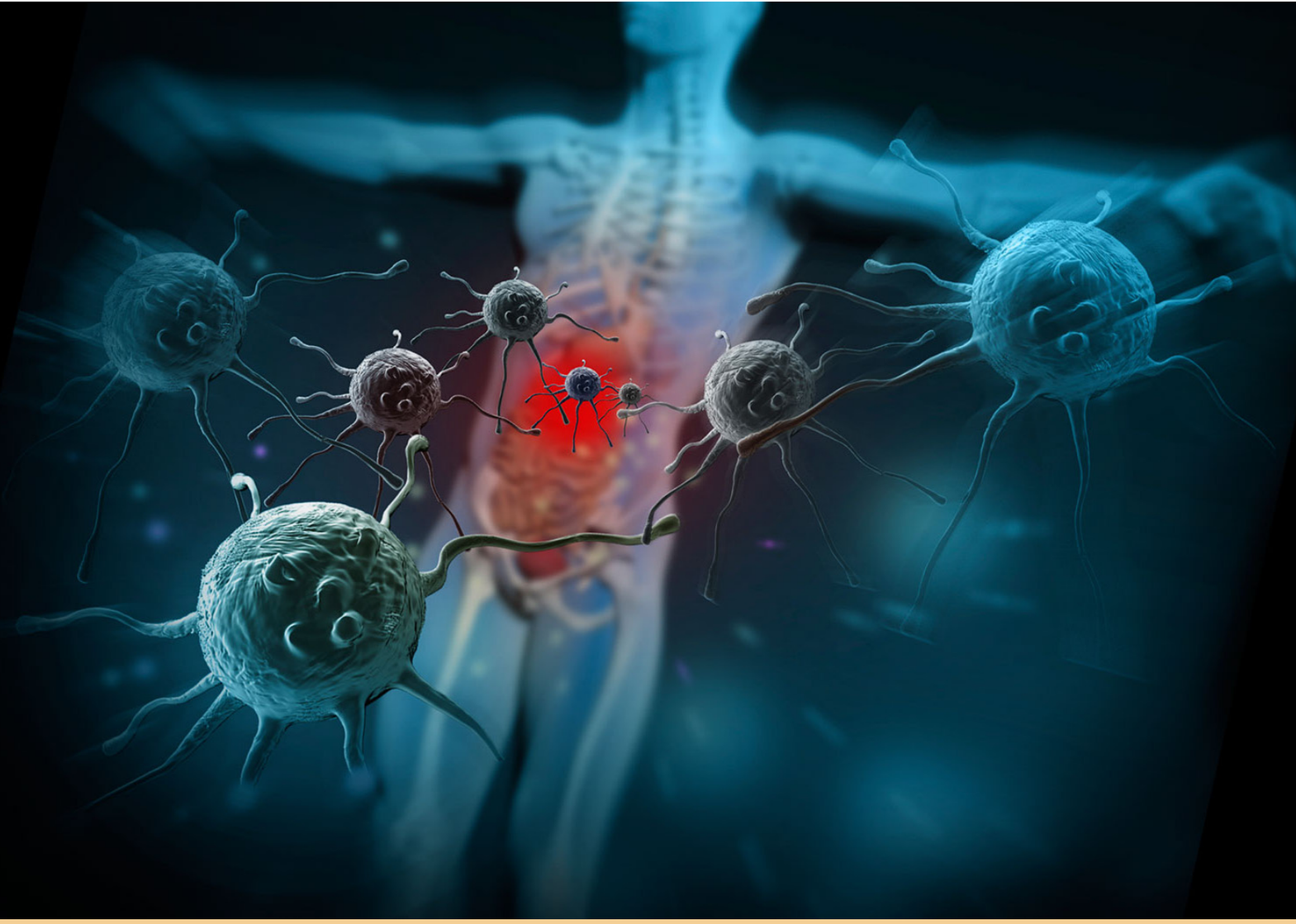

Mafalda Inês Álvares Ribeiro Martins da Silva

Exosomes in Alzheimer's disease - potential biomarkers for pre-clinical diagnosis and their therapeutic role

Monografia realizada no âmbito da unidade de Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pela Professora Doutora Maria Teresa Teixeira Cruz Rosete e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro 2016

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Coimbra, 16 de setembro de 2016.

_____

(Mafalda Inês Álvares Ribeiro Martins da Silva)

A Tutora

_____

(Professora Doutora Maria Teresa Teixeira Cruz Rosete)

A Aluna

_____

(Mafalda Silva)

Agradecimentos

À Professora Doutora Maria Teresa Teixeira da Cruz Rosete, minha tutora, pela disponibilidade e encorajamento.

Ao Pai, à Mãe e ao António Bernardo.

À Carolina.
À Maria Afonso.

Ao Francisco.

Index

Abbreviations

Aβ – β-Amyloid protein

Ach – Acetylcholine

AD – Alzheimer Disease

ApoE – Apolipoprotein E

APP – Amyloid beta Precursor Protein

BACE1 – Beta-site-APP-Cleaving Enzyme 1

BBB – Brain Blood Barrier

CSF – Cerebrospinal Fluid

EC – Enthorinal Cortex

EV – cell-derived Extracellular Vesicles

FAD – Familial AD

ILVs – Intra-Luminal Vesicles

MCI – Mild Cognitive Impairment

MHCI – Major Histocompatibility Complexes Class I

MHCII – Major Histocompatibility Complexes Class II

miR – microRNA

miRNA – microRNA

MVB – Multivesicular Body

MVs – Microvesicles

NFT – Neurofibrillary Tangles

NMDARs – N-Methyl-D-Aspartate Receptors

NP – Neurofibrillary Plaques

OLGs – Oligodendrocytes

PET – Positron Emission Tomography

PS1 – Presenilin 1

PS2 – Presenilin 2

pTau – Hyperphosphorylated Tau protein

SP – Senile Plaques

Resumo

A doença de Alzheimer (DA) é uma doença crónica, progressiva, cada vez mais prevalente e que se inicia com um longo período de latência clínica, durante o qual não são visíveis quaisquer sintomas. Atualmente, o diagnóstico da DA consiste na utilização de técnicas de imagiologia e na medição de bioindicadores nos fluídos corporais. Estas ferramentas de diagnóstico são maioritariamente invasivas e apenas urge executá-las quando se manifestam os primeiros sintomas, que geralmente só se começam a pronunciar em fases tardias da doença. Para ultrapassar estes problemas, muitos investigadores ambicionam identificar biomarcadores menos invasivos que permitam detetar a doença, desde a fase pré-clínica, e monitorizar o seu decurso, de forma a iniciar e a gerir o tratamento, o mais cedo possível. Recentemente, um número considerável de projetos de investigação revelou a presença de alterações nos níveis dos microRNAs (miRNAs) provenientes de exossomas, tanto em cérebros de cadáveres que sofreram da DA, como em fluídos biológicos de pacientes que têm a doença e ainda em modelos de ratos portadores desta neurodegenerescência. De acordo com alguns estudos, os exossomas também poderão ter um efeito terapêutico nesta patologia. Estas descobertas elegem os exossomas como biomarcadores promissores no diagnóstico da DA, e ainda, possíveis intervenientes no seu tratamento. Na presente monografia, foram consultados alguns artigos de revisão a partir dos quais se resumiram os avanços mais recentes focados no potencial de aplicação dos exossomas no diagnóstico, prognóstico, monitorização e tratamento da DA, desde o momento em que os seus sinais fisiopatológicos começam a deflagrar.

Palavras-chave: Alzheimer, Exosomas, miRNAs, Diagnóstico.

Abstract

Alzheimer disease (AD) is chronic, progressive, increasingly prevalent and has a long asymptomatic latency period. Current diagnosis of AD consists mainly in imaging techniques and measurement of bioindicators in the bodily fluids. These diagnostic tools are only undertaken when the first symptoms appear, which corresponds to a late stage of the disease, and some of them are invasive approaches. In order to overcome these faults, many investigators are searching for minimally invasive biomarkers that can detect the disease, since its pre-clinic stages, as well as monitor its course, in order to enable an earlier treatment and initiate disease management. Recently, an increasing body of literature has reported changes in the levels of exosomal microRNAs (miRNAs) either in AD *post-mortem* brain, biofluids from AD patients, or even in AD mouse models. Furthermore, some studies have verified that exosomes may also have a therapeutic role in this pathology. These findings elect exosomes as promising diagnostic biomarkers and therapeutic players in AD. In this document, the author consulted relevant reviews with the aim of summarizing the most recent developments in the potential application of exosomes either in diagnosis, prognosis, monitoring and therapeutic of AD, since its very onset.

Key words: Alzheimer, Exosomes, miRNAs, Diagnosis.

1. Introduction

AD is a pathology that has a deleterious impact in the elderly generations. This scenario gets even worse as the disease is only possible to be detected at late stages and has not a definitive cure. For this reason, I consider the investigation on this issue of paramount importance, and combining with my particular interest in the pathophysiology and pharmacology of neurological disorders, I chose the theme "Exosomes in Alzheimer's Disease – Potential biomarkers for pre-clinical diagnosis and their therapeutic role" as the subject of my Master's degree.

This monography starts with the description of AD, its epidemiology, clinical features, pathophysiology, and scarce available therapies. Thereafter, it will be reported the current diagnostic tools of this neurodegenerative disease, underlining their limitations. Having made this contextualization, shall be explained the urgent need of the introduction of new diagnostic methods that detect the pathology at its onset, to allow the beginning of the treatment at an early phase. This is where the theme of my work emerges, the potential of exosomal miRNAs in clinical diagnosis and treatment of AD. In favour of facilitating the comprehension of this matter, it will be made a previous approach on the characterization of exosomes, their biogenesis, molecular composition and functions. Only then I will get into exosomal miRNAs, their isolation from bodily fluids and detection methods. Subsequently, I will review the results of some studies in which has been performed expression profiling of miRNAs in AD cases vs. healthy controls with the purpose of establishing miRNA expression patterns typical of AD. In addition, there will be presented some evidences that attribute to exosomes both possible neuroprotective and neurotoxic roles in the disease.

In summary, I will try to provide an overview of the latest data of exosomal miRNAs as potential diagnostic biomarkers for AD, and also to explain the neutrodetrimental vs. beneficial and therapeutic role of exosomes in the pathology, based on current literature. At the end, I will conclude which gaps are yet to fill in this research, and what are the future perspectives to overcome these difficulties and to successfully implement these biomarkers in clinical practice.

2. Alzheimer Disease

2.1. Characterization and Epidemiology

Alzheimer's disease (AD) is a neurodegenerative disease and the leading cause of dementia in adult life. It is a complex disorder with both environmental and genetic components (1). Its early onset, when signs and symptoms appear before the age of 65, is associated with genetic mutations in the transmembrane amyloid beta precursor protein (APP), in presenilin 1(PS1) and presenilin 2 (PS2) (2-4). The late onset of this multifactor disease, after 65 years, makes clear that aging is strongly correlated with AD, which suggests a contribution of environmental factors. Consistent with this, some of the major risk factors that are believed to promote advanced AD manifestation are lifestyle (smoking, sedentariness), obesity, dyslipidemia, type 2 diabetes, hypertension, cerebrovascular disease, apolipoprotein allele E4 and other genetic variants (1-6). According to this scenario, AD can be classified in familial AD and sporadic AD (7).

Epidemiological studies notice that currently, over 46.8 million people live with dementia worldwide and predict that this number will reach 131.5 million by 2050, of which 115.4 million with AD (2, 8).

2.2. Signs and Symptoms

The first manifest symptoms appear most likely in the elderly people. The typical clinical conditions are memory loss, decreased ability to learn and retain new information, changes in behavior and personality, intellectual impairments and multiple cognitive abnormalities such as problems with language, difficulties performing familiar tasks and disorientation to time and place (1,2,9,10).

In 2010, the National Institute on Aging and the Alzheimer Association (NIA-AA) established three phases of AD: the pre-symptomatic phase or preclinical phase; the symptomatic predementia phase, often referred as Mild Cognitive Impairment (MCI); and the dementia phase. However, it is of must importance to bear in mind that AD is continuum, and there are no exact transition points that define when a patient has progressed from one phase to another (10).

2.3. Pathophysiology

AD is characterized by an insidious onset and inexorable progression of atrophy in the medial temporal lobe structure (including the hippocampus and enthorinal cortex (EC)) and by cortical thinning, particularly in a set of functionally connected brain regions, known as default network, such as precuneus, posterior cingulate, inferior parietal, lateral temporal and superior frontal cortices (10). Patients with AD reveal patterns of deterioration concordant with their deficits, such as visual variants demonstrating posterior cortical atrophy and those with prominent language deficits evidencing left temporal atrophy (10).

There are many cellular mechanisms described in the literature that are thought to be involved in AD pathogenesis. Considerable reviews associated the disease with increased inflammation, mitochondrial structural and functional abnormalities, oxidative stress, cell cycle deregulation, hormonal imbalance and neuronal death in several brain regions.

In AD patients some mutations have been detected in the gene that encodes Apolipoprotein E (ApoE) conferring to the protein functions promoting oxidative stress (3). Additionally, acetylcholine (Ach), a neurotransmitter very important to memory, is markedly decreased (11). The specific features of AD are the hyperphosphorylation of the microtubule-associated Tau protein which generates the intracellular accumulation of hyperphosphorylated Tau protein (pTau) in neurofibrillary tangles (NFT) (1,2,11); and the deregulation in the splicing of APP leading to its defective proteolysis, responsible for an overexpression of the β-amyloid protein (Aβ) that aggregates in extracellular β-amyloid plaques, also called senile plaques (SP) (1,2,7,11). The brain cortices implicated in memory and complex cognitive processes are especially vulnerable to this amyloid deposition (10). Nonetheless, apart from the brain parenchyma, this event might also occur in the cerebral blood vessels where is known as congophilic angiopathy or cerebral amyloid angiopathy (CAA) (11). Tau neurofibrillary inclusions originate in the EC before the appearance of clinical symptoms and gradually spread to anatomically connected hippocampal region and the neocortex (7).

The sequential enzymatic cleavage of APP in AD (Figure 1) is performed by the beta-site-APP-cleaving enzyme 1 (BACE1) or β-secretase and by the γ-secretase (1-3). Presenilins are a family of transmembrane proteins that constitute the catalytic subunits of γ-secretase. In familial AD (FAD), there is a missense mutation in the *PSEN1* gene, the gene that encodes the PSI protein, resulting in an increase of γ-secretase (10, 12). This enzyme cleaves APP at different positions generating heterogeneous Aβ peptides, like Aβ40, Aβ42 and Aβ43 (7, 11, 12). Aβ isoforms are produced by cooperative activities of both neuron and its associated

astrocytes in different subcellular compartments such as endoplasmic reticulum, Golgi, endosomes and lysosomes (7, 11). As a consequence of the imbalance between production and clearance of Aβ monomer species, its production exceeds the safe limit and neurons start spilling out these soluble peptides to the extracellular surroundings. They can disperse and/or spontaneously aggregate into extracellular soluble oligomers, which convert over time into protofibrils, then into insoluble fibrils, until finally form neurofibrillary plaques (NP) (7). Insoluble plaques are considered quite inert structures, while soluble Aβ oligomers, present in the tissue surrounding the plaques, are highly neurotoxic and correlate with disease severity (7). These harmful species induce oxidative damage to which the oligodendrocytes (OLGs) are particularly sensible, resulting in its destruction. Soluble Aβ forms also have toxic effects on synapses and mitochondria, damage cholesterol rich membranes (such as those found in OLGs and myelin), and promote tau hyperphosphorylation. Furthermore, due to their proximity, the Aβ42 oligomers released by the neuron bind to their neighbor astrocytes (11). The signals from the receptors involved in this connection induce the astrocytes to exocytose glutamate. This discharged neurotransmitter activates the extrasynaptic N-Methyl-D-Aspartate Receptors (NMDARs) of the neurons astrocytes' partners (11). The consequent stimuli trigger Ca2+ which in turn provokes mitochondrial dysfunctionalities, oxidative stress, caspase 3 activation, tau hyperphosphorylation, excess production of nitric oxide and vascular endothelial growth factor (figure 2). The referred cascade of events, product of this neuron-astrocyte-neuron crosstalk, leads to the destruction of dendritic spines and neuronal synapses (11). The degeneration of projecting axons may mediate the diffusion of the pathology through the limbic area and continues along white matter until the rest of the brain (7).

In addition, other signaling pathways are involved. Amyloid deposits induce the recruitment and activation of microglia, which produces and releases proinflammatory cytokines that stimulate the nearby astrocyte-neuron to generate further amounts of Aβ42 oligomers and to promote its dispersal (7, 11).

It is important to bear in mind that despite the recognition of these neuropathological features of AD, the intricacies of its mechanisms still require a clearer definition.

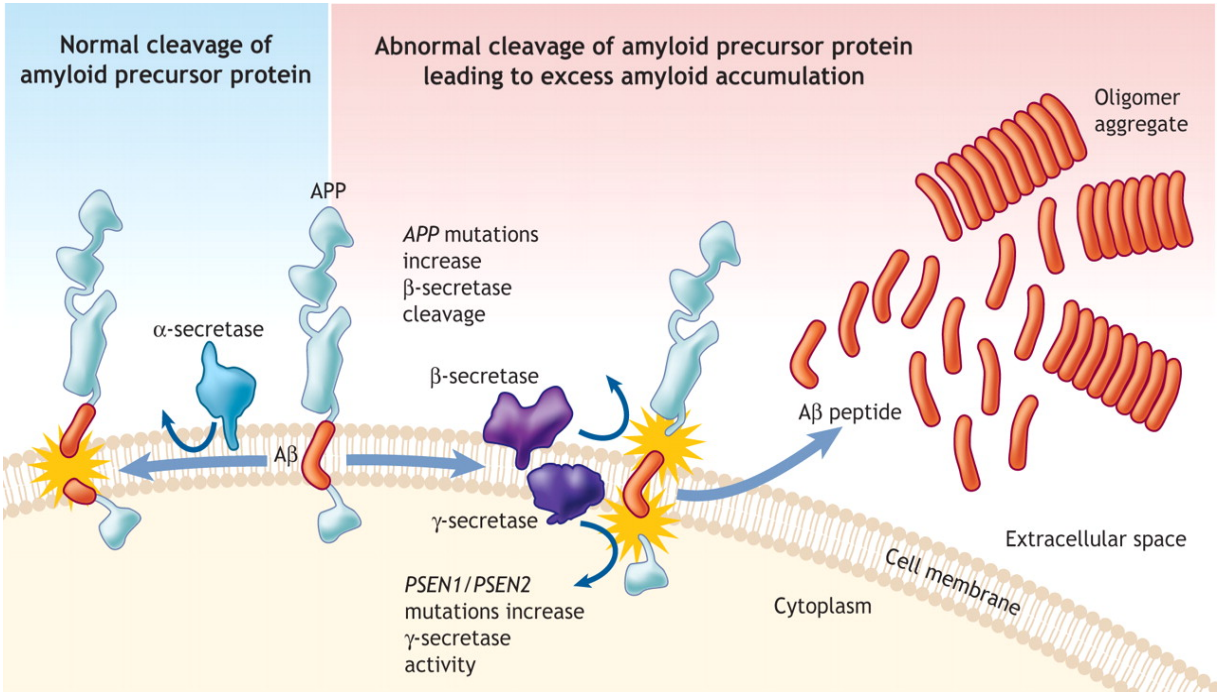

Figure 1 – APP (Amyloid beta Precursor Protein) cleavage in physiological and pathophysiological conditions (13).

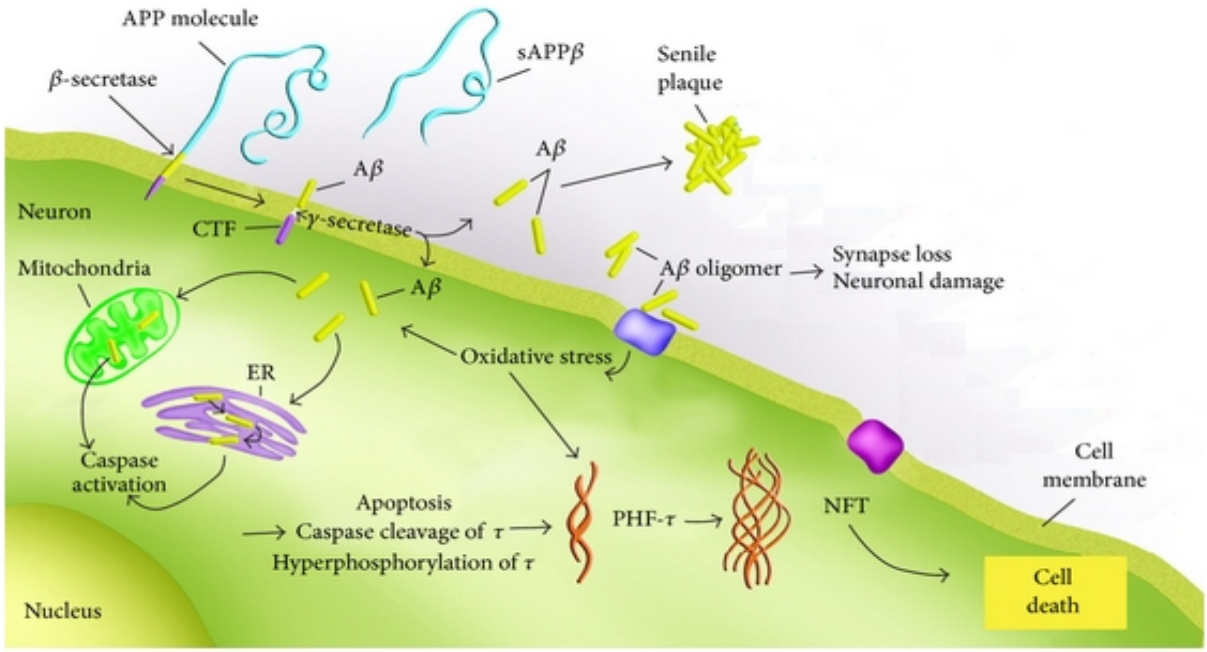

Figure 2 – NFT (Neurofibrilliary Tangles), SP (Senile Plaques), pathophysiological mechanisms and neuronal death (14).

2.4. Therapeutic

Therapeutic strategies have been developed, based on these cellular changes. Current commercially available treatments are AcetylCholinoEsterase Inhibitors (AChEIs) (e.g. rivastigmine, galantamine, donepezil), that increase Ach levels; and NMDARs antagonists (e.g. memantine), which block the exaggerated response to glutamate. These drugs modestly decelerate the progression of symptoms and mainly provide symptomatic relief, but without eradicating the cause of the disease (11). At least, half of the people who follow this treatment does not respond to it (11). Only memantine has shown to be effective for the later stages of the disease (11). Nowadays, there are no drugs or agents that prevent the onset and the progression of AD. The lack of understanding of the whole pathogenic process may be one strong reason for the unavailability of a definitive cure that acts at the root of the disease (11). A complete knowledge about the various pathological pathways of AD is the key to bring new drug candidate targets.

Efforts have been made to develop a successful therapeutic, trough disease-modifying drugs, blockers of the progression of the disease, relying on deep research centered in the discovery of novel pathophysiological events and on a better comprehension of the mechanisms studied before (11).

2.5. Current Diagnosis

Current diagnosis of AD starts with the analysis of the patient's cognitive function in clinical settings (1). This analysis involves a detailed medical history, neurological examinations, a cognitive function testing, a brief psychiatric evaluation and an interview with a knowledgeable informant (2). For instance, some criteria include: impairments in one or more cognitive domains that are greater than one would expect on the patient's age or educational background (10). Nevertheless, this clinical assessment *per se* is not enough to distinguish AD from other types of dementia, so it needs to be further complemented with other techniques.

The measurement of Aβ and pTau levels in the cerebrospinal fluid (CSF) is useful but implicates lumbar puncture, an invasive approach for routine assessment of the disease. Paradoxically, AD is associated with a decrease in CSF Aβ1-42 since represents evidence that Aβ oligomers are polymerizing and depositing in the brain as NP (11). CSF p-Tau and total Tau measures are thought to reflect neuronal injury and neuronal death respectively (10). The combination of elevated CSF P-tau with low Aβ1-42 is assumed to indicate a biomarker signature of AD (10).

Neuroimaging techniques using functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) scans provide more conclusive evidence of amyloid pathogenesis (1,2,10). They possibility the visualization of the amyloid plaques similarly to an autopsy (3,10). However, amyloid positivity, when are observed moderately to severely elevated levels of Aβ deposits trough this approach, does not reliably distinguish AD dementia from MCI, Lewy body disease and from other neurodegenerative diseases. Moreover, both CSF and PET amyloid imaging markers are thought to reflect amyloid accumulation primarily into fibrillary form but may not reveal other forms of Aβ, such as smaller soluble species – so-called oligomeric species, the presumed toxic forms of Aβ (10). Additionally, to increase the diagnosis accuracy, it is also useful to know if there is any major genetic risk factor, such as the ApoE gene (1).

To draw a conclusion, all the results of these different diagnosis methods should be integrated in one unique diagnosis and considered in a full clinical context.

The major concern is that AD begins silently without manifest symptoms. During this period, that may last up to 17 years, the patient doesn't know that has the disease, letting it progress without intervention (1). Current diagnosis is only made when the first clinical symptoms appear, and by then the most common situation is that AD already stands in late stages. Even in very mildly impaired individuals the volumes of medial temporal lobe structures are reduced by 15% to 30% (10).

Accordingly, early diagnosis of AD is of utmost importance in order to identify AD before the onset of irreversible neurological damage and to enhance the effects of therapeutic intervention and disease-modifying drugs (1). Further challenge is to employ less invasive assays and to increase the diagnostic specificity or sensitivity, which can be achieved by combining many biomarkers. Facing this urgent need, researchers are committed to find novel informative, reliable, robust and non-invasive biomarkers that could be add to the platforms that are commonly used in clinical diagnosis (1).

2.6. Future Diagnosis: Emergence of exosomal miRNAs as novel diagnostic biomarkers

Cells release exosomes under physiological and pathological conditions. The biological or pathogenic state of the cell dictates the exosomal vesicles that are released (15). Therefore, the molecular content of exosomes, especially proteins and microRNAs (miRNAs), is a fingerprint of the releasing cell type and its status (15). Accordingly, it was hypothesized that neuronal exosomes could provide a potential source of biomarkers for neurological conditions.

For instance, if exosomes carry constituents of their parental cells, it can be assumed that an exosome secreted by neurons affected by AD will probably contain Aβ or Tau proteins, or even miRNAs with information that regulates the expression of genes involved in AD. In the past two decades, studies have been conducted to find out the potential of exosomes as biomarkers of many diseases, such as AD. These vesicles are isolated mainly from biological fluids and subsequently is carried out the expression profiling of their content in miRNAs. The purpose is to compare the expression profile of those miRNAs in AD patients or mouse models with the one in the healthy controls, and then trying to establish patterns of miRNAs expression characteristic of AD. Some recent advances have been made supporting that exosomal miRNAs hold great promise as novel biomarkers for clinical diagnosis in AD, even in early stages of the disease, which may overcome some of the many difficulties (in both the diagnosis and treatment of AD patients) referred before. This explains the growing interest in the clinical application of exosomes in the diagnosis of many diseases, in this particular case, of AD (1,2).

3. Exosomes

3.1. Characterization

Cells generate membrane vesicles to perform diverse biological processes, such as exocytosis, phagocytosis, apoptosis, among several others. This family includes microvesicles (MVs), apoptotic bodies and exosomes, according to their size, functions and morphological characteristics (1).

So far, there are two types of cell-derived extracellular vesicles (EV): shedding vesicles (MVs also called ectosomes) with a particle size distribution that ranges between 100 and 4000 μm, derived from the cell plasma membrane upon surface bending and budding as a consequence of lipid scrambling and cytoskeletal remodeling; and exosomes, 50-100 nm sized vesicles, of endosomal origin, resulting from exocytosis of multivesicular bodies (MVBs) (2,7,16). Exosomes are nanovesicles that exhibit a round, saucer-like morphology encapsulated by a lipid bilayer (17). Using Cryo-electron microscopy (cryo-EM) to analyze the structure of exosomes it was found that they can have one, two or three membrane vesicles (15).

These nanosized shuttles are secreted by live cells to be released into the extracellular space enrolling in critical biological functions such as immune response, antigen presentation, intercellular communication and the transfer of RNA and proteins (1,18).

3.2. Biogenesis and secretion

The biogenesis of exosomes initiates in the endosomal system where through the endosomal trafficking pathway amounts of intracellular fluid are limited by a membrane generating a small intracellular body, called "early" or "sorting" endosome (15,18). The major role of these acidified compartments is to sort molecules: to be recycled back to the surface; to be transferred to the endocytic recycling compartment; or to be targeted to lysosomes or late endosomes (15). In this last possibility occurs a maturation process in which intra-luminal vesicles (ILVs) are formed by inward budding of the endosomal limiting membrane, randomly engulfing portions of the cytosolic contents (e.g. RNAs) and incorporating transmembrane and peripheral proteins into the invaginating membrane. The ILVs progressively accumulate inside the lumen of the early endosome which maturates and develops into the late endosome, that filled of ILVs starts to be called multivesicular body (MVB) (15,18). The fate of MVBs may vary. When an MVB contains material that must be degraded it fuses with lysosomes and the proteins inside the ILVs sort out and are hydrolyzed. Alternatively, MVBs can fuse with the plasma membrane and therefore release the ILVs into the extracellular environment, which then earn the name of exosomes (15,18).

A cell line can produce different populations of exosomes (1). These vesicles can be secreted by a variety of cells such as mast cells, dendritic cells, reticulocytes, epithelial cells, B-cells and neural cells (1).

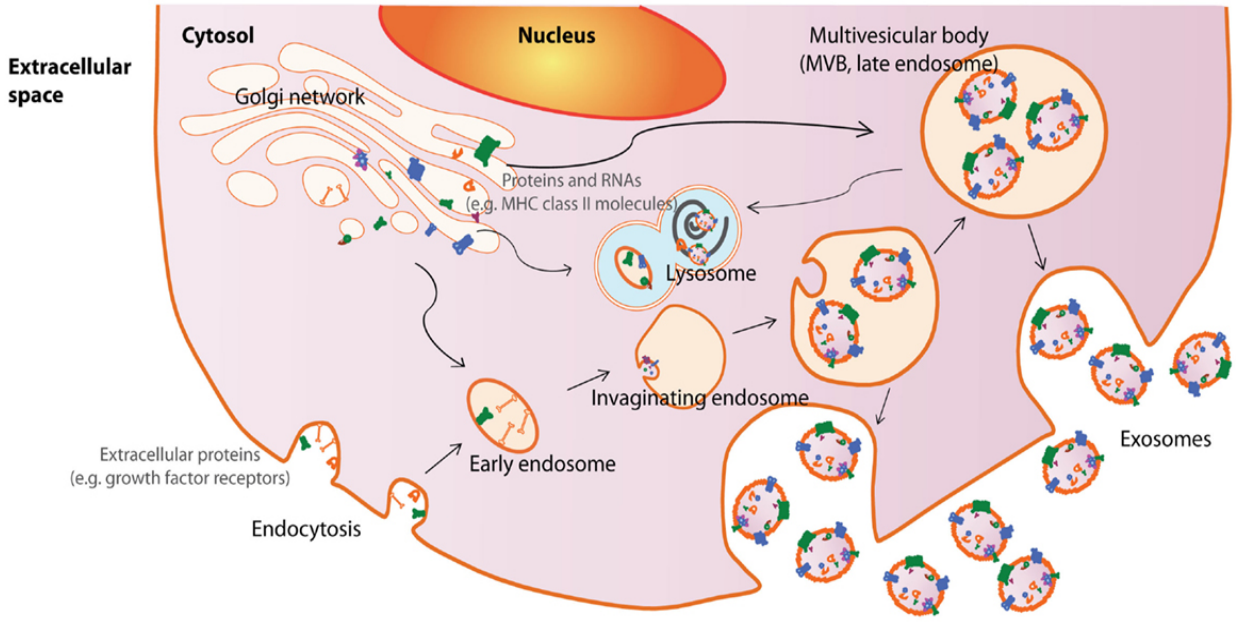

Figure 3 – Biogenesis of exosomes (19).

3.3. Isolation methods

Exosomes are secreted into the peripheral circulatory systems like blood and blood components (serum and plasma), but also are known to be stable in many other common biofluids including urine, breast milk, amniotic fluid, saliva, bile, bronchoalveolar lavage fluid, stools, tears and CSF (1,2,15). For the application of biomarkers in clinical routine settings the preferable sources of exosomes are the body fluids such as serum, cerebrospinal fluid and urine (1).

The ExoCarta is an exosome database that has recorded the appearance of a wide variety of molecules in exosomes, such as proteins, lipids, DNAs, mRNAs, and miRNAs. These vesicles can be isolated based on their size, density and biochemical properties (1).

The current assay used for the isolation of exosomes from biological fluids is based on a two-step differential centrifugation process. Commonly, the method of purifying exosomes involves a serial of centrifugations to remove cellular debris, requiring a final high-speed ultracentrifugation to pellet the exosomes. Cellular debris can also be removed by a filtration process. Another ultracentrifugation step can be performed to eliminate contaminating proteins. Additionally, exosomes can likewise be purified by flotation through a sucrose density gradient or by sucrose deuterium oxide cushions, since they have a specific density (1).

To detect proteins in total populations of exosomes, electrophoresis is the most widely known method, and is followed by Coomassie blue staining and Western Blotting. Chromatography equally works as a purification method and is based on exosome size. These EVs also can be isolated based on their membrane properties, directing antibodies against proteins that are known to be enriched in the exosomal membrane (1).

It has been settled a good manufacturing practice approved method for the isolation of exosomes used for clinical applications, which is based on ultrafiltration and diafiltration, proceeded from a centrifugation on sucrose cushions being the product a highly purified and sterile exosome pellet. Nonetheless, research should keep pursuing a really fast and reliable method to yield purified exosome fractions (1). Regarding a quantitative high throughput analysis of exosomes, a new approach has been developed in recent times combining a Becton Dickinson influx flow cytometer and a fluorescently labeled vesicle with high-resolution flow cytometry (1). Lately, has been used another technique to detect membrane vesicles: the atomic force microscopy (1). The recent advances brought a new tool for high-throughput affinity isolation of exosomes requiring just one benchtop centrifugation step prior to operations (1).

Most of these techniques have as disadvantages the fact that they don't integrate quantification neither detection in line with particle isolation, and also the fact that are limited in the volume of fluid that can be processed (1).

3.4. Molecular Composition

All types of exosomes have the same size, density, lipid bilayer composition and overall composition (1). Consistent with their biogenesis, exosomal proteins typically originate from the endosomes, plasma membrane and the cytosol, and not from the nucleus, mitochondria neither the endoplasmic reticulum. As can be seen in the figure 4, exosomes contain common protein families such as the chaperones (Hsc70 and Hsp90); subunits of the trimeric G proteins; cytoskeletal proteins (actin, tubullin and moesin); endosomal sorting complex required for transport (ESCRT) proteins (Tsg101 and Alix); proteins involved in transport and fusion (Rab2, Rab7, Rab11, and Annexines); and tetraspanin proteins (CD9, CD63, CD81 and CD82) (1,15). Exosomes also share the same composition in sphingolipids, cholesterol and ceramide (1,15). Lipids are often inserted in detergent resistant microdomains (DRMs), suggesting a possible role for DRMs in the traffic of proteins into exosomes (15).

Nevertheless, exosomes can be selectively or randomly enriched in various membrane and cytosolic cell-specific proteins as well as nucleic acids (1). As an example, the major histocompatibility complexes class I (MHCI) and II (MHCII) molecules, displayed in the figure 4, are exosomal proteins characteristic from the exosomes secreted by antigen presenting cells (APC) (1).

According to the Exocarta, 4563 proteins, 194 lipids and 1639 miRNAs have been identified from exosomes of multiple organisms (1).

In summary, exosomes are composed by proteins, lipids, mRNAs and miRNAs (1,15). The cell-specific exosomal components may explain the exosome-specific functions (1).

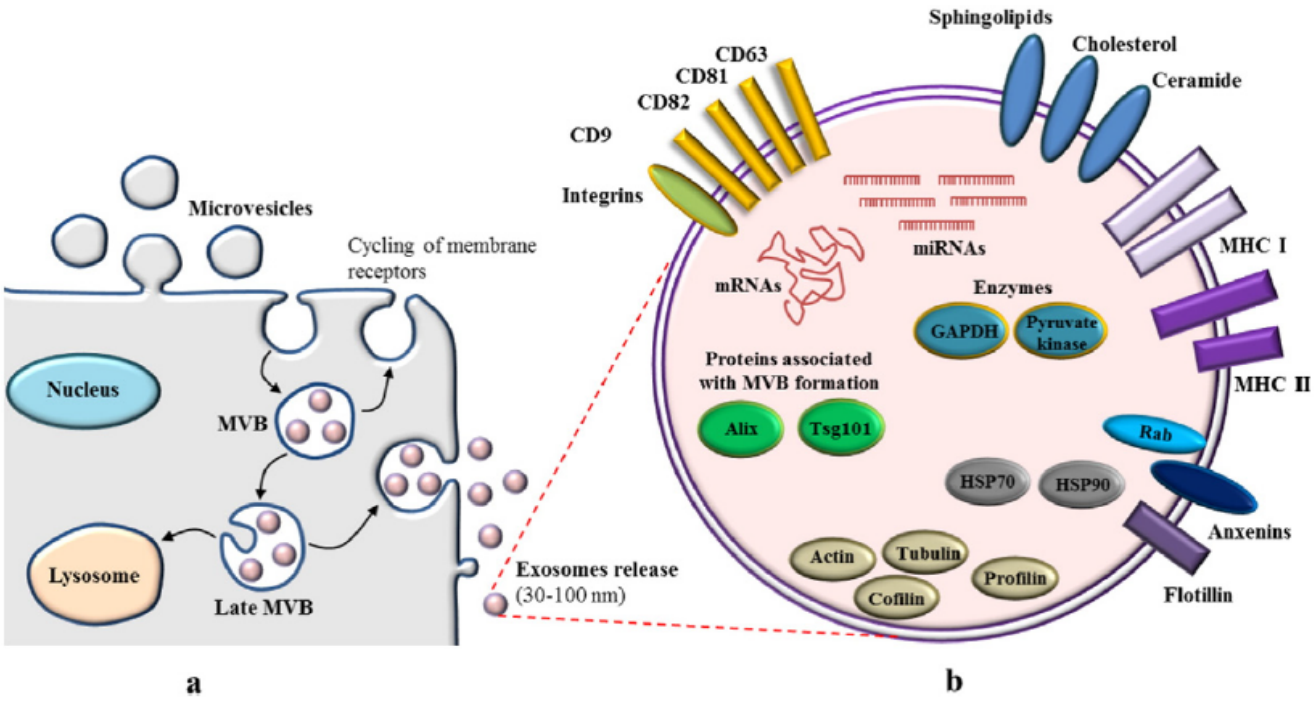

Figure 4 – Molecular composition (1).

3.5. Functions

Exosomes functions are determined by the type of cell who was in its origin, since they contain functional proteins and nucleic acids derived from their parent cell. For instance, exosomes secreted by B lymphocytes, macrophages and dendritic cells contain MHC II molecules on the surface, and so are able to present antigens for CD4 T cells (1). Likewise, cancer cell-derived EVs exhibit functions associated with malignancy.

These nanosized shuttles are released from the host cell and taken up by recipient cells. Serving as transfer vehicles for cytosolic proteins, lipids and RNA, exosomes play an important role in cell-to-cell communication and target cell reprogramming (1). Studies keep demonstrating the diversity of specialized functions that these important mediators of intercellular communication can have: coagulation, waste management by removal of unwanted proteins, adaptive immune responses, and other examples among many.

Also in the Central Nervous System, the neuronal exosomes have particular functions and are involved in intercellular communication. Since it has becoming increasingly clear that exosomes content mirror both physiological and pathological processes of the cell from which they were originated, pathophysiological alterations in AD might be reflected in the number or composition of neuronal exosomes (1,2). In fact, exosomes in pathogenic states mainly contain disease-specific or deregulated miRNAs.

Many miRNAs are expressed in the brain in a brain-specific or brain-enriched manner, being in this way associated with brain functions. Overall, vast majority of miRNAs found in the brain are the key regulators of different biological functions in neurons, such as synaptic plasticity, neurogenesis and neuronal differentiation (2). Therefore, is expected for them to be altered in neurological disorders, such as AD (1). In some studies, miRNAs considered to be implicated in development, cognition, and synaptic plasticity revealed different levels of expression in AD. Increasing evidence indicates that abnormal miRNA expression is a common feature of AD.

Trying to establish patterns of exosomal miRNAs expression in AD, marks the expression profiling of these miRNAs as the novel promising method of diagnosis of this disease, feasible in any stage of the pathology (1).

3.6. Exosomal miRNAs

3.6.1. Characterization of miRNAs

miRNAs are small non-coding RNAs that regulate gene expression. These molecules are involved in various cellular processes such as development, cell proliferation, replicative senescence and aging (2). miRNA biogenesis initiates in the nucleus with the transcription of the primary miRNA transcript, and through diverse processing steps ends in the cytoplasm with the formation of mature miRNA molecules (2).

In the human genome approximately 2000 genes encode multiple miRNAs molecules which target nearly 60% of all human genes in a sequence-specific manner, modulating their expression by mRNA degradation or translation repression (2).

There is a miRbase-21 database released in June 2004 that includes 1881 precursor and 2588 mature miRNAs identified in many human diseases such as cancer, viral infection, diabetes, immunologic diseases and neurodegenerative disorders (2).

3.6.2. Exosomal miRNAs as potential biomarkers for AD

miRNAs are secreted into the extracellular circulatory biofluids in five different modes: 1 - bound to high-density lipoprotein (HDL) particles in a non-vesicle form; 2 - forming a complex with Ago2 proteins; 3 - placed in exosomes; 4 - encapsulated in micro-vesicles (MVs); and 5 - accumulated in apoptotic bodies (2). Studies of miRNAs have focused on exosomes as diagnostic molecules for several reasons: 1- exosomes in pathogenic states mainly contain disease-specific or deregulated miRNAs; 2- exosomes have the potential to cross the brain

blood barrier (BBB) through transcytosis, hence, they can easily pass through endothelial cellular layers and circulate in biofluids, which is important in neurodegenerative or brain-related disorders; 3- in the circulatory system, the packaging of miRNAs in vesicles protects them from degradation by RNases; 4- studies have been undertaken to remove non-exosomal miRNAs in healthy cells (2, 15).

That said, it has been proven that miRNAs operate in the modulation of key genes involved in genetic and sporadic AD. The comprehension of how miRNAs influence the beginning of this degenerative disorder and the development of tools to allow their detection may implement the early diagnosis of this disease. Also, the study of the role of deregulated miRNAs in the alteration of translation or expression levels of disease-related transcripts may also bring new informations about the progression of the disease. Therefore, the determination of the patterns of exosomal miRNAs along AD evolution may reveal previously undescribed regulatory mechanisms of the disease and enables the characterization of its status. AD features are only known in late stages of disease, prior to the first cognitive signals. Hence, research since the earlier stages emerges increasingly beneficial (1). Through follow-up from the first amyloid deposits in the brain, to symptomatic MCI and dementia stages until death (and brain autopsy) it is possible to acquire more insight into biological and cognitive processes of AD (20).

3.6.3. Detection techniques and application in clinical settings

miRNAs found to be highly expressed in the brain are also detected abundantly in body fluids such as serum, urine and CSF, all of them presenting a good source of material for the diagnosis of AD. CSF is a biofluid produced in the choroid plexus of the brain that circulates through the inner ventricular system, across the BBB and is absorbed into the bloodstream. Its role in the inter-cerebral transportation of miRNAs explains why these molecules, when released from the neuronal tissue, also can be found in the blood (2). In the extracellular circulation, exosomal miRNAs are quite stable, which makes them outstanding peripheral biomarkers. Additionally, their circulatory form also represents a clinical advantage for early diagnosis, since they can be assessed in a blood draw, which is minimally invasive (2). For the identification of exosomal miRNAs as potential biomarkers for AD applied in clinical routine settings, serum is the most suitable circulatory biofluid (2).

After isolation from exosomes, the detection of miRNAs is held through expression profiling using laboratory techniques such as quantitative Real Time Polymerase Chain Reaction (qRT-PCR), microarrays and the next-generation deep sequencing technologies. Currently, high-throughput next-generation sequencing (NGS) is being used to obtain a miRNA profile in

biological fluids. Genome-Wide Analysis, immune-assay platforms, single-end sequencing on Hiseq 2000 (Illuumina) and Solexia sequencing are other useful tools. Also, a machine learning model was built using the mature miRNA expression data in order to predict whether an individual sample belonged to the AD group or control group (1,2,13,21).

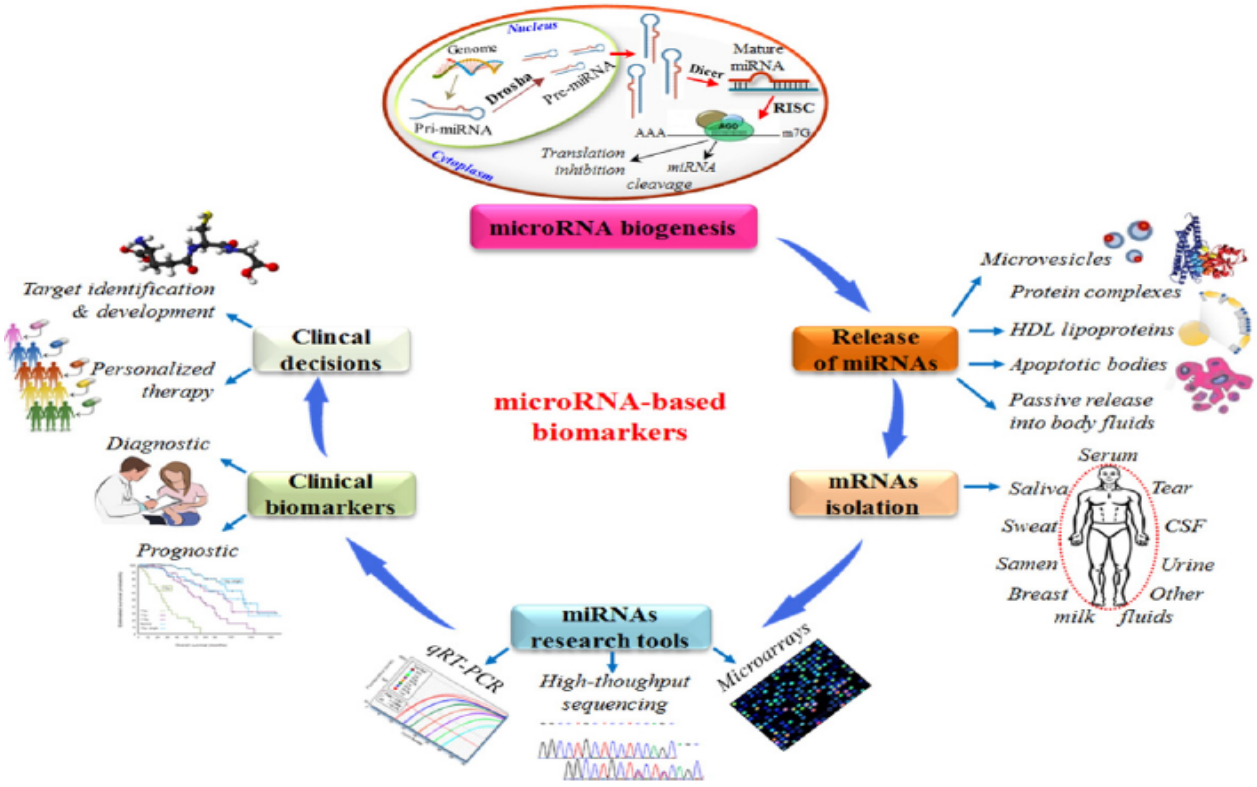

Figure 5 – miRNA-based biomarkers (1).

3.6.4. Experimental studies

An increasing number of studies has been conducted attempting to establish a pattern of miRNA (miR) deregulation characteristic of AD. Depending on each study, the miRNAs were isolated from distinct bodily fluids, such as CSF, serum, blood, and extracellular fluid (ECF); from neuronal live or post-mortem tissues; and even from peripheral blood mononuclear cells (PBMC) - whether in exosomes or in other associations -, and the techniques employed for the detection of their expression levels varied (2).

Kumar and Reddy, in 2016, discussed 12 studies about the assessment of the circulatory miRNAs, from 1268 participants with MCI or AD dementia, frontotemporal dementia (FTD) and vascular dementia (VD). This group also included inflammatory neurological disease controls (INDCs) and healthy or negative controls (NC). Neurologically healthy controls,

confirmed by medical history and laboratory, Mini-Mental State and general examinations, couldn't suffer from significant diseases (e.g. type 2 diabetes mellitus, coronary heart disease, hemorrhagic stroke, cancer, glaucoma, etc.) (2).

A total of 100 miRNAs were identified and validated in different subject groups. Of the 100 miRNAs, 54 miRNAs were upregulated and 46 were downregulated in the AD and MCI patients. The authors also noticed that only six miRNAs - miR9, miR-125b, miR-146a, miR-181c, let-7g-5p and miR-191-5p - were reported by multiple investigators (2). The table 1 summarizes the compiling data described before.

Table1- Deregulation of miRNAs (miR) in Alzheimer Disease and Mild Cognitive Impairment in 12 different studies (2).

Sources	Study groups	Sex ratio (Male vs Female)	miRNA detection methods	Up-regulated miRNAs	Down-regulated miRNAs	References
Blood	NC = 22 AD = 48	M = 11, F = 11 M = 23, F = 25	Small RNA sequencing dataset	miR-26b-3p, miR-28-3p, miR-30c-5p, miR-30d-5p, miR-148b-5p, miR-151a-3p, miR-186-5p, miR-425-5p, miR-550a-5p, miR-1468, miR-4781-3p, miR-5001-3p, and miR-6513-3p	let-7a-5p, let-7e-5p, let-7f-5p, let-7g-5p, miR-15a-5p, miR-17-3p, miR-29b-3p, miR-98-5p, miR-144-5p, miR-148a-3p, miR-502-3p, miR-660-5p, miR-1294, and miR-3200-3p	Satoh et al. (2015)
PBMC	NC = 16 AD = 16	NA	Microarray	miR-34a, miR-181b	–	Schipper et al. (2007)
Serum	NC = 7 MCI/EAD = 7 AD = 7	M = 4, F = 3 M = 1, F = 5 M = 2, F = 5	QRT-PCR	–	miR-137, miR-181c, miR-9, miR-29a, miR-29b	Geekiyanage et al. (2012)
Serum/CSF	NC = 18 AD = 22 FTD = 10 INDCs = 8	M = 13, F = 5 M = 8, F = 14 M = 2, F = 8 M = 2, F = 8	miRNA PCR array & TaqMan QRT PCR assay	–	miR-125b, miR-23a, miR-26b	Galimberti et al. (2014)
Serum	NC = 155 AD = 105	M = 75, F = 80 M = 57, F = 48	QRT-PCR	miR-9	miR-125b, miR-181c	Tan et al. (2014)
Serum	Cohort 1 NC = 50 AD = 50 Cohort 2 NC = 155 AD = 158	M = 25, F = 25 M = 25, F = 25 M = 70, F = 85 M = 78, F = 80	*Discovery* Sequencing *Validation* QRT-PCR	miR-3158-3p, miR-27a-3p, miR-26b-3p, miR-151b	miR-36, miR-98-5p, miR-885-5p, miR-485-5p, miR-483-3p, miR-342-3p, miR-30e-5p, miR-191-5p, let-7g-5p, let-7d-5p	Tan et al. (2014)
Serum	Cohort 1 NC = 48 AD = 48 Cohort 2 NC = 75 AD = 79	NC M = 65, F = 58 AD M = 72, F = 55	*Discovery* Solexa Sequencing *Validation* RT-qPCR assay	–	miR-31, miR-93, miR-143, miR-146a	Dong et al. (2015)
Serum exosomes	Cohort 1 HC = 23 MCI = 3 AD = 23 Cohort 2 HC = 36 MCI = 8 AD = 16	M = 13,F = 10 M = 2, F = 1 M = 9, F = 13 M = 14, F = 21 M = 4, F = 4 M = 5, F = 10	*Discovery* Sequencing *Validation* QRT-PCR	miR-361-5p, miR-30e-5p, miR-93-5p, miR-15a-5p, miR-143-3p, miR-335-5p, miR-106b-5p, miR-101–3p, miR-425-5p, miR-106a-5p, miR-18b-5p, miR-3065-5p, miR-20a-5p, miR-582-5p	miR-1306-5p, miR-342-3p, miR-15b-3p	Cheng L et al. (2014)
Plasma	Cohort 1 HC = 20 MCI = 9 AD = 11 Cohort 2 NC = 17 AD = 20	M = 10, F = 8 M = 3, F = 6 M = 6, F = 5 M = 9, F = 8 M = 10, F = 10	*Discovery* nCounter miRNA assay (Nanostring) *Validation* TaqMan QRT-PCR	miR-323b-5p, miR-545-3p, miR-563, miR-600, miR-1274a, miR-1975	let-7d-5p, let-7g-5p,miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p, miR-545-3p,	Kumar et al. (2013)
Plasma exosomes	NC = 35 AD = 35	M = 18, F = 17 M = 15, F = 20	Illumina deep Sequencing	miR-548at-5p, miR-138-5p, miR-5001-3p, miR-659-5p	miR-185-5p, miR-342-3p, miR-141-3p, miR-342-5p, miR-23b-3p, miR-338-3p, miR-3613-3p	Lugli et al. (2015)
CSF, ECF	NC = 6 AD = 6	NA	Microarray	miR-9, miR-125b, miR-146a, miR-155	–	Alexandrov et al. (2012)
CSF	NC = 28 AD = 22	M = 14, F = 14 M = 9, F = 13	Open Array QRT-PCR	miR-146a, miR-100, miR-505, miR-4467, miR-766, miR-3622b-3p, miR-296	miR-449, miR-1274a, miR-4674, miR-335, miR-375, miR-708, miR-219, miR-103	Denk et al. (2015)

NC: Negative Controls; AD: Alzheimer disease; M: Male; F: Female; miR: miRNA; PBMC: Peripheral Blood Mononuclear Cells; MCI: Mild Cognitive Impairment; FTD: Frontotemporal Dementia; INDCs: Inflammatory Neurological Disease Controls; HC: Healthy Controls.

Below, the table 2 describes the associated brain functions of some miRNAs that were shown to be deregulated in AD.

Table 2 – miRNA brain function

Study	miRNA (miR) type	miRNA Brain function
(1)	miR-9, -328, -298	Targeting of BACE1
(1)	miR-124, -101, -137, -16, -153	Regulation of APP mRNA alternative splicing
(1)	miR-128	Neuronal differentiation and development
(1)	miR-134	Synaptic development, maturation and/or plasticity
(1)	miR-107	Neuronal differentiation, targeting of BACE-1
(1)	miR-9, miR-125b, miR-146a and miR-155	Pro-inflammation
(2)	miR-9 and miR-181c	Neurogenesis and microglial mediated neuronal injuries
(2)	miR-146a	Regulation of immunological and inflammatory responses in humans
(2)	miR-125b, miR-191, -5p, let-7g-5p	Regulation of cell differentiation, proliferation and metastasis
(15)	miR-138	Human memory performance, regulation of dendritic spines, phosphorylation of tau protein, effects related to cognition

It remains difficult to predict whether the observed changes in human miRNA levels are a cause or consequence of the neurodegenerative process. Hence, the study of miRNA expression profiles in AD mouse models might help to address these challenges. Mice are considered to be genetically homogenous in nature when compared to humans, providing unique tools to study miRNA-regulated gene pathways in AD development. In fact, some of the experiments revealed miR-9, miR-181c, miR-30c, miR-148b, miR-20b, miR-29 and miR-106b as some examples of miRNA downregulated in both human and mouse AD brains (1). miR-146a expression was increased in human AD brain and also in five different mouse

models (1). The current finding data from AD mice suggests that miRNA alterations equally occur in AD humans, yet some discrepancies still exist (1).

Because miRNAs are often regulated in groups, researchers have analyzed not only single changes in miRNA expression levels, but changes in how pairs of miRNAs were correlated with each other across individual samples, both beyond the entire dataset as well as differentially in the AD group vs. controls. Correlation analysis is complementary to expression analysis, since two miRNAs may be highly correlated regardless of whether either one shows any mean change across groups. Cases in which pairs or modules of miRNAs where highly correlated in AD group but not in controls, or vice versa, may suggest changes in the regulatory network that occur in AD (21).

The ultimate aim is to fully integrate these miRNA expression data with other data (cognitive, imaging, other biomarkers) and hence there would be obtained more informative and robust biomarkers.

3.7. Neurotoxic role of exosomes in AD

The role of exosomes in the propagation of diseases has been the focus of many studies. Their small size and high stability enables them to travel over relatively long distances independently within the body, which induces us to believe that they can be involved in the delivery of pathogenic particles form one cell to another (7,15). Consistent with this intercellular signaling, there is substantial evidence that exosomes are exploited by prions or virus to promote viral dissemination (1,15). Identically, they may also contribute to the spread of misfolded proteins in many neurodegenerative diseases, such as Aβ and pTau in AD (3,21).

The proposed mechanism is that exosomes, through cell-to-cell signaling, deliver their functional contents from the donor cells to the recipient cells, potentially impacting the ones with which interact, that in turn can influence other downstream cells. miRNAs deregulated in AD can be transferred from the parental cell into the surrounding ones and directly target disease-related transcripts to alter their translation or expression levels (15). Several researchers believe that some miRNAs are specifically targeted to be released by these vesicles (1).

Accordingly, accumulating evidence reveals that some miRNAs regulate Aβ production, NFT formation and neurodegeneration by spotting genes responsible for Aβ metabolism and genes involved in the dysregulation of Tau, contributing to toxicity (1).

Through proteomic analysis in CSF of AD cases were observed increased levels of exosomes associated to pTau and Aβ species (7,15,16). These proteins were also found through immunocapture of neuron-derived exosomes from blood samples (7,15,16). Furthermore, it was reported the presence of some exosome-specific proteins, such as Alix and flotillins, in amyloid plaques of AD mice brains and post-mortem human brains. All these observations support that exosomes might be involved in the propagation of AD misfolded proteins and in trafficking of Aβ aggregates to NFP during disease progression (15,16).

Moreover, secretases, such as β- secretase, have been identified in neuronal exosomes, which indicates that abnormal cleavage of APP originating Aβ peptides could occur within these vesicles. In fact, all exosomes isolated from the brain tissue of AD patients and APP transgenic mice, have shown to be enriched with APP C-terminal fragments (7,15,16).

Taking into account these findings, Dinkins and coworkers injected a known blocker of exosomes secretion in an AD mouse and the result was the decrease of amyloid plaque formation, which lead them to believe that exosomes stimulate Aβ aggregation (7).

Recently, Nigro and coworkers observed that microglia are activated by exposition to Aβ1-42 and shed harmful EV's *in vitro* (16). The researchers speculated that when microglia are significantly surrounded by amyloid deposits, their pathways of phagocytic degradation of excessive Aβ become saturated. Consequently, microglia assume a reactive form: overproduce neurotoxic EVs to release excess of Aβ species and promotes formation of soluble neurotoxic Aβ1 -42 species from extracellular insoluble aggregates (7, 16). Microglial EVs propagate damage to surrounding OLGs and neurons, behaving as potential drivers of neuronal damage by spread of Aβ pathology in the brain (16). A similar mechanism can boost tau propagation to neurons, through phagocytosis of tau protein by microglia and subsequent secretion in exosomes, that are released in the extracellular space (16). The observation of an increase of microglial EVs in AD patients with significant hippocampus atrophy and altered value of AD soluble proteins, suggests that there is a significant correlation between number of microglial EVs and AD, and thereby elects EV's as potential predictive biomarkers and/or therapeutic targets for AD (7,16). However, it still remains unclear whether the increased secretion of microglial EVs is the cause or the response of the disease.

In summary, all these evidences suggest a potential role of EV's, including exosomes, in the pathophysiology of the disease.

3.8. Neuroprotective role and Therapeutic potential of Exosomes

Although some studies prove the neurotoxic role of exosomes in AD, others demonstrate their beneficial effects against the pathology. There are evidences that exosomes appear to bind the neurotoxic Aβ species, neutralizing them, which contributes to decrease their aggregation (15). Yuyama and co-workers hypothesized that exosomes may have the ability to trap Aβ and promote its clearance by neighboring microglia (7). The results of some studies *in vitro* in primary cortical cells, as well as in mouse models, corroborated this possibility of exosomes as scavenger of neurotoxic soluble Aβ peptides (7,16). Aβ sequestration depends of a PrP, a known Aβ receptor in neuronal exosomes surface, which bind these oligomers with high affinity (7). Also other molecules at the vesicle surface, such as sphingolipid ceramide and/or glycosphingolipids, mediate this interaction between EVs and Aβ (7,16). In view of this, authors suggested that exosomes could have therapeutic potential to prevent plaque deposition in AD (16).

In FAD, the presence of PS2 mutations leads to a decreased ability of neurons to secrete cystatin C, APP, and their toxic metabolites through exosomes. A reduction of exosomal cystatin C, a neuroprotective growth factor as well as an inhibitor of Aβ aggregation, might result in increased Aβ assembly (7). According to this hypothesis, FAD patients could benefit from exosomes administration. Katsuda and coworkers found that exosomes originated from human adipose tissue-derived mesenchymal stem cells (hADSCs) carry enzymatically active neprilysin, the most important Aβ-degrading enzyme in the brain. Through co-culture experiments, this group verified that mesenchymal stem cells-derived exosomes, loaded in neprilysin, decreased the Aβ levels (22). Taking this into account, it was proposed that neprilysin-bound exosomes could have a neuroprotective role against AD (16,22).

Also the incorporation of siRNA for the knockdown of the BACE1 protein into exosomes, injected systematically but targeted to specific cells in the brain, is a potential therapeutic for AD (15). Furthermore, some studies revealed that exosomes derived from Schwann cells can promote axonal regeneration and reverse nerve injury, other added-value of these vesicles in AD (15).

3.9. Neuroprotective vs Neurotoxic role of EVs

The image below summarizes the neuro-protective and neuro-degenerative role of EV's, including exosomes, in AD. 1- It begins when the APP is cleaved and generates

monomeric Aβ42 forms which exit the neuron and aggregate to form soluble Aβ42 oligomers. These are converted into insoluble fibrils, the main components of amyloid plaques. 2- Microglia internalizes fibrillary and soluble Aβ42 species to undertake phagocytose. 3- The excess of Aβ42 can be re-secreted with microglial ectosomes in a neurotoxic form. 4- Microglia-derived ectosomes also promote formation of soluble Aβ42 peptides from extracellular insoluble aggregates. These soluble Aβ oligomers bind to neuronal dendrites and to OLGs and mediate synaptic dysfunction and spine loss. 5,6- Astrocytes and neurons overproducing Aβ species want to get rid of them and release them through exosomes. On the other hand, contrarily to microglial ectosomes, exosomes released by these cells stimulate aggregation of Aβ42 soluble oligomers in inert insoluble fibrils, thereby preventing toxic effects. Neuronal exosomes can also mediate neuroprotection by promoting Aβ42 clearance by microglial cells. Furthermore, these vesicles, when containing normal Aβ levels, may also have neuroprotective factors and act as scavengers of synaptotoxin Aβ species (7).

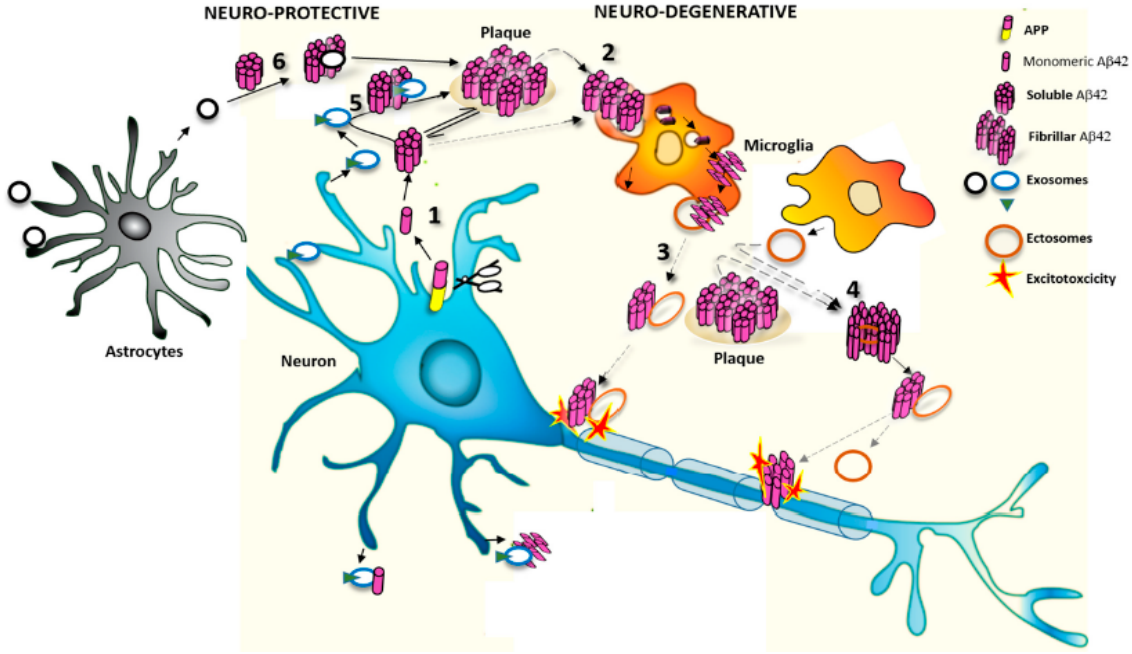

Figure 6 – Neuroprotective vs. Neurotoxic role of exosomes and ectosomes in AD (7).

How EVs influence the complex process of Aβ assembly remains controversial: whether they promote or counteract the deleterious action of Aβ is still a matter of debate (7). Findings about exosome neurotoxic role are indeed counterbalanced by data evidencing their neuroprotective effect.

In conclusion, up to now is believed that detrimental or protective role of EVs, including exossomes, in AD depends of the functional phenotype of their parent cells.

4. Conclusion

Coming to the end of this work, is possible to acknowledge that exosomal miRNAs have emerged as powerful tools for solving many difficulties in both the diagnosis and treatment of AD patients. Exosomes contain a source of miRNAs that provides insights on disease states inside cells. Exosomal miRNAs levels in AD are altered and they might have an expression pattern disease-specifc. They can be found within biological fluids, wich makes them outsanding peripheral biomarkers. Accordingly, they have proven to be suitable markers for diagnostic applications, once they can be assessed non-invasely through a blood draw. Researchers are trying to establish an exosomal miRNA signature so that expression profiling of exosomal miRNAs can be the novel approach to predict the onset of the disease, to enable its monotoring and to indentify new therapeutic targets. Some studies have also evidenced that exosomes may have neurodetrimental *versus* neuroprotective roles in AD, as well as a therapeutic potential.

The emergence of a sensitive, non-invasive and affordable tool for early diagnosis of AD brings the possibility of population screening to identify undiagnosed AD individuals without symptoms or cognitive complaints. The priority of the screening should be a subgroup of people with certain risk factors (e.g., age, genetic risks) (20). Recently, the International Association of Gerontology and Geriatrics (IAGG) group concluded, based on age as a risk factor, that all individuals from 70 and older should be screened every year (20). Next to the accomplishment of a screening technique remains the crucial challenge of implement disease-modifying drugs that can block the progression of AD. Further research is needed in novel therapeutic targets, because otherwise the early detection of AD becomes less worthwhile. Nonetheless, it is important to underline that drug development can profit from the improvement of detection techniques.

Despite of existing evidences on the exosomes potential, there remain many limitations. To date, the potential role of EVs in AD is object of debate and evidences for either beneficial or detrimental action have been reported. The interaction of distinct EVs populations with different Aβ forms and their impact on Aβ assembly and cell-to-cell spreading still needs a better definition.

Moreover, regarding the exosomes diagnostic potential, normalization of miRNA data is a challenging task and differential miRNA expression may not accurately reflect miRNA deregulation in neuronal tissues. Although it is known that miRNAs are altered in the brains of AD patients, their functional implications remain unclear. Many subjects are still under investigation, such as the complementary roles of transcription factors, the specific

targets of pathogenic miRNAs in AD and the selective involvement of miRNAs in different regions of the brain. The mechanisms of miRNAs origin, localization and regulation of cellular function are not completely understood. It remains difficult to predict whether the observed changes in miRNA levels are a cause or a consequence of the neurodegenerative process.

Additionally, heterogeneity in miRNAs normalization strategies could introduce some degree of variation in miRNAs expression results. Different methodological approaches (eg.: miRNA extraction protocols), with varied criteria, using different blood fractions (whole blood, plasma, serum) might explain differential miRNAs expression from study-to-study. The complexity of miRNA biogenesis, miRNA secretion as well as other genetic, epigenetic and environmental factors that are highly variable among different human populations (lifestyle, age, gender, body mass index, status of ApoE and BACE) also influence miRNA expression.

Furthermore, labelling "Ad" or "Control" basing only on clinical diagnosis at the time of blood draw, rather than the gold standard of autopsy confirmation, opens the possibility of some of the control subjects have had been in subclinical stages leading to AD, and some subjects diagnosed as AD might have suffered some other form of dementia instead.

Due to the inconsistency in miRNA profiling data in different studies, miRNAs as biomarkers for neurological diseases cannot be used yet. A more uniform methodological process is urgently demanded. Findings warrant replication, repetition over times and extensive follow-up with a larger cohort of patients and controls and both *in vitro* and animal models at different stages of AD progression. This is the only way to have better neuropathological validations and robust statistical power in order to develop a credible tool for AD diagnosis.

Much work remains to be done to standardize exosomes as biomarkers in AD, enabling their optimal use in clinical settings and establishing validated normative values and calibrations metrics to be easily interpretable for clinical physicians. At the same time, the look in the future focus on unveiling the potential role of exosomes in therapeutic of AD.

This is the path to launch exosomes as the novel approach for prognostic, diagnostic, disease monotoring and therapy of AD.

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