Diagnostic and therapeutic potential of exosomal miRNAs in Alzheimer's disease

Ida Manna*, Selene De Benedittis, Enrico Iaccino, Andrea Quattrone, Aldo Quattrone

Alzheimer's disease (AD) is a primary cause of dementia. AD is a neurodegenerative disorder, characterized by synapses loss, extracellular amyloid plaques composed of the amyloid-β peptide (Aβ) and intracellular aggregates of hyperphosphorylated tau protein. AD is a complex disease linked to multiple interacting factors, both environmental and genetic, which can contribute to the onset and severity of the disease. Longitudinal studies have highlighted several cardiovascular risk factors that can increase the risk of AD. The genetic landscape of AD has changed dramatically in recent decades. Early studies identified mutations in the amyloid precursor protein gene (*APP*) as well as proteins that are involved in the enzymatic cleavage of APP to toxic β-amyloid (Aβ), namely presenilin-1 and presnilin-2. However, these mutations were found in familial cases of early-onset AD, while the causes of sporadic late-onset AD are still unknown. The latest advances in Genome-wide Association Studies (GWAS), sequencing, and bioinformatics have begun to unravel the complex genetic architecture of the sporadic form of AD. GWAS were able to uncover common variants with high frequency in the population that individually carried low risk (Robinson et al., 2017). The advent of next-generation and third-generation sequencing platforms shows great promise in further unravelling the genetics of AD. Exome sequencing has been gradually optimized to identify mutations in protein-coding regions, and genome sequencing detects potential disease-causing mutations in non-coding sections of DNA. It has been suggested that underlying the base of neurodegenerative diseases, there is, also, an involvement of epigenetic mechanisms able to influence the expression of genes without altering the DNA sequence, including methylation, non-coding RNAs such as microRNA, and chromatin remodeling (Fenoglio et al., 2018). All these findings radically changed the understanding of AD pathology. In fact, the understanding of the genetic and epigenetic mechanisms and the biological pathways underlying AD has and will continue to have significant benefits also for the search for new therapeutic targets. Clinical symptoms of AD are assessed by instrumental and cognitive examinations, associated with the patient's medical history, to establish a "probable AD" diagnosis (McKhann et al., 1984). To complete these assessments, five biomarkers of AD, divided into two categories, were validated in clinical practice. The first category of biomarkers concerns the dosage of the Aβ protein, i.e. the decrease in the concentrations of the $A\beta_{42}$ protein in the cerebrospinal fluid (CSF). A second biomarker uses positron emission tomography (PET), a neuroimaging technique to measure Aβ deposition by calculating the absorption and retention of a tracer. These techniques are well correlated, and have been validated by post mortem examination. The second category of biomarkers concerns neurodegeneration: a first kind of biomarker is the total tau protein and phosphorylated tau assay in the patient's CSF, which increases during the course of the disease; a second kind of biomarker is the use of structural magnetic resonance imaging to measure increased atrophy during AD. A third category of biomarkers is hypometabolism in disease as measured by [18F] fluorodeoxyglucose PET imaging. Again these correlate well with post mortem outcomes (Lashley et al., 2018). In summary, definitive diagnosis of AD is only possible with a *post mortem* examination of brain tissue showing senile plaques and neurofibrillary tangles. Diagnosis, even at an early stage, is now performed by tests on CSF and with PET, but these tests are expensive or invasive. To date, there are no peripheral AD biomarkers used in clinical practice, so, considering the invasive nature of lumbar puncture for CSF sampling and the cost of neuroimaging, there is an absolute need to have specific biomarkers for early diagnosis of AD. In this regard, recent works have shown that high-precision tests for plasma $A\beta_{42}/A\beta_{40}$ detection are predictive for the accumulation of Aβ in the brain. Therefore, the development of blood-based Aβ biomarkers is of great interest (Schindler et al., 2019). So overall, identification of cost-effective biomarkers and use of more accessible biofluids, such as blood, could represent valid peripheral biomarkers for the AD diagnosis.

Circulating miRNAs as potential biomarkers: One class of molecules that are increasingly implicated in AD are small non-coding microRNAs (miRNAs). miRNAs are more abundant in the central nervous system (CNS) and are critical for neuronal development and functions including neurogenesis and synaptic plasticity. miRNAs act in the cell as post-transcriptional regulators of protein expression and, furthermore, by virtue of their multi-targeting nature, they can regulate complex molecular pathways. There is a growing body of evidence that highlights the regulatory role of miRNAs in the pathogenesis of AD. Indeed, as mentioned in our previous review (Manna et al., 2020), several works highlighted the involvement of many miRNAs in AD pathogenesis, for example the group of miR-15/107 that is implicated in APP processing, and let-7 family involved in inflammation. Furthermore, the first large-scale analysis of AD dysregulated miRNAs, established by profiling the hippocampus and the prefrontal cortex of a cohort of patients with late-onset AD (LOAD) and 23 controls, showed a small subset of altered miRNAs in both brain areas of AD patients. Among these, the most dysregulated during the disease phases was miR-132-3p. Indeed, as the disease progresses, neurons present a low amount of miR-132-3p and an accumulation of hyperphosphorylated tau, indicating the downregulation of miR-132-3p as a new disease biomarker (Manna et al., 2020). Recently, Herrera-Espejo et al. (2021) published GWAS functional single nucleotide polymorphisms (SNPs) in miRNAs. They demonstrated with *in silico* analysis a possible functional effect of these SNPs in miRNA levels and in the regulation of pathways of relevance for the development of LOAD.

Given their presence in body fluids as plasma, serum, CSF and urine, miRNAs are easily detectable using laboratory techniques such as quantitative real-time PCR (qRT-PCR), microarrays, and the most recently developed deep sequencing technologies. Moreover, miRNAs have been found enriched in exosomes, where are less subject to the process of degradation because they are protected by the RNases present in biological fluids. Exosomes are nano-sized biovesicles, generally 30–40 to ~150nm in diameter, released into surrounding body fluids upon fusion of multivesicular bodies and the plasma membrane (Shi et al., 2019). These circulating nanoparticles have emerged as important mediators in cell communication, transferring proteins, lipids, DNA and RNA species (miRNA, mRNA, tRNAs, etc.) between cells (Cheng et al., 2014). Interestingly, once produced, the exosomes have specific markers on their surface, i.e. molecular "labels" that identify their origin. Remarkably, exosomes can also traverse the blood-brain barrier (BBB), raising the possibility that exosomes in the peripheral circulation may carry a signature of CNS pathology. These features mean that circulating exosomes can serve as biomarkers for CNS disorders (Shi et al., 2019). Thus, dysregulation of miRNAs in circulating CNS-derived exosomes may be proposed as putative biomarkers. The up-regulation and down-regulation of numerous exosomal miRNAs has been associated with AD. Indeed, it has been reported that whole blood, plasma, serum and CSF derived exosomes carry potential miRNA biomarkers in AD (**Figure 1**).

Our recent review summarizes the results of all the work reported, so far, in the literature on the profile of exosomal miRNAs in patients with AD (Manna et al, 2020). Although these studies highlight a potential use of exosomal miRNAs as diagnostic biomarkers, the main limitation that emerges from critical analysis of the works present in the literature and included in our review, is the no overlap between the different exosomal miRNAs found dysregulated across the studies. The non-reproducibility that appears to be a major drawback of exosomal miRNAs studies, could be due to the large number of variables, such as the nature of the biological liquid (serum/plasma *vs*. CSF), the methods for exosome isolation, the methods of miRNAs quantification (such as microarrays and RNA sequencing) and data analysis. We believe, despite the above limitations, that the choice of very standardized parameters (patient recruitment, gender, inclusion and exclusion criteria, drug therapies, normalization and statistical data analysis), and longitudinal analysis of larger patient cohorts is fundamental to use exosomal miRNAs as diagnostic biomarkers of disease in clinical practice.

Future prospects: potential use of exosomal miRNAs in AD treatment: miRNAs represent a target for controlling endogenous levels of gene expression. The growing knowledge on miRNAs functions, considering their small size and the network of proteins they regulate, increases the interest in the development of new therapies. Today, the efforts made for the development of miRNA-based therapeutics, are mainly directed towards the production of both mimic miRNAs and antagomiRs. Mimic miRNAs replace the miRNAs reduced in the pathology, on the contrary, antagomiRs are used to suppress the function of specific miRNAs, whose overexpression is involved in pathology (Bonneau et al., 2019). As reported by Bonneau et al. (2019), several pharmaceutical and biotech companies have launched miRNA projects in their development miRNAs-based therapeutics. The encouraging results from recent clinical trials allow us to imagine that miRNA-based pharmacological approaches could, in the future, meet the requirements for successful

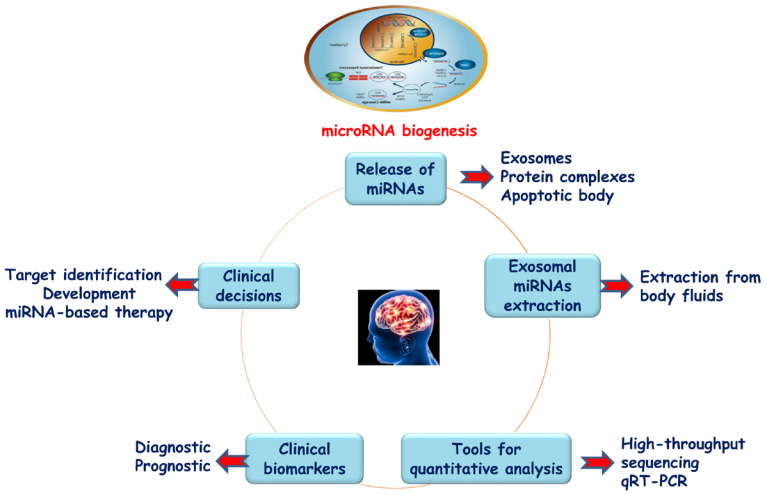

Figure 1 | **Exosomal miRNAs as biomarkers and therapeutic strategies in Alzheimer's disease: overview of fields of research.**
Diagram in Figure 1 represents miRNA biogenesis, modes of their secretion into body fluids, RNA extraction and quantitative approaches, miRNA-based biomarkers, and in future, their possible therapeutic use.

therapeutic outcomes. Despite this potential, the development of miRNA-based and miRNA-targeting therapeutics still needs time for the overcoming of two major challenges: stability and delivery. For this reason most of the molecules are still in the preclinical phase, and only some of them are in the clinical evaluation phase (Bonneau et al., 2019). About delivery of gene-based therapy, it is important to deliver the drug to the correct place without its degradation and elimination. It is known that exosomes perform a cargo function, transporting various biomolecules, as proteins, functional mRNAs and miRNAs, thus mediating, also long-distance cell-to-cell communication processes. Because of this feature, and their other peculiarities, they are considered a potential approach for AD therapy. In addition to finding a potential new approach for treating AD, considering that BBB greatly hinders the penetration and accumulation of drugs in the brain, with reduced therapeutic effects, it is also important to seek a strategy that allows drugs to pass through the BBB. Wang et al. (2019) demonstrated that curcumin transporter exosomes exhibited highly effective BBB crossing mediated by transcytosis mechanisms, observing an inhibition of tau phosphorylation and a great potential for improvement drug target delivery and recovery of neuronal functions in the AD therapy. Recently, exosomes have been considered a suitable vehicle for drug delivery due to their excellent characteristics, like their natural targeting capacity. The exosomes showed targeted capabilities during the drug delivery process by an active ligand receptor-mediated targeting process. Indeed, exosomes inherit some peptides from their parental cells that could specifically combine with target cell receptors, thus accelerating the accumulation of exosomes in target tissues (Wang et al., 2019). Moreover, exosomes can be engineered using specific ligands on their surface in order to provide them targeting capability, and they can be used as nanovesicles delivering siRNAs/miRNAs to the CNS. To realize a tissue specific delivery, Alvarez-Erviti et al. (2011) engineer the exosome to express the central nervous system-specific rabies viral glycoprotein (RVG) peptide. Targeting was achieved by engineering the self-derived dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron specific RVG peptide. Intravenous injection of RVG-targeted exosome in mice delivered siRNA to neurons, microglia, and oligodendrocytes, demonstrating a significant reduction of Aβ deposits in the brain of animals. This result suggests an *in vivo* therapeutic potential of a RNA-therapy exosome delivered and highlights another feature of exosomes, their poor immunogenicity. Indeed, exosome deriving from self-derived cells offers the advantage of a well-tolerated vehicle from a toxicological and immunological point of view (Alvarez-Erviti et al., 2011). Another one intrinsic characteristic of exosomes could provide an aid in AD therapy. As demonstrated by Yuyama et al. (2014), the exosomes act as scavengers of extracellular Aβ by trapping the Aβ on surface glycosphingolipids, and transport it into microglia in AD mouse brain. They demonstrated that intracerebral administration of exosomes results in CNS Aβ reduction in CNS of the AD animal model (Yuyama et al., 2014).

Conclusion: Finally, the observation that exosomal miRNAs can be dysregulated in patients with AD makes them attractive candidates for monitoring disease. More studies are needed to confirm their use in clinical practice as disease biomarkers. Furthermore, correction of miRNA expression using miRNA agonists or antagonist oligonucleotides, despite the complexity of these approaches, suggests that it could be a promising tool for the future development of miRNA-based therapy for neurodegenerative diseases.

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