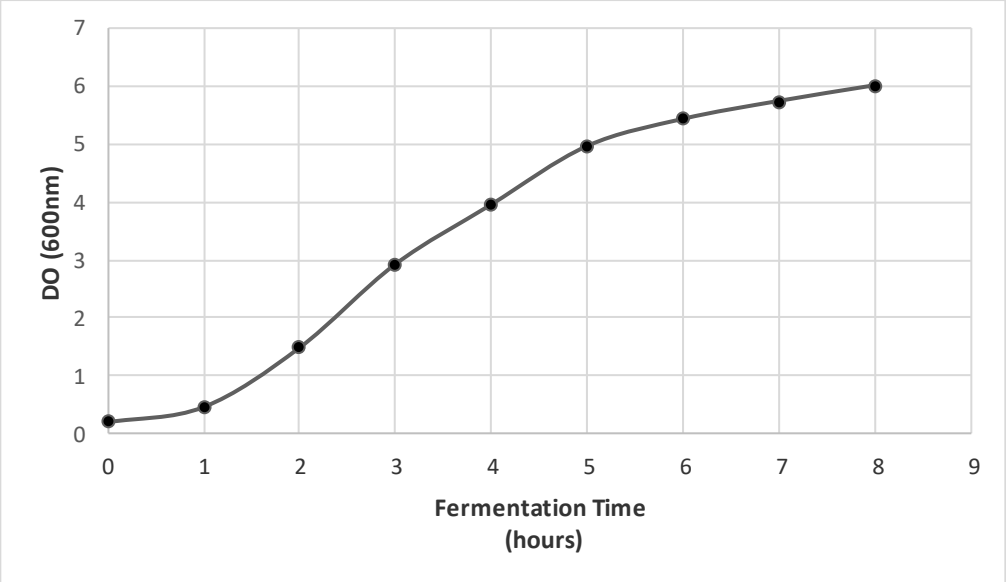


Figure 12. Growth curve obtained of *E. coli DH5a* bacteria modified with plasmid DNA pBHSR1-RM containing the sequence of human pre-miR-9 or -29b.

Comparing a growth curve of *E. coli DH5a* obtained in this study with the growth curve of our previous work, as shown in figure 13, it can be affirmed that both growth curves have the same profile. Where the lag phase is seen in the first hour, the log phase from the first hour until the sixth hour and the beginning of the stationary phase in the sixth hour until the eighth hour. So, it was verified that the fermentation occurred as expected and without any contamination. The time to interrupt the cell growth was established in accordance with our previous evidence by Pereira *et al.*, 2017 (figure 13), where it was found that this time enable the recovery of a higher concentration of pre-miR-29b, what can indicated a considerable number of copies of pDNA, and almost no dead cells, at this fermentation point.

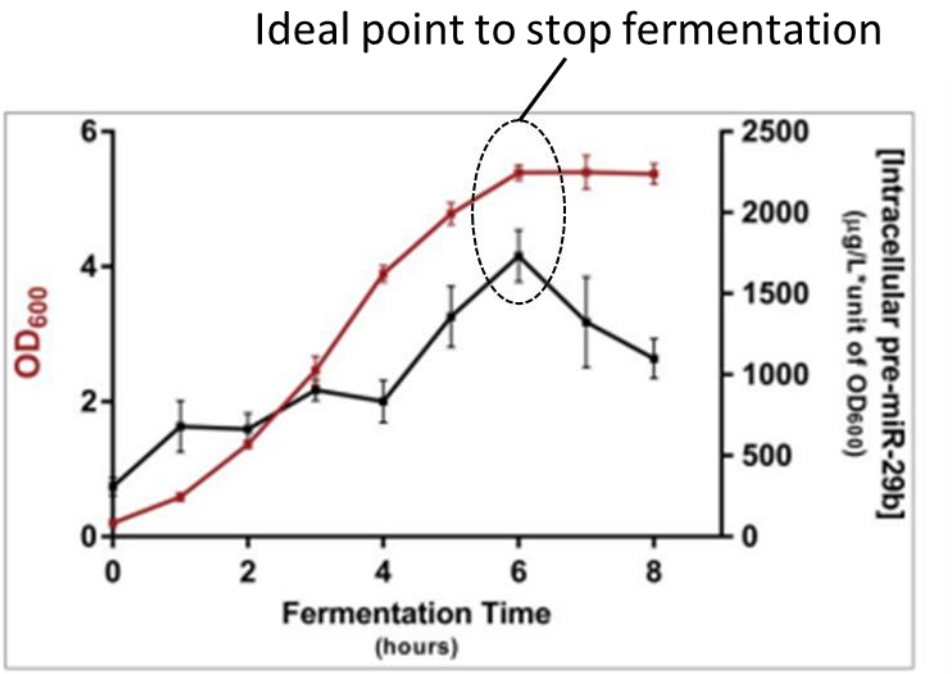


Figure 13. Growth curve for *E. coli DH5a* bacteria modified with plasmid pBHSR1-RM containing the sequence of human pre-miR-29b and production of the pre-miR-29b (Adapted from Pereira *et al.*, 2017).

4.2 Isolation of plasmid DNA from *E. coli DH5a* cells

The recombinant plasmids DNA - pBHSR1-RM-pre-miR-9 encoding the sequence for pre-miR-9 and pBHSR1-RM-pre-miR-29b encoding the sequence of pre-miR-29b - were isolated from the *E. coli DH5a* cells using NZYMiniprep Kit. The main objective is the recovery of pure pDNA to be further used in the production of pre-miRNAs by *in vitro* transcription. In this way, after the recovery of pDNAs, their integrity and purity were evaluated by 1% agarose gel electrophoresis, as represented in figure 14.

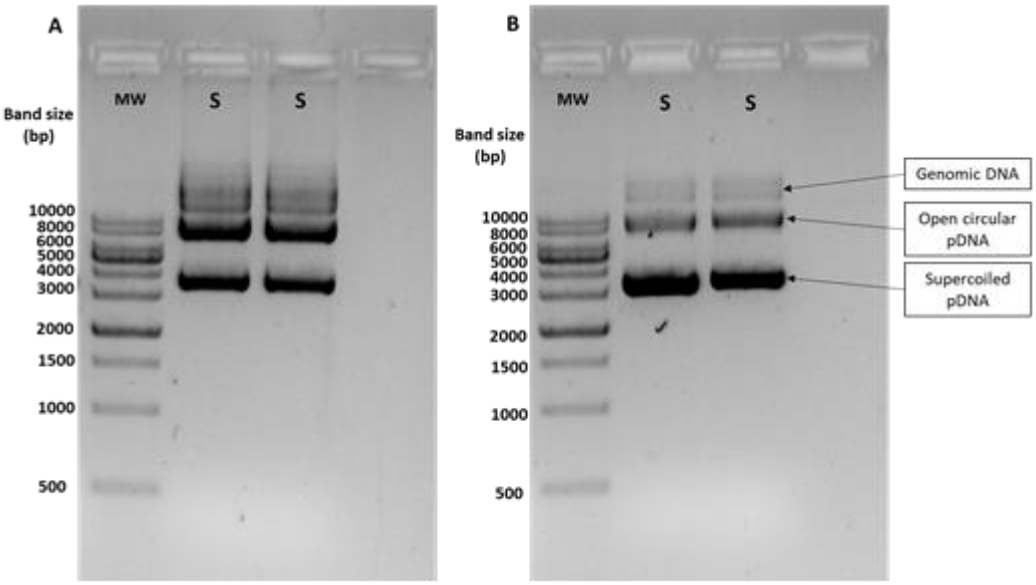
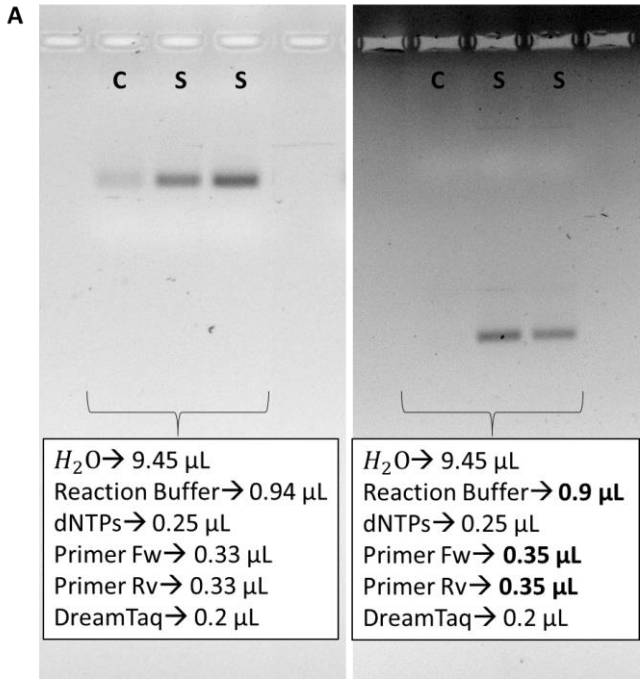


Figure 14. Isolation of plasmid pBHSR1-RM containing the sequence of human pre-miR-9/-29b from *E. coli* DH5α cellular pellet. A- 1% gel electrophoresis of the sample with the pDNA encoding to the pre-miR-9 sequence (two samples are represented – duplicate). B- 1% gel electrophoresis of the sample with the pDNA coding to the pre-miR-29b sequence (two samples are represented – duplicate). Where the S corresponds to the obtained samples isolated from the *E. coli* DH5α cells.

Analyzing the figure 14, it is possible to visualize 3 different bands in each sample. The first band corresponds to genomic DNA (high molecular weight) and two bands correspond to the different isoforms of pDNA (open circular and supercoiled) with molecular weight between 4000 and 8000 bp, as indicated in the figure. The goal of this step was to obtain the two pDNA isoforms, so the recovery of genomic DNA was not intended. However, form the electrophoresis it is verified some contamination with genomic DNA, which suggests that this kit is not sufficiently efficient on the purification of the pDNA from an *E. coli* pellet corresponding to 100 mL of culture. To accomplish a better purification, without changing the isolation Kit, maybe it could be reduced the number of cells, to get better performance. Decreasing the quantity of culture, we would have fewer cells and the lysis and purification method would be more efficient. However, with this approach another problem could appear, that could be related to the lower pDNA quantity recovered, which could compromise the production of the pre-miRNAs. So, because the genomic DNA present in the samples was in a smaller percentage when compared to the pDNA, and as in the next step this small concentration was not going to interfere in the amplification with the specific primers to the target sequence, it was decided to continue the experiments with these samples.

4.3 Pre-miRNA sequences amplification by PCR

With the two plasmids isolated, each one with the sequence for pre-miR-9 or pre-miR-29b, the next step in the process was to amplify each DNA sequence by PCR. For that, it was necessary to optimize the PCR experimental conditions, namely the volume of the reagents, annealing temperature of each pair of primers and concentration of the plasmid DNA, in order reaching a good and a specific amplification. In this regard, first it was tested the volume of the components of the master mix. The goal was to obtain a specific amplification, avoid non-specificity or dimers formation in the control. Negative control is a PCR reaction with all reagents except plasmid DNA. Thus, the effect of the volumes of the master mix components was evaluated. As shown in figure 15, several attempts were required to achieve the conditions where no amplification was verified in the control, trying several volumes for the dNTPs (one availability restricts polymerase action), for the reaction buffer (contains magnesium - polymerase cofactor) and for the primers (enable the amplification of all single strand). For pre-miR-9 it was necessary to decrease reaction buffer volume, restricting RNA polymerase action and avoiding non-specific amplification in the negative control. In turn, the amount of primers was adjusted to not affect sample amplification. Thus, the volumes chosen were: 0.45 µL of nuclease-free water, 0.9 µL of reaction buffer, 0.25 µL of dNTPs, 0.35 µL of primers and 0.2 µL of DreamTaq. For pre-miR-29b, the amplification was lower and the volume of each compound in the mixture had to be increased. The volume was optimized for: 8.2 µL of nuclease-free water, 1.6 µL of reaction buffer, 0.3 µL of dNTPs, 0.6 µL of primers and 0.2 µL of DreamTaq.



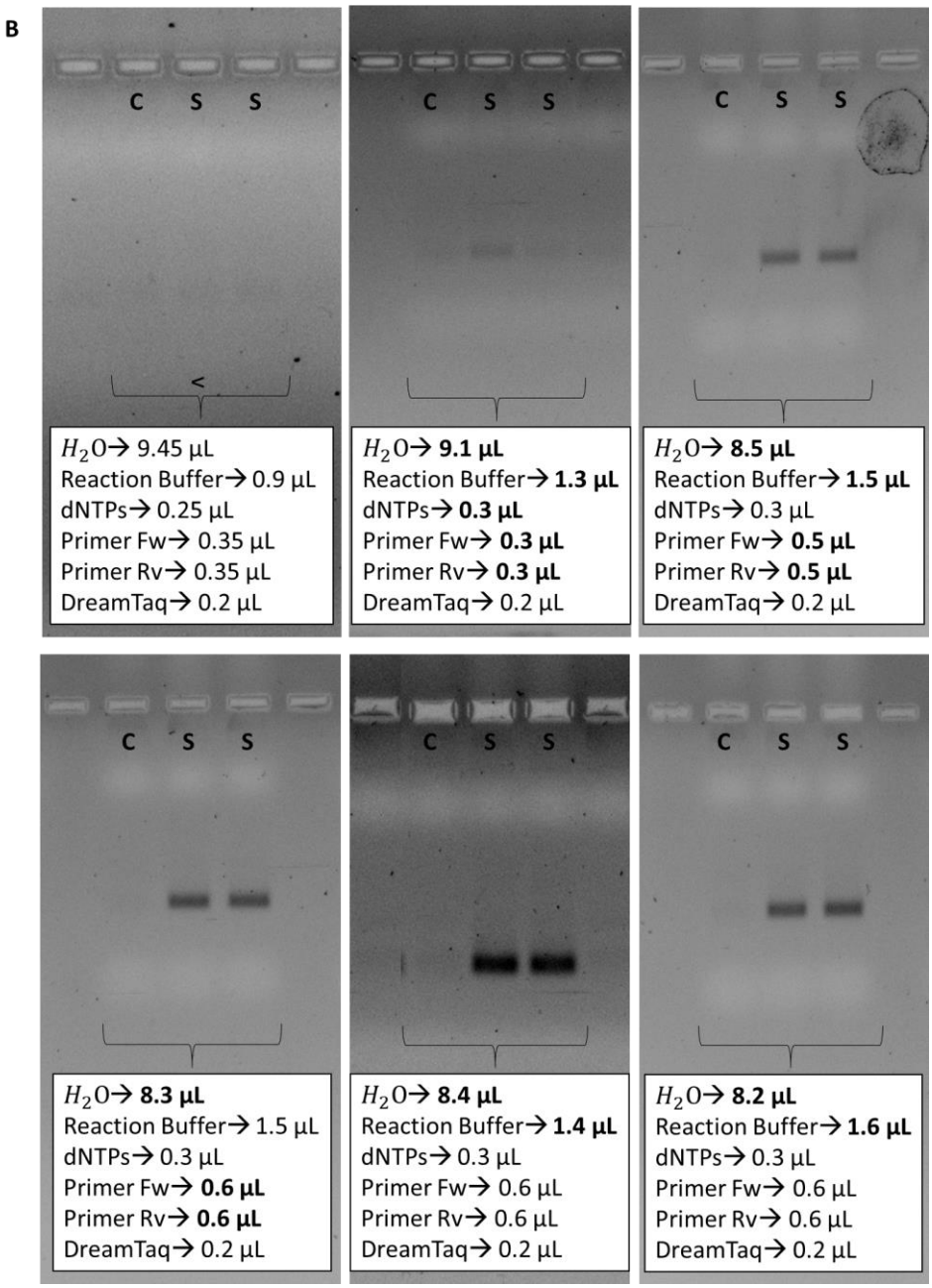


Figure 15. Optimization of the master mix components used in the PCR technique. A- 1% agarose gel electrophoresis of the pre-miR-9 PCR products with different volumes of the master mix components. B- 1% agarose gel electrophoresis of the pre-miR-29b PCR products with different volumes of the master mix components. Where C corresponds to the negative control of the PCR reaction and S corresponds to the obtained samples of the PCR reaction (master mix and DNA template).

Once, the volume for each component of the PCR mixture was established, the second step consisted to increase the amount of sequence amplified but maintaining the control without amplification. For that, different annealing temperatures between 60 ˚C to 70 ˚C were tested. This temperature is important because is in this PCR step where primers bind to the DNA template. The right annealing temperature allowed an increase in the specificity and a decreased in the non-specific amplifications, leading to an increase in the target amplification.

Analyzing the figure 16, it is possible to see that temperatures above 66 ˚C no occurred amplification, and the most probable explanation is that the primers were not annealing to the template. For the pre-miR-9 PCR products (figure 16A), the enzyme begins to amplify unspecific sequences below 65 ˚C and, so it was chosen 66˚C, as annealing temperature. For pre-miR-29b (figure 16B), below 65 ˚C starts to appear amplification in the negative control probably due to the fact that the primers are forming dimers, so it was selected 65 ˚C as annealing temperature. The annealing temperature below the amplifications are not specific.

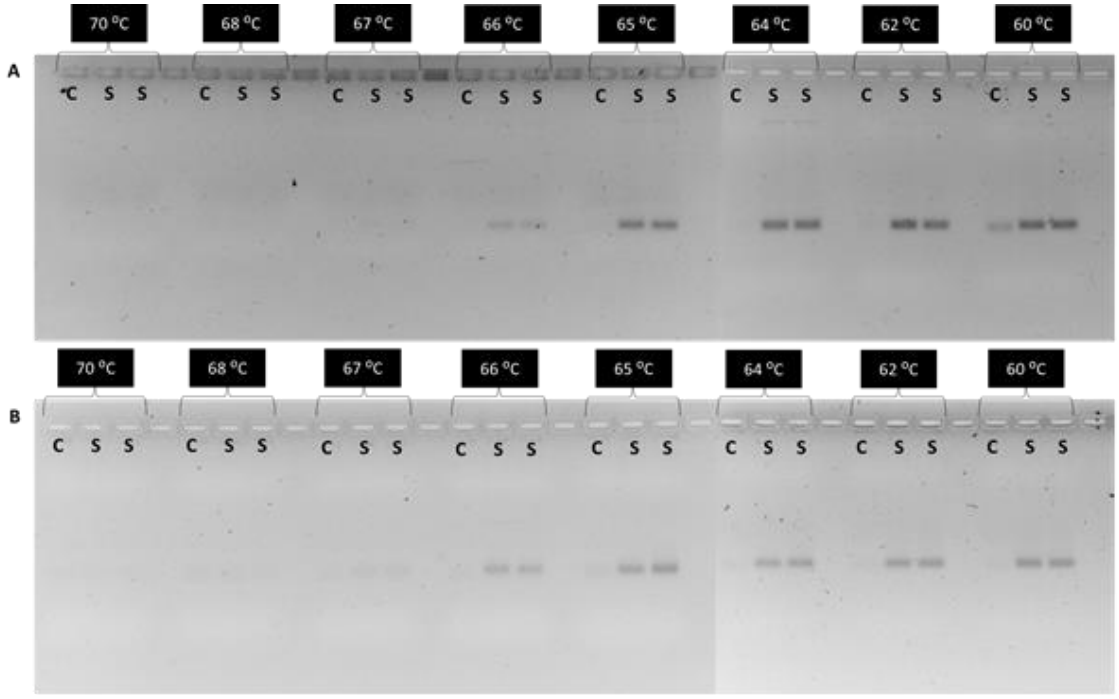


Figure 16. Effect of the annealing temperature on the PCR reaction. A- 1% agarose gel electrophoresis for the pre-miR-9 PCR with different annealing temperatures. B - 1% agarose gel electrophoresis for the pre-miR-29b PCR with different annealing temperatures. Where the C corresponds to the control with all the components of the master mix without plasmid DNA and S correspond to the samples where is included the master mix and the DNA template.

The cycles number in the PCR reaction was also tried, namely 25 and 30 cycles, to understand if increasing the amplification cycles, it would be possible to increase the quantity of the target sequence. In the pre-miR-9 amplification, a number of 30 cycles resulted in an amplification in the control and, in the case of the pre-miR-29b, it was not observed any difference (results not shown). So, it was selected to perform the PCR with the 24 cycles.

Finally, the amount of DNA to add to the reaction mix was also optimized. For that, it was performed PCR reactions with three distinct dilutions of the pDNA isolated from *E. coli* DH5α, namely 1:100, 1:1000 and 1:10000. The amount of DNA template included in the PCR mixture can influence the amplification, by inhibiting it due to excess of pDNA, leading to unspecific amplification, or lack of template. Once more, the main goal was to obtain a good amplification without any unspecific amplifications. As shown in figure 17A, for pre-miR-9 the more pDNA the more amplification, but it can also be observed that it starts to amplify other sequences in the pDNA, so it was chosen dilution 1:100 because represented the better result in terms of specific amplification. For pre-miR-29b (figure 17B) the smaller dilution was the best one since the amplification level was greatly affected by higher dilutions. The final conditions optimized for amplification of the pre-miR-9 and pre-miR-29b sequences are summarized in table 8 and the profile of the amplification achieved under these conditions is presented in figure 18.

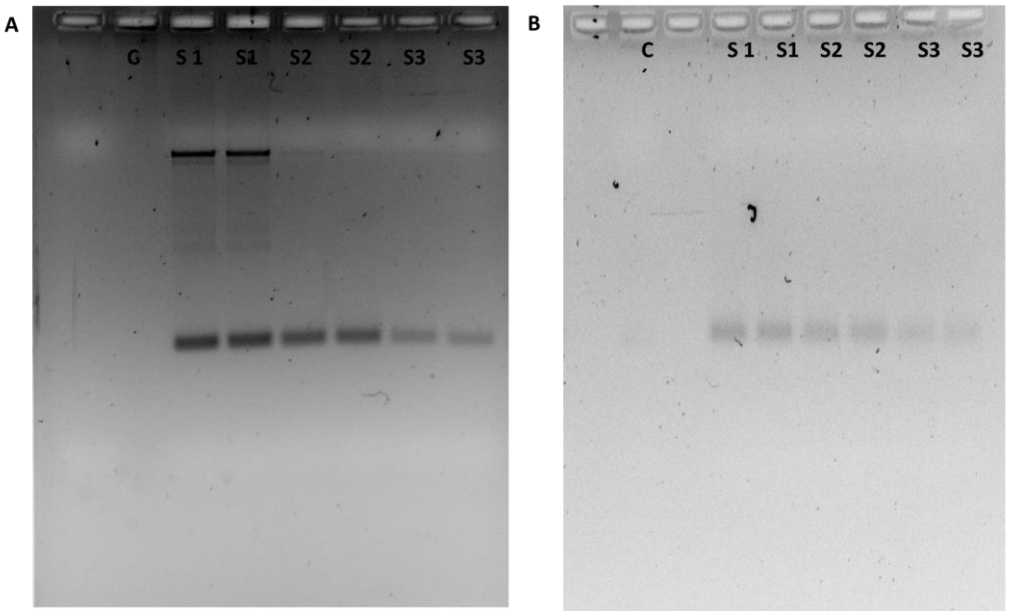


Figure 17. Optimization of the DNA concentration used for the PCR technique. A- 1% agarose gel electrophoresis for pre-mir-9 using three different dilutions. B - 1% agarose gel electrophoresis for pre-miR-29b using three different dilutions. The C corresponds to the negative control with all the components of the master mix (except template), S1 correspond to dilution 1:100, S2 to dilution 1:1000 and S3 to dilution 1:10000 of the DNA template in the samples.

Table 8. Summary of the final conditions for PCR after optimization for pre-miR-9 and pre-miR-29b.

		Pre-miR-9	Pre-miR-29b
Master Mix	H_2O (µL)	9.45	8.2
	Reaction Buffer (µL)	0.9	1.6
	dNTPs (µL)	0.25	0.3
	Primer Fw (µL)	0.35	0.6
	Primer Rv (µL)	0.35	0.6
	DreamTaq (µL)	0.2	0.2
Annealing temperature (˚C)		65	66
DNA dilution		1:1000	1:100

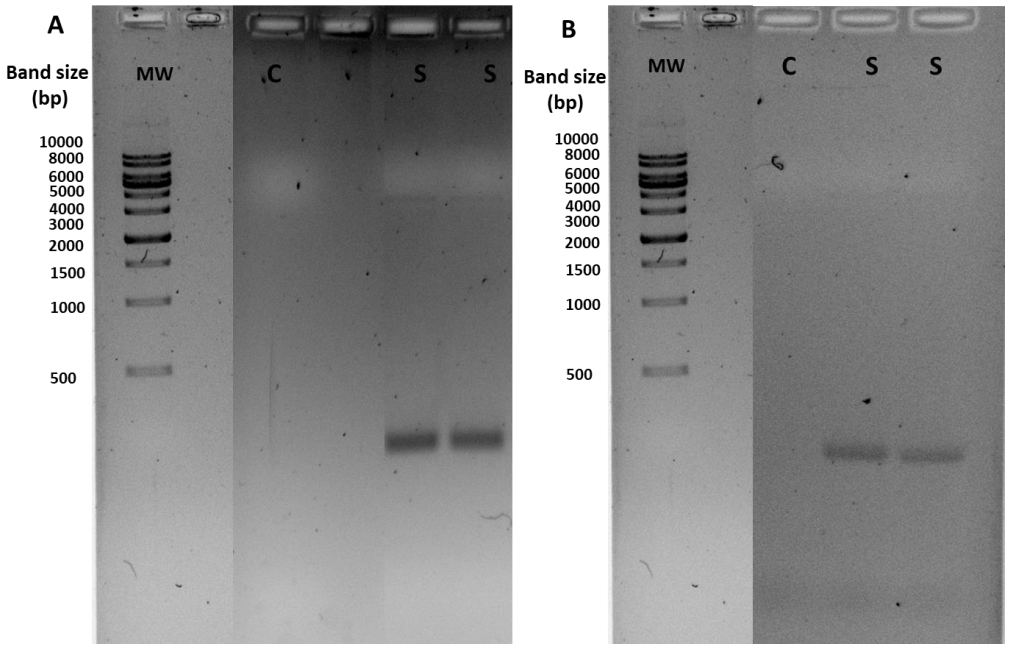


Figure 18. Final amplification of the pre-miRNAs, after optimization of the PCR conditions. A- 1% agarose gel electrophoresis for pre-miR-9 amplification. B - 1% agarose gel electrophoresis for pre-miR-29b amplification. The C corresponds to the negative control and S corresponds to the samples containing DNA template.

Despite all attempts, one of the problems in the optimization of the PCR for the pre-miR-9 was that this pair of primers were not specific enough and keep on amplifying the plasmid, which is visible by the faint band of higher molecular weight. However, because the quantity was almost inexistent, that amplification was not considered.

4.4 Purification of the PCR products

After amplification, PCR products were then purified using the NucleoSpin Gel and PCR Clear-up Kit. For the confirmation that the conditions in the kit were the right ones and that the purified samples of pre-miR-9 and -29b were maintained intact, a 1% agarose gel electrophoresis was performed after purification.

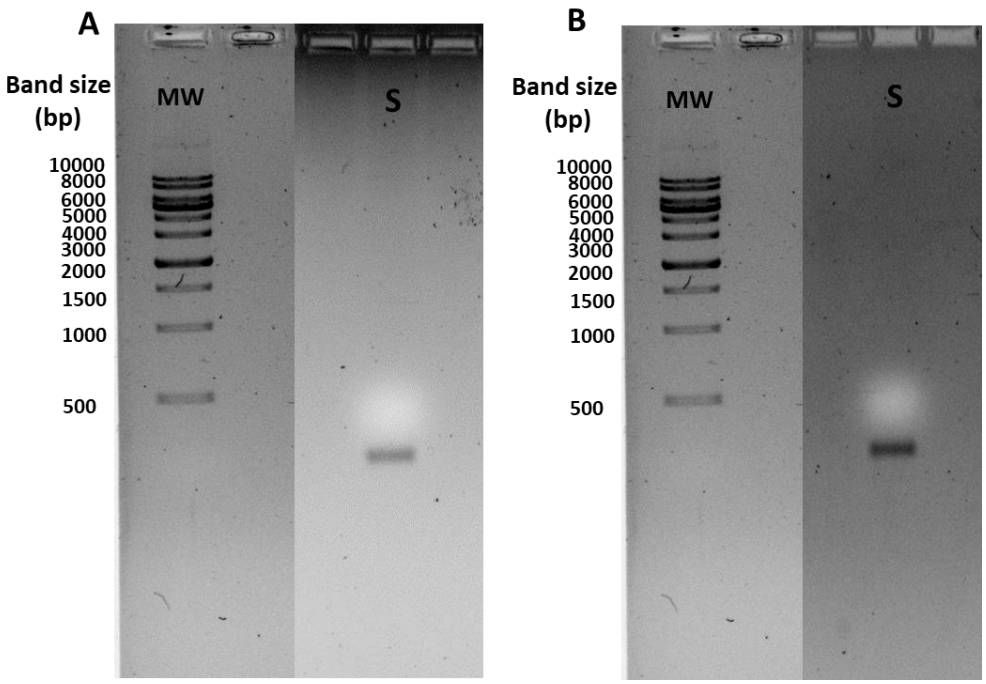


Figure 19. Electrophoresis after the purification of PCR products. A- 1% agarose gel electrophoresis of the purified pre-miR-9. B - 1% agarose gel electrophoresis of the purified pre-miR-29b. The S corresponds to the purified samples.

The figure 19 is representative of the purification process, and its analysis allows to confirm that the sequences amplified remain intact after purification and the presence of the pDNA template in the pre-miR-9 PCR reaction (figure 19A) was almost nonexistent, which solved the problem encountered in the optimization step.

4.5 *In vitro* transcription of pre-miRNAs

Completed this process, the samples were then transcribed to RNA using in vitro transcription kit. For the confirmation that the conditions in the kit were the appropriate for the pre-miR-9 and -29b transcription and to verify if a suitable and intact transcripts samples was obtained, the analysis by agaroses gel electrophoresis was also performed (figure 20).

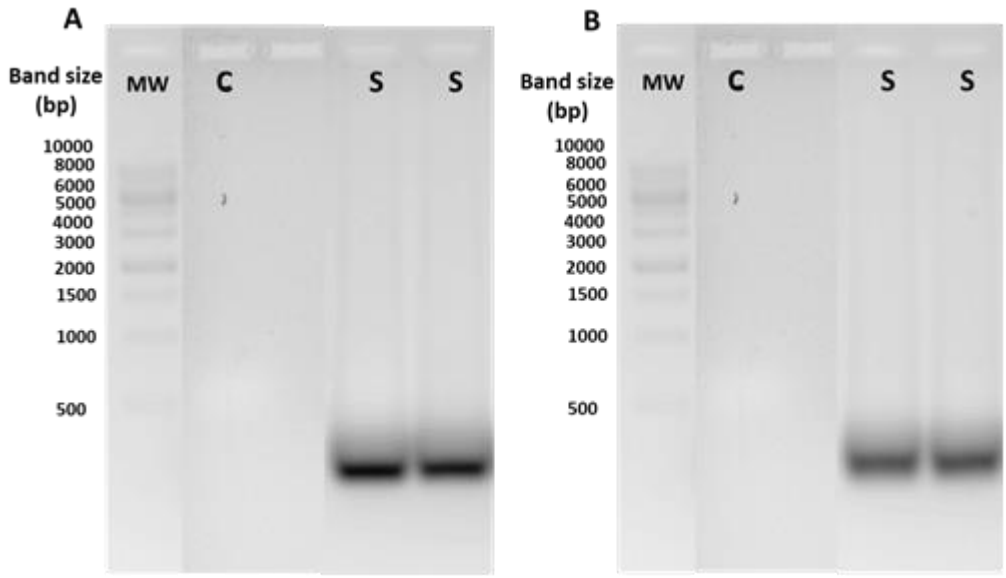


Figure 20. Electrophoresis of the pre-miR-9 and pre-miR-29b transcripts obtained by *in vitro* transcription. A- 1% agarose gel electrophoresis of the pre-miR-9 transcript. B - 1% agarose gel electrophoresis of the pre-miR-29b transcript. The C corresponds to the negative control and S corresponds to samples.

After analyzing the electrophoresis, figure 20, it is possible conclude that the pre-miRNAs PCR sequences were successfully transcribed to RNA and that all DNA present in the samples was degraded by the DNAse I.

At the end of the transcription, some of the samples were purified and concentrated. The figure 21 shows the result of this purification and concentration step, and it becomes clear the increased intensity of the pre-miRNAs bands, which is extremely important for the transfection experiments. Moreover, it was proved the purity and integrity of the transcripts, suggesting their suitability to proceed the study and evaluate their impact on the expression of target proteins associated to AD.

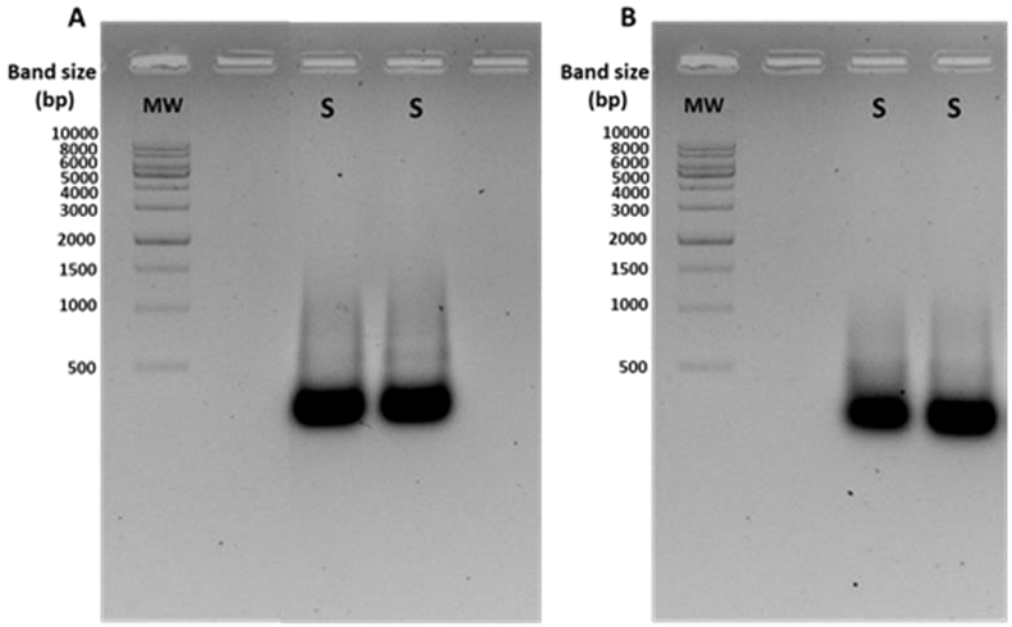


Figure 21. Electrophoresis of the concentrated and pure RNA transcripts. A - 1% agarose gel electrophoresis of the purification and concentration of the pre-miR-9. B - 1% agarose gel electrophoresis of the purification and concentration of the pre-miR-29b. Where S corresponds to the purified samples.

4.6 Analysis of the mRNA expression by RT-qPCR

N2a695 cells were transfected with the different miRNAs, namely synthetic miR-9/-29b and pre-miR-9/-29b obtained by *in vitro* transcription, were analyzed by RT-qPCR for the determination of mRNA expression levels. The cells were lysed, and the total RNA was extracted using TRIzol. After total RNA extraction the RNA samples were subjected to an agarose gel electrophoresis, to verify the absence of DNA contamination and to assess its integrity. In this type of extraction, the electrophoresis profile is the presence of 3 bands, corresponding to rRNA 28s, rRNA 18s and RNA of low molecular weight, by decreasing order of molecular weight. The integrity of the RNA is evaluated by the integrity of the rRNA because these are the most abundant in the eukaryotic cells and can be easily seen in the agarose electrophoresis. Following this and by observing figure 22, it can be said that RNA extracted from N2a695 cells is intact and without DNA contamination.

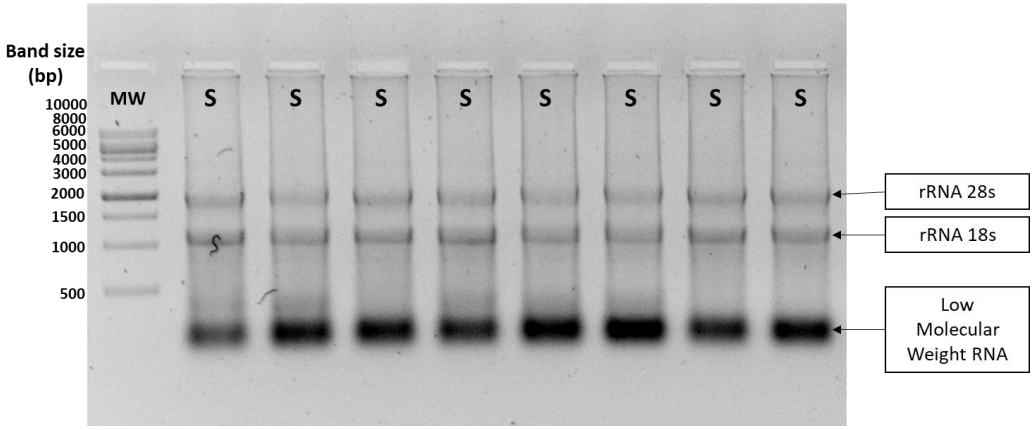


Figure 22. Electrophoresis to verify the integrity of total RNA extracted from the transfected N2a695 cells. Where S correspond to the total RNA extracted from eukaryotic cells.

To evaluate and compare the effect of miR-9/-29b and of the precursor forms, pre-miR-9/-29b on the regulation of the expression of proteins related with AD pathological hallmarks, N2a695 cells were transfected, also using two different transfection agents. With this study we intended to compare, on one hand the influence of using the mature form of miRNA, or the precursor form, the pre-miRNA, on the proteins expression, also inferring about the most suitable method to prepare the miRNA/pre-miRNA, chemically synthesized vs enzymatically prepared. On the other hand, it was also expected to study the effect and influence of the transfection agent, lipidic- or polymeric-based, on the protection and delivery of the RNA. RT-qPCR was applied with the main objective of evaluating the differences in mRNA expression of key proteins involved in the pathological pathway of AD, like APP, BACE1 and PS1.

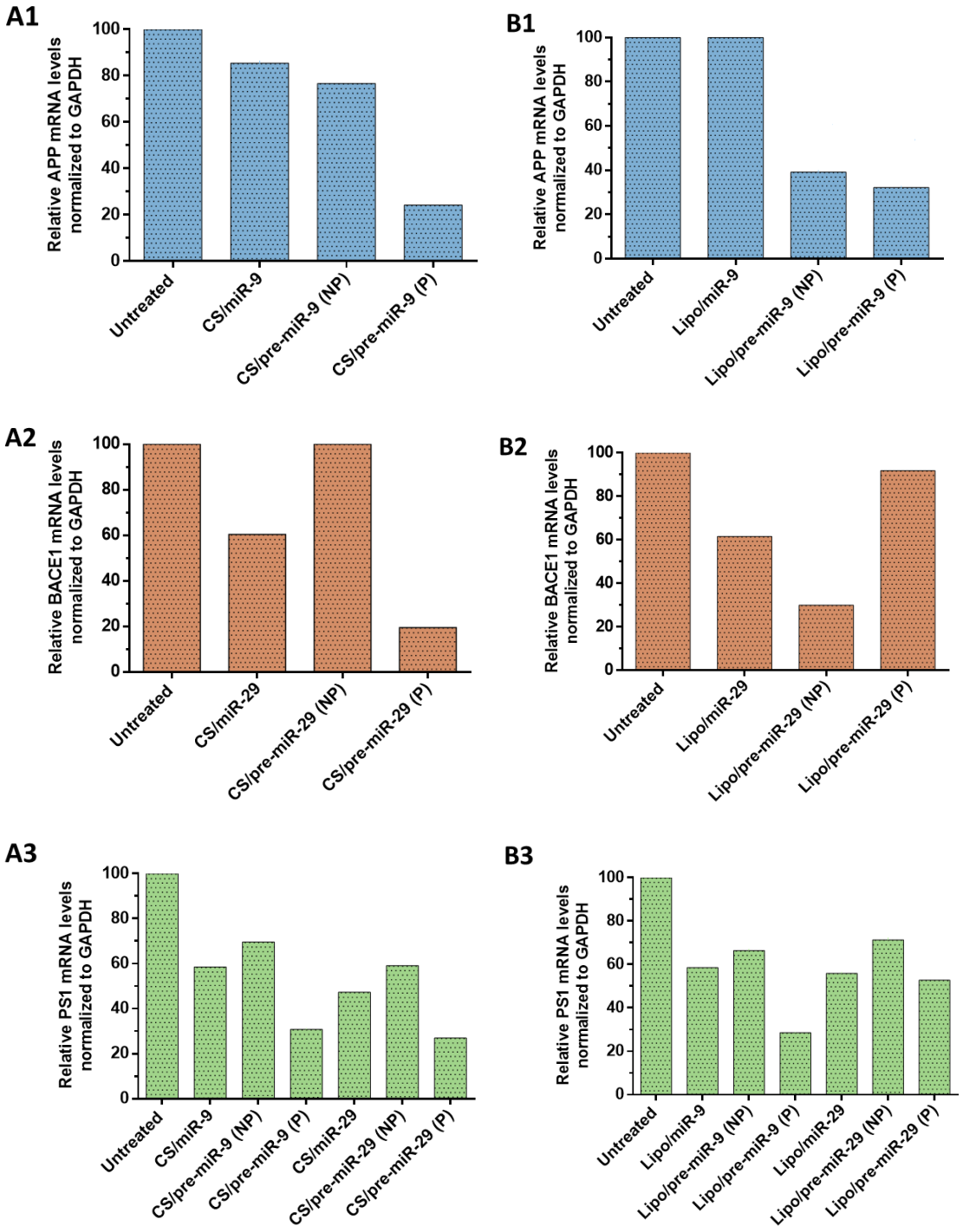


Figure 23. RT-qPCR analysis of the expression levels of APP, BACE1 and PS1 mRNA from N2a695 cells transfected with different types of miRNAs and pre-miRNAs. A – Cells transfections performed with chitosan-nanoparticles, where A1 corresponds to the APP mRNA levels, A2 corresponds to the BACE1 mRNA levels and A3 corresponds to the PS1 mRNA levels; B – Cells transfections performed with Lipofectamine 2000, where B1 corresponds to the APP mRNA levels, B2 corresponds to the BACE1 mRNA levels and B3 corresponds to the PS1 mRNA levels; NP means non-purified transcript and P means purified transcript.

Analyzing the mRNA levels, it was verified a reduction on the APP mRNA levels only when the cells were transfected with miR-9 (figure 23 A1 and B1), and no effect was observed after transfection with miR-29 (results not shown). The results showed about 70% of APP

mRNA silencing in the cells transfected with pre-miR-9 purified (with both transfection agents), while only a reduction of around 15% was achieved with the synthetic miR-9. Actually, from the revision of the literature it was not possible to find many studies correlating the levels and influence of miR-9 in APP. One example of this possible influence is described on the work of Hong and collaborators (Hong *et al.*, 2017), where it is mentioned that in APP/PS1 AD mice (which are transgenic mice co-injected with a vector encoding APP and PS1 mutants), miR-9 was found to be significantly downregulated, suggesting a relationship between these proteins and miR-9. However, further studies are needed to understand if the results seen in this work correspond to a direct or indirect influence of miR-9 in the APP, and also to verify which can be the consequences of this silencing on the pathological mechanism of AD. APP protein plays a vital role in regulating metals and iron transport in the brain, however, its cleavage may sometimes be unregulated and produce Aß peptides that are toxic to the brain. Therefore, APP silencing should be well understood to ensure that these physiological functions are not affected.

Contrarily to the results for APP, for BACE1 the effect on its mRNA expression was only verified for the cells transfected with miR-29b (figure 23 A2 and B2). The silencing of BACE1 mRNA by pre-miR-29b was also seen in previous studies developed by our research group (Pereira *et al.,* 2016), where N2a695 cells were transfected with recombinant pre-miR-29b and synthetic miR-29b, resulting in around 75% and 40% of BACE1 mRNA silencing, respectively. Also, a decrease of 40% of BACE1 mRNA expression induced by miR-29b (with both transfections agents) was verified in the present work, what is in total agreement with the previous published results. Other researchers also described a possible relationship between this miRNA and BACE1 protein (Yang *et al.*, 2015), this work described, by correlation analysis, that miR-29c, cluster with miR-29b, was downregulated in peripheral blood of patients with AD and also found upregulated BACE1 levels. The results for the pre-miR-29b are not in agreement between methods of transfections but the 80% silencing induced by the pre-miR-29b purified can be related with the same tendency seen in our previous publish study (Pereira *et al.*, 2016).

For the PS1 mRNA expression, both miR-9 and miR-29b provoked a decrease in the mRNA levels, which is in line with the results obtained in our previous studies and to the evidence shown in the study done by Maes *et al.*, 2009, where was suggested that miR-9 can have an influence in the regulation of PS1 expression. In this study, synthetic miR-9 induced 40% of silencing and synthetic miR-29b allowed a ~50% reduction of the PS1 mRNA levels. However, the pre-miR-9 and pre-miR-29b purified induced the highest percentage of silencing, with 70% for the pre-miR-9 purified and ~71/50% for the pre-miR-29b. In general, the influence of miR-9 and miR-29b found in this work, it is in line with the evidence shown in the study done by Krichevsky *et al.*, 2003, where it was suggested that miR-9 may influence the regulation of PS1 expression. Krichevsky and collaborators showed that PS1 null mice, miR-9 was decreased and had several brain defects. Maes *et al.*, 2009 also predicted that

miR-9 has PS1 as target, suggesting that positive regulation of PS1 could be associated with low levels of miR-9 expression.

Relatively to the methods used for the miRNAs production, our results seem to indicate that, in general, the pre-miRNAs produced enzymatically (*in vitro* transcription after production of a recombinant vector) and further purified, and delivered in precursor form, led to a more significant silencing effect (around 70%), when compared with the synthetic miRNAs (around 40%), delivered in the mature form. One possible explanation is that the mature form can be detected by the cells as aberrant and consequently be destroyed and eliminated, impairing its function. Other justification already proposed is the possibility that the recognition and processing within the cell to be more efficient when delivering a hairpin shape RNA (Tsutsumi *et al.*, 2011), and thus the pre-miRNA could be more rapidly recognized and processed by the cell machinery within the biogenesis route, to lead the formation of mature and more active miRNA. Moreover, even between the pre-miRNAs it was observed a difference on the results achieved with the purified transcript (around 70%) in comparison to the non-purified pre-miRNAs (around 35%). These results can be explained by the fact that when non-purified sample is used, some contaminants like nucleotides and aberrant pre-miRNAs are present and can also be encapsulated and delivered to the cells. This can reduce the rate of encapsulation and delivery of the right pre-miRNA, limiting its action on the cells and, in addition, these aberrant forms can lead to some toxicity or off-target effects, affecting the results. Thus, the results clearly demonstrate the importance of properly purify and deliver the miRNAs to reach the best performance or potential therapeutic effect.

Despite the specific effect of each miRNA on the different mRNAs levels, a general result was verified concerning the transfection agents used. Globally, the chitosan-nanoparticles appears to have a better performance for encapsulation and delivery than the lipofectamine, maybe due to the chitosan-nanoparticles being derivate from natural material, giving them low toxicity, and being able to encapsulate large amounts of genetic material. With the use of lipofectamine, some results were not coherent, and some variability was found, maybe due to the properties of lipofectamine, namely the toxicity that can be associated to this agent (Pereira *et al.*, 2017). For a better comparison of these two agents, or even others, more experiments must be done.

4.7 Analysis of the protein expression by Western blot

To evaluate the effect of the different types of miRNAs used on the transfection of the N2a695 cells, in the protein expression (APP, BACE1 and PS1), the western blot technique was used. The main bands obtained were approximately at 95 kDa for APP protein, at 70 kDa

for BACE1 and at 50 kDa for PS1. The band at 42 kDa was correspondent to the ß-actin, used as housekeeping, to normalize the quantity of protein in the samples (figure 24).

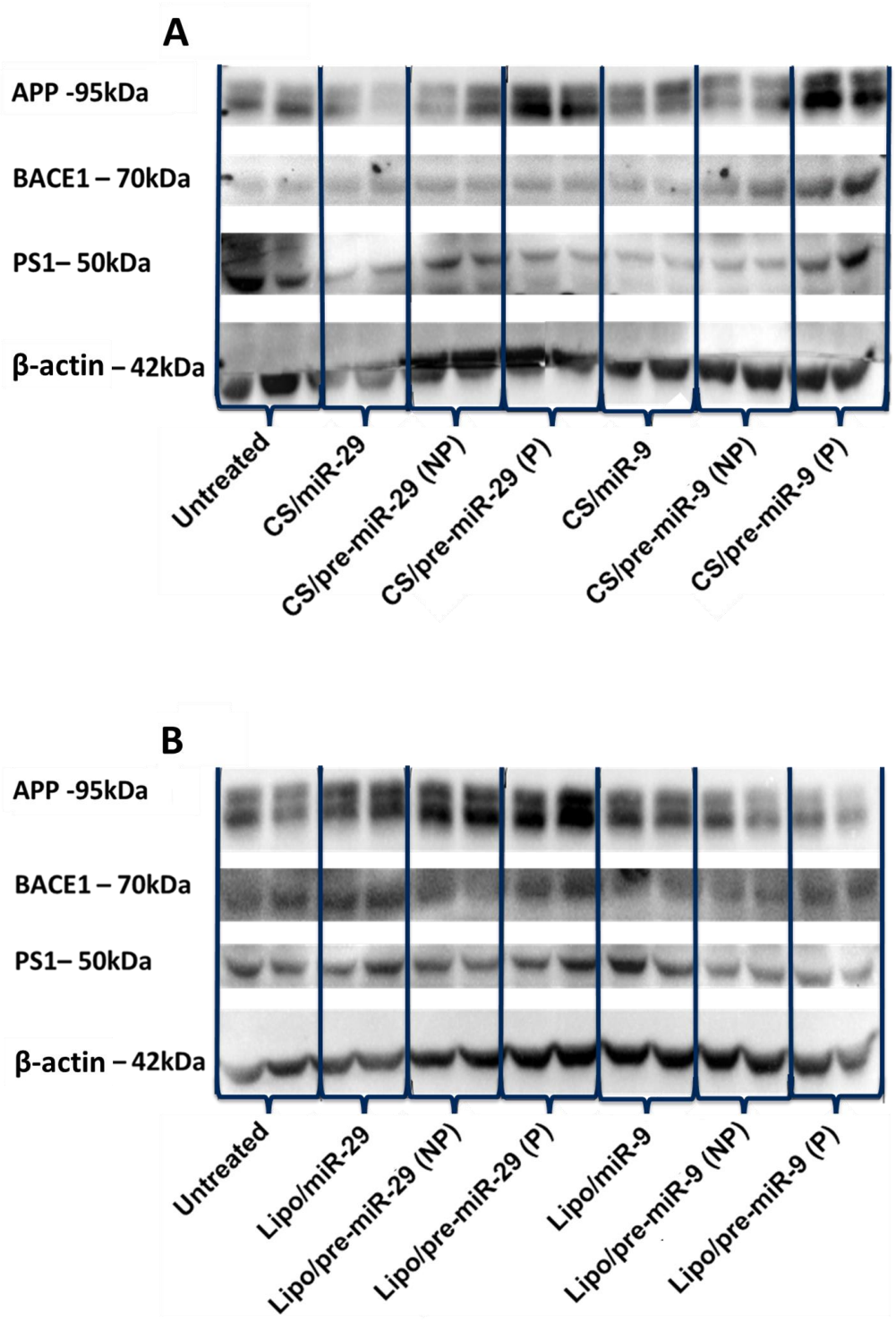


Figure 24. Western blot analysis of APP, BACE1, PS1 and ß-actin from N2a695 cells transfected with different types of miRNAs and pre-miRNAs. A- Western blot for the transfection done with chitosan-nanoparticles; B- Western blot for the transfection done with Lipofectamine 2000; NP means non-purified transcript and P means purified transcript.

By observing the figure 24A, although not an obvious effect was achieved, which limits the quantitative analysis, the results seem to suggest that for the APP, synthetic forms of miR-29b and miR-9 lead to a slight decrease in the protein expression. However, as the results are not totally evident, more experiments should be done to confirm, also because regarding the effect on the mRNA levels of APP, only the miR-9 seemed to have some influence. Considering the PS1 it is better visualized the silencing effect induced by all tested miRNAs and pre-miRNAs, when compared to the control, what is also in agreement with the results presented for the mRNA levels. Now, focusing on the results obtained for the transfection with Lipofectamine 2000 (figure 24B), in general, it is not possible to describe a decrease in the proteins expression, except for the APP and PS1 in the samples corresponding to the pre-miR-9 purified and unpurified, where it seems to be present a slight decrease on the proteins bands intensity. The BACE1 protein bands are not well defined which makes it difficult to analyze the results but there seems to be no silencing in both methods of transfection. Compering the transfection agents, by the results it can't be said with certainty which one is the best, but the results suggest that the chitosan-nanoparticles was more efficient on the transfection, what, in this case, could corroborate the results obtained for the RT-qPCR experiments. The discrepancy between these results and techniques may have different causes. A possible cause for this can be degradation of the pre-miRNA somewhere between the production process and the transfection into the N2a695 cells, once the RNA is an extremely vulnerable molecule and slight variations in temperature are enough to alter the molecular structure. Another, explanation can be the lower transfection efficiency, most likely in the lipofectamine transfections due to the need of a better optimization for the delivery of pre-miRNA. Thus, the consequence of low miRNA levels within the cells would be a limitation on the target mRNAs inhibition, and consequently it would not be sufficient to have a more pronounced effect on proteins expression. Also, the stability of cells could influence these results, which could explain the irregularities seen in the housekeeping band. The N2a695 cells are cells mutated to produce APP that is favorable to be cleaved by the amyloid pathway, with the goal to replicate the process of AD. This makes them more likely to suffer mutations with the manipulations and transfections, also affecting the results of transfection and silencing evaluation. All these limitations need to be overcome, and so, more experiments are required to confirm the results and conclude about the real effect of each sample on the expression levels of target mRNA and proteins. However, even considering these difficulties, this work gives some important insights about the relevance of controlling the RNA purity and stability to reach better biological outputs. It becomes really evident the difference in the biological effect induced by miRNAs or pre-miRNAs, which is also related to the production method, emphasizing the need to explore more robust processes for the RNA production when its therapeutic application is envisioned.

Chapter V

Conclusion and Future perspectives

5. Conclusion and Future perspectives

AD is one of the neurodegenerative diseases that affect most people in the world, having just few therapies to relive symptoms, and all of them with adverse side effects. Presently there are not known effective treatments to delay or stop the disease progression. This fact is due to the complex and interconnected pathologic mechanism that can directly or indirectly result in the hallmarks of this disease. Nevertheless, one thing is known, that proteins like APP, BACE1, PS1, Aß and Tau are elevated and have an important active role in the pathology of AD. An alternative to all the failures in the attempt to created efficient treatments for AD was the used of miRNAs that have the ability to regulate protein expression by the decrease or blockage of their target mRNA translation. Another fact in favor of this line of thought is that miRNAs show to be dysregulated in the brain of AD patients and have been used as biomarkers to detect the early stages. So, the idea that miRNA can be used as therapeutic agents is based on the thought that if some miRNAs levels are decreased and some proteins are overexpressed when the pathology is present, and considering that miRNAs can regulate proteins levels, maybe the re-establishment of the miRNAs levels could regulate the proteins expression, and consequently the hallmarks of the disease. This led to try to find the targets of the miRNAs unregulated in AD, so they could be used as therapeutic vehicles to reset the levels of both miRNAs and proteins, back to the balance found in a healthy brain.

So, the main goal in this work was to try to find any evidence that showed that miR-9 and miR-29b, both downregulated in AD, have influence in three of the main proteins present in amyloid pathway. In general, in the results of this work it was observed that the miR-9 had an effect in the mRNA silencing of the APP, while the miR-29b showed better effect in decreasing the mRNA expression of the BACE1. The PS1 was the protein that showed that can be regulated by both miRNAs, in both mRNA and the protein levels. Which of the miRNAs have a more significant effect in the PS1 is difficult to tell because more studies are needed for a better understanding. Between all the types of miRNAs used for the transfections, the enzymatically and purified pre-miRNAs, in general, showed the best results in silencing the mRNA. Relatively to the transfection agents, both enabled the delivery of miRNA, although the results seem to be more reliable between techniques when using chitosan-nanoparticles.

In conclusion, it can be said that the goal of this work was achieved because it was shown that both miR-9 and miR-29 already identified by other groups as regulators of APP, BACE1 and PS1, showed to have an influence, mainly, in the mRNA expression. Still, the work has many points to be confirmed and methods that need to be tested again to be said with certainty which methods of delivery and production of miRNA can lead to better results, and in specific which miRNA can be associated to the regulation of proteins, that can be related with the potential AD treatment.

Thus, for future perspectives is intended to repeat the experience with more replicates and with co-transfection of both miRNAs, to improve the results by western blot technique, using other techniques to confirm results or exclude error that maybe exist and test the effect in the Aß levels and hyperphosphorylated Tau.

Chapter VI

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6.References

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