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Review

miRNAs as biofluid markers for diagnostics of Alzheimer's disease: recent status and perspectives

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Abstract. After many decades of research in the field of neurodegeneration, we have no effective cure for Alzheimer's disease (AD), a major form of dementia. It is mainly due to the lack of early, reliable and sensitive biomarkers and incomplete understanding of disease mechanisms at molecular level. Several recently employed biomarkers, especially their combinations, can discriminate advanced stages of AD from other forms of dementia or neuropathy. They do not provide much information on molecular mechanisms of disease, rather they reflect the amount of key histopathological markers in the diseased brain. This review is focussed on novel class of potentially very promising AD biomarkers: extracellular miRNAs in body liquids, such as cerebrospinal fluid and blood. Those have a great potential not only to indicate the presence of AD, but more importantly, to reflect the molecular mechanisms playing a role early at the beginning of the pathogenic pathways consequently leading to AD. We believe this comprehensive review on deregulated miRNAs in AD can be a good source of information for thorough *in silico* analyses aiming to identify, develop and validate of miRNAs as "diseases mechanism engaged" candidate biomarkers. Having such molecules could bring us closer to the goal – successful treatment of AD.

Key words: Alzheimer's disease — miRNA — Biomarker — Diagnostics — Cerebrospinal fluid — Blood

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; BACE1, beta-secretase 1; CSF, cerebrospinal fluid; DGCR8, DiGeorge syndrome critical region 8; FTD, frontotemporal dementia; MCI, mild cognitive impairment; miRNA, microRNA; MVB, multivesicular bodies; pri-miRNA, primary microRNA transcript; pre-miRNA, precursor microRNA; RISC, RNA-induced silencing complex; qPCR, quantitative real time polymerase chain reaction; NGS, next generation sequencing; TRBP, trans-activation responsive RNA-binding protein.

Introduction

Alzheimer's disease (AD) is characterized by progressive impairment of memory and other cognitive functions, resulting in complete dependency on caregivers and ultimately in deterioration of patients vital brain functions. According to world health organization there are about 50 million people living with dementia worldwide as of December 2017. Approximately 60–70 % of them suffer from AD. Predictions suggest that this numbers will rise up to 82 million and 152 million in 2030 or 2050, respectively (http://www.who.int). These figures signify the importance of having a reliable and specific early diagnosis of AD. However, molecular biomarkers that could identify AD-specific pathogenic changes in preclinical stage are still lacking, although they would speed up the quest for disease modifying therapy of AD, which is still missing as well.

AD is a complex multifactorial disease. Although major risk factors of AD include age and positive family history of dementia, the genetic, epigenetic and environmental influ-

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ences are interlinked and go hand in hand and influence the course of the pathogenesis. Comorbidities such as diabetes, hypertension, high cholesterol level and brain traumatic injuries play also an important role in manifestation of sporadic AD (Zhou and Xu 2012; Tolppanen 2013). A combination of these influences may predispose an individual to increased cognitive decline.

Another level of complexity is the fact that clinical symptoms of AD are similar to other types of dementia like frontotemporal dementia, human prion disease, Huntington's disease, Lewy Body dementia, Parkinson's disease dementia or vascular dementia. Hence definitive confirmation of AD can only be performed after pathological examination of brain (Nelson et al. 2012). Interestingly, post-mortem examination often reveals mixed pathologies in the brain of patients. Senile plaques and neurofibrillary tangles can often be found in close proximity with other brain inclusions like TDP43 and Lewy bodies. The extent to which these other pathologies can contribute to the AD is not yet known (Lashley et al. 2018). Although major and promising initiatives exist to provide the ways for early diagnostics based on biomarkers (Frisoni et al. 2017) it still holds true that no reliable peripheral biomarker for AD recently exists.

Currently microRNAs (miRNA), discovered 25 years ago (Lee et al. 1993), have gained a prominent attention as a potential biomarker for pathogenesis of various diseases and were recently identified as molecules which function to control posttranscriptional gene expression also in AD (Reddy et al. 2017a).

There is a general agreement and hope that *via* identification of deregulated miRNA in pre-symptomatic AD we will be able to better understand the molecular changes initiating AD pathogenesis well before the clinical onset of AD. Obviously, specific miRNAs regulating certain signaling pathways are directly engaged in disease pathogenesis. We hypothesize that miRNAs can convey the message from diseased brain cells to the peripheral fluids (either in cerebrospinal fluid (CSF) or blood where they are quantifiable.

miRNA in peripheral fluids are remarkably stable (De Guire et al. 2013) and therefore have significant potential as means of intercellular communication (Chen et al. 2012). The information they carry is transferred from diseased brain cells to the CSF, as well as the peripheral blood (Reddy et al. 2017b). Several miRNAs, differentially present in the blood and CSF of AD patients when compared to healthy controls therefore represent a great source of diagnostically relevant information, which can be reached *via* non-invasive methods.

In this review, we provide comprehensive and up to date information on miRNAs differentially present in blood and CSF of AD patients (summarized in Table 1 and 2). Deciphering the role of miRNA in specific AD cases, decoding their multiple RNA targets may bring about better understanding of molecular mechanism leading to development of AD. Though *in silico* analyses of miRNA profiles in the field of neurodegeneration are very sparse and the roles of specific miRNAs as AD biomarkers are yet to be validated, the information presented here can be useful for experimental research, development and validation of miRNAs, either as biomarkers and potentially also as therapeutic targets for treatment of AD.

Membrane vesicles as transfer vehicles

Pathological and disease specific molecular signatures in the forms of proteins, nucleic acids or other biomolecules can escape from the brain in free form or *via* internalization in membrane vesicles (Figure 1). After the crossing of blood-brain or brain-CSF barriers they can be found in peripheral tissues. Generally the membrane vesicles originate in endosomes and plasma membranes of the most mammalian cells and are secreted to the extracellular space. These vesicles provide certain degree of protection to the molecules they contain. They circulate in all human body fluids including blood, urine, saliva, amniotic fluid, tears, milk and CSF. Exosomes and ectosomes represent the two major groups of vesicles, which are classified based on their size and origin.

Exosomes are membrane vesicles with the size of 50–100 nm. They arise during endocytosis after which they are transferred through endosomes and are clustered in multivesicular bodies (MVB). MVBs are in fact late endosomes loaded with intraluminal vesicles, which upon fusion with lysosomes undergo degradation. However, when MVB fuse with plasma membranes, intraluminal vesicles are released to extracellular space as exosomes by the process of exocytosis (Cocucci and Meldolesi 2015). The membrane of the exosomes is rich in cholesterol, sphingomyelin and membrane proteins as integrins and tetraspanins (Thery et al. 2002). Typical surface markers for exosomes are molecules CD63 and CD61 (Cocucci and Meldolesi 2015).

Ectosomes are comparably larger vesicles produced by huge variety of mammalian cells including neurons, astrocytes, microglia, endothelia, immune cells, cancer cells, stem cells and blood cells (Cocucci and Meldolesi 2011). Their size varies from 100 to 1000 nm. They are formed directly at the plasma membrane and are released to the extracellular space (Turola et al. 2012). TyA and C1q proteins are typical ectosomal markers (Cocucci and Meldolesi 2015). Moreover, ectosomes contain also large amounts of phosphatidylserine on the membrane surface when compared to exosomes. This is probably related to the different origin of these vesicles (Cocucci and Meldolesi 2011). Despite the different biogenesis of exosomes and ectosomes their functions are quite similar. Exosomes and ectosomes are

Table 1. MicroRNAs dysregulated in peripheral fluids of AD patients

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change	*p* value	Ref.
			Patients	Controls				
let-7f-5p	BMC	Microarray	16 mild AD	16	–	↑	ns	*1*
		TaqMan qPCR	10 AD	10	RNU24			
let-7i-5p	CSF	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↑	0.019	*2*
miR-9	serum	SYBR Green qPCR	105 probable AD	150	cel-miR-39	↑	0.002	*3*
		SYBR Green qPCR	7 sporadic AD	7	cel-miR-39 miR-22 miR-126 miR-191	↓	<0.05	*4*
			7 MCI	7			<0.05	
	CSF	Hybridization miRNA array LED-Northern dot blot	3 AD	3	miR-183	↑	<0.01	*5*
		Hybridization miRNA array LED-Northern dot blot	5 AD	5	miR-183	↑	<0.05	*6*
		SYBR Green qPCR	18 AD	18	–	–	0.001	*7*
	plasma	TaqMan qPCR	50 AD	50	miR-874	↑		*8*
miR-15a	plasma	TaqMan qPCR	3 AD	6	miR-132	↓	<0.05	*9*
	CSF	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↑	0.005	*2*
miR-17-3p	blood	Illumina HiSeq 2000 sequencing	49 AD	55		↓	3E-11	*10*
miR-23a	serum	TaqMan qPCR	15 AD	12	cel-miR-39 U6 snRNA SNORD61 SNORD68 SNORD72 SNORD95 SNORD96a	↓	<0.001	*11*
miR-26b	serum						<0.01	
	CSF						<0.001	
miR-27a-3p	CSF	SYBR Green qPCR	20 AD	19	miR-320a miR-101-3p	↓	0.008	*12*
			15 AD	18			0.015	
miR-29a	serum	SYBR Green qPCR	7 sporadic AD	7	cel-miR-39 miR-22 miR-126 miR-191	↓	<0.05	*4*
			7 MCI	7			<0.05	
		TaqMan qPCR	84 AD	62	U6 snRNA	↓	<0.01	*13*
	CSF	TaqMan qPCR	18 AD	20	miR-16 miR-24 U6 snRNA	↑	<0.001	*14*
		TaqMan qPCR	10 AD	10	U6 snRNA miR-24	↑	<0.05	*15*
miR-29b	serum	SYBR Green qPCR	7 sporadic AD	7	cel-miR-39 miR-22 miR-126 miR-191	↓	<0.05	*4*
			7 MCI	7			<0.05	
	BMC	TaqMan qPCR	28 AD	25	RNU48	↓	0.002	*16*
	CSF	TaqMan qPCR	10 AD	10	U6 snRNA miR-24	↑	<0.05	*15*
miR-29c-3p	CSF	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↓	0.009	*2*

(continued)

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change	*p* value	Ref.
			Patients	Controls				
miR-34a	BMC	Microarray	16 mild sporadic AD	16	–	↑	not shown	*1*
		SYBR Green qPCR	10 AD	10	5S rRNA			
	plasma	TaqMan qPCR	10 AD	10	U6 snRNA miR-24	↓	<0.05	*15*
	CSF							
	BMC	TaqMan qPCR	78 AD	85	cel-miR-39 miR-16	↑	<0.05	*17*
	plasma						<0.01	
	plasma	TaqMan qPCR	21 preclinical AD	21	miR-106a-5p miR-17-5p cel-miR-39	↓	<0.01	*18*
miR-34c	BMC	TaqMan qPCR	25 AD	27	cel-miR-39 miR-16	↑	<0.05	*17*
	plasma						<0.01	
miR-99b	plasma	TaqMan qPCR	50 AD	50	let-7e	↑		*8*
miR-125b	CSF	TaqMan qPCR	37 AD	31	miR-30c	↑	0.04	*19*
		TaqMan qPCR	18 AD	20	miR-16 miR-24 U6 snRNA	↑	<0.05	*14*
		TaqMan qPCR	10 AD	10	U6 snRNA miR-24	↓	<0.05	*15*
		Hybridization miRNA array LED-Northern dot blot	3 AD	3	miR-183	↑	<0.01	*5*
	CSF	TaqMan qPCR	15 AD	12	cel-miR-39	↓	<0.001	*11*
	serum				U6 snRNA SNORD61 SNORD68 SNORD72 SNORD95 SNORD96a		0.009	
	serum	SYBR Green qPCR	105 AD	150	cel-miR-39	↓	4.86E-7	*3*
		TaqMan qPCR	84 AD	62	U6 snRNA	↓	<0.001	*13*
miR-128	monocytes	SYBR Green qPCR	34 sporadic AD	37	U6 snRNA	↑	<0.05	*20*
	lymphocytes	SYBR Green qPCR	34 sporadic AD	37	U6 snRNA	↑	<0.05	*20*
miR-135a	serum	TaqMan qPCR	38 AD	30	cel-miR-39	↓	0.02	*21*
	CSF		5 AD	5			0.015	
miR-137	serum	SYBR Green qPCR	7 sporadic AD	7	miR-22 miR-126	↓	<0.05	*4*
miR-142-5p	blood	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↑	0.043	*2*
miR-146a	plasma	TaqMan qPCR	10 AD	10	U6 snRNA miR-24	↓	<0.05	*15*
	CSF							
	CSF	Hybridization miRNA array LED-Northern dot blot	3 AD	3	miR-183	↑	<0.01	*5*
		Hybridization miRNA array LED-Northern dot blot	5 AD	5	miR-183	↑	<0.01	*6*
		TaqMan qPCR	20 AD	22	U6 snRNA	↓	0.0005	*22*
miR-151a-3p	blood	Illumina HiSeq 2000 sequencing	49 AD	55		↑	6E-12	*10*
miR-155	CSF	Hybridization miRNA array LED-Northern dot blot	3 AD	3	miR-183	↑	0.01	*5*
		Hybridization miRNA array LED-Northern dot blot	5 AD	5	miR-183	↑	<001	*6*
miR-181b	BMC	Microarray	16 mild sporadic AD	16		↑	not shown	*1*
		SYBR Green qPCR	10 AD	10	5S rRNA			
miR-181c	serum	SYBR Green qPCR	105 probable AD	150	cel-miR-39	↓	3.34E-5	*3*

(continued)

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change	*p* value	Ref.
			Patients	Controls				
miR-193b	serum exosomes	TaqMan qPCR	51 AD	unknown	U6 snRNA	↓ in AD	<0.05	*21*
			43 MCI	unknown			<0.05	
			51 AD	43 MCI			<0.05	
	CSF exosomes		7 AD	7			<0.05	
miR-194-5p	blood	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↓	0.028	*2*
miR-200a	BMC	Microarray	16 mild sporadic AD	16		↑	not shown	*1*
		SYBR Green qPCR	10 AD	10	5S rRNA			
miR-200b	serum	TaqMan qPCR	38 AD	30	cel-miR-39	↓ in AD	0.007	*21*
			38 AD	31 MCI			0.025	
	CSF		5 AD	5			0.001	
miR-206	serum	SYBR Green qPCR	128 Amnestic MCI to AD conversion follow up		cel-miR-39	↑	<0.001	*23*
miR-210	serum	SYBR Green qPCR	26 AD	30 MCI	U6 snRNA	↓ in AD	<0.01	*24*
	CSF		26 AD	30 MCI			<0.01	
miR-222	CSF	TaqMan qPCR	37 AD	31	miR-30c	↑	0.006	*19*
miR-223	serum	TaqMan qPCR	84 AD	62	U6 snRNA	↓	<0.01	*13*
miR-329	plasma	TaqMan qPCR	50 AD	50	miR-181a	↑		*8*
miR-339-5p	BMC	SYBR Green qPCR	45 AD	41	U6 snRNA	↑	0.003	*25*
miR-342-3p	serum	Illumina HiSeq 2000 sequencing	50 AD	50		↓	4.63E-28	*26*
		SYBR Green qPCR	158 AD	155	cel-miR-39		9.19E-16	
	plasma exosomes	Illumina HiSeq2500 sequencing	35 AD	35	Raw sequence counts normalized per million mappable reads to human genome	↓	0.0007	*27*
miR-384	serum	TaqMan qPCR	45 AD	50	U6 snRNA	↓ in AD	<0.05	*21*
	plasma		45 AD	32 MCI			<0.05	
	CSF		7 AD	7			<0.05	
miR-425-5p	BMC	SYBR Green qPCR	45 AD	41	U6 snRNA	↑	<0.001	*25*
miR-455-3p	serum	SYBR Green qPCR	11 AD	18	U6 snRNA	↑	0.007	*28*
	blood B-lymphocytes	SYBR Green qPCR	6 Sporadic AD	10	U6 snRNA	↑	0.044	*29*
miR-501-3p	serum	TaqMan qPCR	36 AD	22	cel-miR-39-3p miR-451a	↓	0.00002	*30*
miR-519	serum	TaqMan qPCR	84 AD	62	U6 snRNA	↑	<0.01	*13*
miR-545-3p	plasma	TaqMan qPCR	21 preclinic. AD	21	miR-106a-5p miR-17-5p cel-miR-39	↓	<0.05	*18*
			21 AD	21			<0.01	
miR-590-5p	blood	SYBR Green qPCR	10 AD	10	Synthetic RNA Sp6, DNA Sp3 spike-in control	↑	0.002	*2*
miR-598	CSF	SYBR Green qPCR	18 AD	18	-	-	<0.001	*7*
miR-639	BMC	SYBR Green qPCR	45 AD	41	U6 snRNA	↓	0.04	*25*

↑ up-regulated; ↓ down-regulated; BMC, blood mononuclear cells; CSF, cerebrospinal fluid; AD, Alzheimer's disease. MCI, mild cognitive impairment. References in the table: (*1*) Schipper et al. 2007; (*2*) Sorensen et al. 2016; (*3*) Tan et al. 2014a; (*4*) Geekiyanage et al. 2012; (*5*) Alexandrov et al. 2012; (*6*) Lukiw et al. 2012; (*7*) Riancho et al. 2017; (*8*) Sheinerman et al. 2017;(*9*) Bekris et al. 2013; (*10*) Keller et al. 2016; (*11*) Galimberti et al. 2014; (*12*) Sala Frigerio et al. 2013; (*13*) Jia and Liu 2016; (*14*) Muller et al. 2016; (*15*) Kiko et al. 2014; (*16*) Villa et al. 2013; (*17*) Bhatnagar et al. 2014; (*18*) Cosin-Tomas et al. 2017; (*19*) Dangla-Valls et al. 2017; (*20*) Tiribuzi et al. 2014; (*21*) Liu et al. 2014; (*22*) Muller et al. 2014; (*23*) Xie et al. 2017; (*24*) Zhu et al. 2015; (*25*) Ren et al. 2016; (*26*) Tan et al. 2014b; (*27*) Lugli et al. 2015; (*28*) Kumar et al. 2017; (*29*) Kumar and Reddy 2018; (*30*) Hara et al. 2017.

important for intercellular communication without direct contact between the cells. They transport different specific proteins, enzymes, adhesive molecules, cytoskeletal proteins, mRNAs and also miRNAs. Interestingly, exosomes do not merely replicate the miRNA profile of the cell from which they originate and can contain a specific set of miRNAs of a different expression level, to function as effective regulators of signalling pathways and physiological processes in recipient cells (Hunter et al. 2008).

Membrane vesicles are secreted by different cell types, carrying information about the biological changes in the cells from which they originate and were shown to pass the blood-brain barrier to periphery (Shi et al. 2016). Neuronal cells release ectosomes and exosomes, using the same mechanisms for generation of extracellular vesicles and for spreading from brain cells in normal and pathological conditions. Damage to the brain barriers, such as the blood brain barrier (cerebral vasculature), brain-CSF barrier (pia arachnoid, neuroependyma) and blood-CSF barrier (choroid plexus) is a major factor increasing the permeability during the disease that results in increased transfer of molecules and vesicles from and into the brain. This damage is also highly associated with neuroinflammation, which is an associated pathological entity in neurodegeneration (Stolp et al. 2013). Pathophysiological changes in the neurovascular unit and increased permeability of the blood brain barrier occur early in AD (van de Haar et al. 2016), supporting the idea of leakage of brain derived molecules including miRNAs into peripheral fluids and suggesting a possibility to detect their altered levels in early stages of the disease.

Determination of the level of specific miRNAs, circulating in the body fluids could be potentially used in the future as a diagnostic biomarker for clinical practice. Extracellular vesicles are in the scope of interest also due to their role in the spreading of β-amyloid and tau pathology in the brain. For example, amyloid beta (Aβ42) was shown to accumulate in multivesicular bodies and to be released from the cells *via* exosomes (Takahashi et al. 2002). Furthermore, it was reported that pathological tau protein is also present and transferred by exosomes and ectosomes (Saman et al. 2012). It was also shown that accumulation of pathological tau protein in the cells leads to its higher secretion *via* exosomes (Dujardin et al. 2014).

Protein based assays for diagnostics of Alzheimer's disease

As of now the most frequently used biomarkers for AD diagnostics are assays for quantification of amyloid beta 42 (Aβ42) or Aβ42/Aβ40; total and phospho-tau in CSF or blood (reviewed in Song et al. 2016; Lewczuk et al. 2017; Jack et al. 2018). Since the senile plaques are made of aggregates mainly consisting of truncated Aβ (Aβ42), development of Aβ42-specific ELISA has improved the specificity of AD diagnostics (Jarrett et al. 1993). Quantification of Aβ42 in CSF of AD patients revealed significant reduction in the level of Aβ42 isoform (Motter et al. 1995). The lower concentration of Aβ42 in CSF of AD patients is explained by the utilization of Aβ42 as a building element of amyloid plaques in the brain (Strozyk et al. 2003). Mild decrease in levels of Aβ42 in the CSF was observed in patients with frontotemporal dementia (FTD), vascular dementia and amyotrophic lateral sclerosis (ALS) (Sjogren et al. 2000, 2002; Riemenschneider et al. 2002), when compared to subjects with psychiatric disorders such as depression, or other chronic neurological disorders such as Parkinson disease or progressive supranuclear palsy (Sjogren et al. 2000, 2002; Holmberg et al. 2003). All these data indicate that ability of Aβ42-ELISA to discriminate between AD, other forms of dementias and neurological disorders is limited. However, studies suggest that decrease in Aβ42/Aβ40 ratio in the CSF is of higher informative value than the levels of Aβ42 alone (Shoji et al. 1998; Fukuyama et al. 2000; Lewczuk et al. 2015).

In contrast to decreased Aβ42 levels, the level of total tau protein, the major composite of neurofibrillary tangles is elevated in the CSF of AD patients. However, increased level of total tau protein is also observed during normal aging or head trauma, which limits tau dependent diagnostic techniques (Burger nee Buch et al. 1999; Blennow et al. 2016). However, it was estimated that total tau concentration in CSF of AD patients is about 3-fold higher when compared to age-matched controls. Cumulative diagnostic parameters for total tau protein in CSF from 2500 AD patients and 1400 controls showed a mean specificity of 90% and 81% sensitivity to AD (Blennow and Hampel 2003).

Pathophysiology of AD is associated with several post-translational modifications, among others the phosphorylation of tau on the "disease-specific" epitopes. Several immunological assays based on monoclonal antibodies specific for phosphorylated tau protein have been developed that could be potentially used as biomarkers of AD diagnostics or progression of AD. These tests quantify the level of tau proteins phosphorylated at amino acid: T181, T231, S199 and S396/S404 (Ishiguro et al. 1999; Kohnken et al. 2000; Vanmechelen et al. 2000; Hu et al. 2002). For example, tau protein phosphorylated at threonine 231 (p-tau 231) in comparison with total tau is better biomarker for early AD in patients with mild cognitive impairment (Kohnken et al. 2000). The level of p-tau 231 decreases during the progression of the disease, which makes this marker a candidate for monitoring progression of the disease. Moreover, p-tau 231 was suggested as an appropriate marker for discrimination of AD from frontotemporal dementia and depression with specificity of up to 92.3% and sensitivity between

Table 2. MicroRNA panels in peripheral samples of AD patients

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change in expression	*p* value	Ref.
			Patients	controls				
miR-26b-3p miR-28–3p miR-30c-5p miR-30d-5p miR-148-5p miR-151a-3p miR-186–5p miR-425–5p miR-550a-5p miR-1468 miR-4781-3p miR-5001–3p miR-6513–3p	blood	Illumina HiSeq 2000 sequencing	48	22		↑	0.008 0.037 0.0068 0.0088 0.0112 0.0021 0.0071 0.0114 0.0071 0.0056 0.0002 0.0056 0.0021	*1*
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 miR-3200–3p	blood	Illumina HiSeq 2000 sequencing	48 AD	22		↓	0.0203 0.0071 0.0002 0.0119 0.0071 0.0119 0.0203 0.0021 0.0013 0.0071 0.0334 0.0088 0.0113 0.0088	
let-7d-3p miR-26a-5p miR-151a-3p miR-1285-5p miR-5010-3p brain-miR-112 brain-miR-161	blood	Illumina HiSeq 2000 sequencing	48 AD	22		↑	< 0.05	*2*
let-7f-5p miR-26b-5p miR-103a-3p miR-107 miR-532-5p						↓		
let-7d-3p miR-151a-3p miR-5010-3p brain-miR-112 brain-miR-161	blood	SYBR Green qPCR	94 AD	21	RNU48	↑ in AD	not shown	
let-7f-5p miR-26a-5p miR-26b-5p miR-103a-3p miR-107 miR-532-5p miR-1285-5p						↓ in AD		

(continued)

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change in expression	*p* value	Ref.
			Patients	controls				
miR-100 miR-146a miR-296 miR-505-5p miR-766 miR-3622b-3p miR-4467	CSF	TaqMan qPCR array	22 AD	28	U6 snRNA ath-miR-159a kshv-miR-K12-1-5p	↑	<0.05	*3*
miR-103 miR-219 miR-335 miR-375 miR-708 miR-1274a miR-4449 miR-4674						↓	<0.05	
miR-1291	CSF	TaqMan qRT-PCR array	16 AD	16	U6 snRNA	↑	0.02	*4*
miR-143-3p miR-142-3p miR-328-3p miR-193a-5p miR-19b-3p miR-30d-5p miR-340-5p miR-140-5p miR-125b-5p miR-223-3p						↓	<0.01 <0.01 0.01 <0.01 0.03 0.04 <0.01 0.02 0.02 <0.01	
miR-485-5p	CSF exosomes	TaqMan miRNA arrays	28 AD	27	U6 snRNA	↑	0.0269	*5*
miR-16-2 miR-29c miR-132-5p miR-136-3p miR-151 miR-331-5p						↓	0.0136 0.0436 0.0188 0.0133 0.0063 0.0404	
miR-483-5p miR-486-5p miR-200a-3p miR-502-3p	plasma	SYBR Green qPCR	20 AD	24	miR-185-5p miR-128-3p miR-130b-3p miR-15a-5p miR-425-3p	↑	<0.01 <0.001 <0.05 <0.01	*6*
miR-30b-5p miR-142-3p						↓	<0.01 <0.01 <0.01	
miR-128 miR-132 miR-874	plasma	TaqMan qPCR	50 MCI	50	miR-491-5p	↑	1.51E-16	*7*
miR-134 miR-323-3p miR-382					miR-370		2.29E-12	

(continued)

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change in expression	*p* value	Ref.
			Patients	controls				
let-7d-5p let-7g-5p miR-15b-5p miR-142-3p miR-191-5p miR-301a-3p miR-545-3p	plasma	TaqMan qPCR	11 AD	20	ath-miR-159a miR-106a	↓	0.0001 0.001 0.001 0.0001 0.002 0.0006 0.03	*8*
let-7d-5p let-7g-5p miR-15b-5p miR-142-3p miR-191-5p miR-301a-3p miR-545-3p			20 AD	17			<0.0001 <0.0001 <0.0001 <0.0001 <0.0001 0.07 0.01	
miR-106b-3p	serum	SYBR Green qPCR	31 mild AD, 52 moderate AD, 38 severe AD	86	miR-451 cel-miR-39	↑ in all AD groups vs controls	<0.001	*9*
miR-26a-5p miR-181c-3p miR-126-5p miR-22-3p miR-148b-5p						↓ in all AD groups vs controls	0.007 <0.001 <0.001 <0.001 <0.001	
miR-6119-5p miR-1246 miR-660-5p miR-26a-5p			31 mild AD, 38 severe AD			↑ in severe AD vs mild AD	<0.001 <0.001 <0.001 0.014	
miR-6119-5p miR-1246			31 mild AD, 52 moderate AD			↑ in moderate AD versus mild AD	<0.001 0.08	
miR-1246 miR-660-5p			52 moderate AD, 38 severe AD			↑ in severe AD versus moderate AD	<0.001 <0.001	
miR-6119-5p miR-1246 miR-660-5p			52 moderate AD, 38 severe AD	86		↑ in severe AD and moderate AD vs controls	<0.001 <0.001 <0.001	
miR-660-5p			52 moderate AD	86		↑ in moderate AD vs controls	0.381	
miR-31 miR-93 miR-143 miR-146a	serum	Solexa sequencing	10 pooled AD sera	7 pooled control sera		↓	<0.0001	*10*
		TaqMan qRT-PCR	127 AD	123	serum volume			
miR-let-7d-5p miR-98-5p miR-191-5p miR-342-3p miR-483-3p miR-885-5p	serum	Illumina HiSeq 2000 sequencing	50 AD	50		↓	<0.0001	*11*
		SYBR Green qPCR	158 AD	155	cel-miR-39			

(continued)

miRNA	Source	Method	Size of cohort (*n*)		Normalisation	Change in expression	*p* value	Ref.
			Patients	controls				
miR-20b-5p miR-106b-3p miR-146a-5p miR-195-5p miR-497-5p	serum	SYBR Green qPCR	65 AD	60	not published	↑	<0.05	*12*
miR-19b-3p miR-29c-3p miR-93-5p miR-125b-3p						↓		
miR-15a-5p miR-18b-5p miR-20a-5p miR-30e-5p miR-93-5p miR-101-3p miR-106a-5p miR-106b-5p miR-143-3p miR-335-5p miR-361-5p miR-424-5p miR-582-5p	serum exosomes	Ion Torrent seguencing	23 AD	23	The number of reads of each miRNA were normalised to reads per million across all samples	↑	0.02 6.85E-3 9.53E-3 1.10E-4 9.26E-3 0.01 0.03 0.02 0.02 0.01 2.26E-3 5.7E-4 0.02	
miR-15b-3p miR-342-3p miR-1306-5p						↓	0.04 1.04E-3 0.01	*13*
miR-15a-5p miR-18b-5p miR-20a-5p miR-30e-5p miR-93-5p miR-101-3p miR-106a-5p miR-106b-5p miR-143-3p miR-335-5p miR-361-5p miR-424-5p miR-582-5p	serum exosomes	TaqMan qPCR	16 AD	36	cel-miR-39 miR-451	↑	not shown	

↑ up-regulated; ↓ down-regulated; AD, Alzheimer's disease. References in the table: (*1*) Satoh et al. 2015; (*2*) Leidinger et al. 2013; (*3*) Denk et al. 2015; (*4*) Lusardi et al. 2017; (*5*) Gui et al. 2015; (*6*) Nagaraj et al. 2017; (*7*) Sheinerman et al. 2013; (*8*) Kumar et al. 2013; (*9*) Guo et al. 2017; (*10*) Dong et al. 2015; (*11*) Tan et al. 2014b; (*12*) Wu et al. 2017; (*13*) Cheng et al. 2015.

57.7–90.2% (Buerger et al. 2002, 2003). It is also shown that a strong increase in the levels of p-tau 181 in AD patients is observed when compared to FTD patients (Vanmechelen et al. 2000). Tau phosphorylated at serine 199 (p-tau 199) is also the candidate for specific diagnostics of AD. In AD patients, the level of p-tau 199 was found increased when compared with subjects suffering from other type of dementia or healthy controls. However, the assay showed only about 85% of sensitivity and specificity (Itoh et al. 2001). The ultrasensitive ELISA technique for the detection of serine 396 and serine 404 showed 400-fold and about 1300-fold higher sensitivity when compared to standard ELISA test (Hu et al. 2002).

Introduction of digital, single molecule assays based on Quanterix platform was important milestone, since this assay allows to detect tau protein and Aβ42 as low as picogram *per* millilitre range in the CSF and blood (Song et al. 2016; Motamedi et al. 2018). Simultaneous determination of tau

and beta amyloid levels in CSF resulted in a better diagnostic value than those gained using quantification of the single molecules.

It is noteworthy that perhaps due to multifactorial nature of the disease, Aβ42 and tau protein still remain the only widely acceptable biomarkers of AD (Jack et al. 2018). Although increased levels of total tau and phosphorylated isoforms of tau protein are observed in the CSF of patients suffering of Alzheimer's disease, the accurate diagnosis of AD demands additional biomarkers (Vandermeeren et al. 1993;

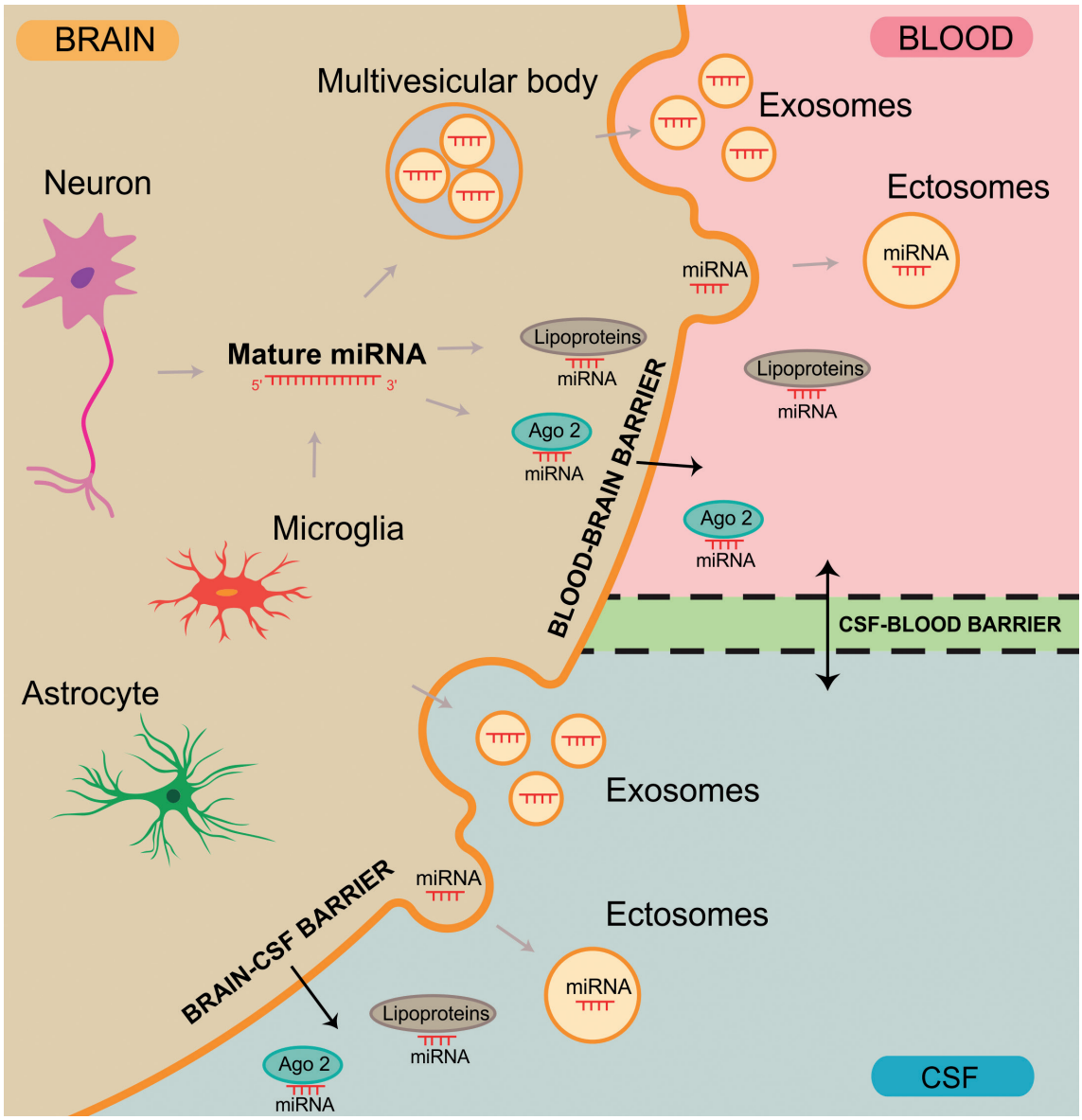

Figure 1. Leakage of miRNAs from brain into blood and cerebrospinal fluid (CSF). Mature miRNA can be secreted by neuronal and glial cells *via* different pathways. Exocytosis involves encapsulation of mature miRNAs into exosomes that are generated as multivesicular bodies and released to extracellular space upon their fusion with plasma membrane. Another mechanism of miRNA secretion includes their encapsulation into ectosomes that are generated directly from the plasma membrane. Importantly, the vesicles provide a certain degree of protection to the molecules they contain. Besides the vesicular pathways miRNAs can leave the secreting cells and spread in a complex with lipoproteins or bound to Argonaute proteins (Ago2). Brain derived miRNAs, either in the vesicles or bound in the protein complexes are present in CSF and circulate in blood stream; however, they were detected also in urine, saliva, amniotic fluid, tears and milk. Peripheral fluids therefore can serve as a good source of brain specific biomarkers reflecting metabolism of neurons and glial cells, which can be specifically modified already in early stages of the neurodegenerative diseases.

Blennow et al. 1995; Mori et al. 1995; Vigo-Pelfrey et al. 1995; Ishiguro et al. 1999; Kohnken et al. 2000; Vanmechelen et al. 2000; Sjogren et al. 2001; Hu et al. 2002).

Clearly, a combination of several biomarkers will bestow higher sensitivity and specificity for differential diagnosis of AD. The identification of novel peripheral fluids-based biomarkers and development of better imaging techniques is currently of the highest importance for improvement of diagnostics of AD especially at the early stages of the disease. Use of quantitative biomarkers as important measures is recently reflected in the novel screening criteria, where diagnostics of neurodegenerative disorders critically relies on biomarkers from peripheral fluids, which may precede the pathological changes seen *via* imaging or neuropsychological examination (Lewczuk et al. 2017).

In such a situation the miRNAs are recently emerging as a promising and entirely novel class of biomarkers for diagnosis of AD. The immanent feature of miRNAs, their ability to transfer epigenetic information intercellularly, a specific regulation of protein translation in target cells and tissues provides added value to these biomolecules.

Nucleic acid based diagnostics of Alzheimer's disease – miRNA

Biogenesis and basic features of miRNAs

miRNAs are class of non-coding RNAs which regulate gene expression on post-transcriptional level. Biogenesis of miRNA starts in cell nucleus, where miRNA-coding sequences are transcribed by RNA polymerase II or III to primary transcripts (pri-miRNAs) (Lee et al. 2004). These pri-miRNAs have stem loop structure with 5' cap and 3' polyA tail and typically encode one or several different miRNAs. pri-miRNA is digested by enzyme complex DGCR8-Drosha to 60–70 nucleotides long stem loop precursor miRNA (pre-miRNA) (Gregory et al. 2004). Precursor miRNA is transported to the cytoplasm by Exportin-5-Ran-GTP complex and further digested by Dicer to miRNA duplex (21–23 nucleotides) (Lund et al. 2004). Cleaved duplex is incorporated into inactive RISC complex (Dicer-Ago2-TRBP) where 3'-5' miRNA strand is being degraded while mature 5'-3' miRNA strand becomes a part of the active RISC complex which binds to the target mRNA.

Based on the level of complementarity of mature miRNA to the target mRNA it can either inhibit translation of target mRNA or lead to degradation of miRNA:mRNA complex. Incomplete base pairing between miRNA and target mRNA at dominant positions (miRNA nucleotides 2–8 and 13–16) leads to inhibition of protein synthesis on ribosomes, while complete base pairing blocks translation as well as induces deadenylation of mRNA that causes faster degradation of mRNA prior effective translation into the protein.

MicroRNAs as diagnostic molecules are studied throughout many disciplines in human medicine. In the field of oncology, miRNAs have already been proposed to be used as prognostic biomarkers for certain types of leukemia (Avigad et al. 2016) and prostate cancer (Sharova et al. 2016). Furthermore, diagnostic test based on detection of 8 miRNA panel is currently available for classification of the four main types of lung cancer (Gilad et al. 2012).

Advantage of miRNAs is their good stability in peripheral fluids. This protection against degradation in RNAses-rich environment is secured by their encapsulation in vesicles, preservation by Ago2 proteins and lipoproteins (Arroyo et al. 2011). It was found that endogenous plasmatic miRNAs are more stable compared to exogenous miRNAs added to the plasma (Tsui et al. 2002).

miRNA as candidates for AD diagnostics

Normal brain development and its function depend on miRNAs which maintain balance between transcriptome and proteome. Brain-specific miRNAs are important for neuronal development, synaptic plasticity, and they affect physiological processes associated with neurodegeneration (Bian and Sun 2011). miRNAs are deregulated in AD. The first study on abnormal miRNAs expression in AD pathology was published in 2007. miR-9, miR-125b and miR-128 were up-regulated in the hippocampus of AD patients compared with age-matched control subjects (Lukiw 2007).

Body fluids represent easily accessible and preferred source for analysis of potential biomarkers for AD. miRNAs from the brain can pass through blood-brain barrier to periphery where they signalize changes that occur in the central nervous system during the disease (Karnati et al. 2015). Number of studies have reported miRNAs as stable degradation-resistant in body fluids and their expression profiles reflect changes under different physiological and pathological conditions (Carlsen et al. 2013; Katoh 2014; Kumar et al. 2013). They can be useful as non-invasive biomarkers in diagnosis, prognosis, and response to the treatment. Typical sources for analysis of circulating miRNAs are CSF, plasma, blood serum, saliva and urine. The blood-derived fluids and CSF are the well-studied in the field of neurodegenerative disorders. Stability of circulating cell-free miRNAs is very high since they can be protected by ectosomes, exosomes and apoptotic bodies, or associated with lipoproteins or Argonaute protein family which make them resistant to RNases in the extracellular environment (Turchinovich et al. 2011). Serum miRNAs are stable after being exposed to the conditions that would normally degrade RNAs, such as low or high pH levels, high temperature (Cortez et al. 2011). Collection of CSF is an invasive procedure and could be traumatic for patients. During the process of collection, CSF can be contaminated with blood

which could potentially affect the level of specific miRNAs, but on the other hand expression profiles of many miRNAs are stable and contamination with the blood cells have been shown to have a minimal impact (Muller et al. 2014). An advantage of CSF is that it contains more brain-specific miRNAs than blood as it is in direct contact with the brain tissue. However, the miRNA concentration in CSF is lower than in blood serum or plasma. On the other hand, collection of the blood from the patients is much more convenient and standardized, which makes blood a preferable body fluid in the research of biomarkers.

AD is characteristic by dysregulation of many miRNAs which are involved in the regulation of the expression of tau protein, amyloid-β production through processing of APP with BACE1, in oxidative stress, apoptosis and other pathways associated with AD. In some cases the expression of miRNAs is regulated by epigenetic modifications such as DNA methylation and histone modification, which change with aging (Fraga 2009; Chouliaras et al. 2011). Hypermethylation and hypomethylation of miRNA promoters alter their expression level and changes in the DNA methylation were observed in many neurodegenerative diseases, including the AD (Van den Hove et al. 2014).

Candidate miRNA molecules that were identified so far as dysregulated in peripheral fluids of AD patients are listed in Tables 1 and 2.

MiR-107 is one of the candidate molecules eventually suitable for AD diagnostics. Studies analysing CSF and temporal cortex from AD patients reported significant decrease of miR-107 in comparison with controls (Nelson and Wang 2010; Wang et al. 2008). Expression of this miRNA was measured in 4 different groups of patients: elderly non-demented with negligible AD-type pathology, non-demented with incipient AD pathology, mild cognitive impairment with moderate AD pathology and AD patients. The results showed a significant down-regulation of miR-107 in the early stage of the disease (Wang et al. 2008). Expression levels of miR-107 reflect the progression of the disease which makes it a potential marker for classification of AD stages.

Compared to the single miRNAs being identified as potential biomarkers the strategies for higher resolution were designed using miRNA panels as better predictors with higher specificity and sensitivity to distinguish AD from other neurodegenerative or neuropsychological diseases.

Discrimination analysis of 1178 miRNAs in the CSF of 22 AD patients and 28 controls identified panel of three miRNAs (miR-100, miR-103 and miR-375) that were in combination with CSF protein markers able to positively classify controls and AD patients with 96.4% and 95.5% accuracy (Denk et al. 2015).

In another study, a panel of four candidate miRNAs (miR-31, miR-93, miR-143 a miR-146a), suitable for differentiating AD and vascular dementia was identified (Dong et al. 2015). Based on the analysis of blood sera from 127 AD patients, 30 patients with mild cognitive impairment and 30 patients with vascular dementia it was observed that AD subjects had significantly lower expression level of this miRNA panel. Expression level of miR-93 and miR-146a were significantly elevated in MCI patients compared to healthy controls (Dong et al. 2015).

Whole genome analysis of miRNAs in the blood sera from 158 AD patients and 155 healthy controls identified a panel of significantly down-regulated miRNAs (miR-98-5p, miR-885-5p, miR-483-3p, miR-342-3p, miR-191-5p, let-7d-5p) in patients with AD. This panel of 6 miRNAs showed 80.6% sensitivity and 68.3% specificity (Tan et al. 2014b). However, the miR-342-3p alone had 81.5% sensitivity and 70.1% specificity. Notably, in this case the panel doesn't bring higher resolution when compared to individual miRNA profile.

Panel of miRNAs from the family miR-132 (miR-128, miR-132, miR-874) and miR-134 (miR-134, miR-323-3p, miR-382) was identified for diagnosis of early AD with mild cognitive impairment. Analysis of the biomarkers from the miR-132 family reached specificity of 96–98% and sensitivity of 84–94%, while those from miR-134 family reached sensitivity of 76–88% and specificity of 80–90%. Combination of all three miRNAs from miR-132 family reached high sensitivity and specificity – 96% for both. Analysis of three miRNAs from the miR-134 family reached 80% sensitivity and 94% specificity. Interestingly, identified miRNAs successfully detected cognitive changes in the majority of patients in the pre-symptomatic state of the disease, i.e. 1–5 years before the clinical diagnosis (Sheinerman et al. 2013).

Another study identified a panel of seven miRNAs (let-7d-5p, let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p and miR-545-3p) that was able to differentiate AD patients from normal controls with more than 95% accuracy. Circulating plasma miRNA expression was measured in 11 AD and 20 control patients. In the first step 654 human miRNAs were measured, out of which 12 miRNAs had differential expression in AD samples. Out of these, down-regulation of seven miRNAs was confirmed using qPCR. Further validation was done on independent cohort of 20 AD and 17 control patients, resulting in positive correlation across the two independent cohorts (Kumar et al. 2013). However, validation of data using larger cohort would add more confidence to these findings.

Profiling of peripheral samples from AD patients revealed frequent appearance of dysregulated miR-29 family members. mir-29a and miR-29b were identified as down-regulated in blood serum and mononuclear cells and up-regulated in CSF samples (Geekiyanage et al. 2012; Villa et al. 2013; Jia and Liu 2016), while miR-29c is under-expressed in CSF of AD patients (Sorensen et al. 2016).

On the other hand, higher expression levels of miR-34a and miR-34c is present in peripheral blood mononuclear cells of the AD patients, while miR-34c also showed elevation in the plasma of AD patients in comparison with controls. Analysis of miR-34c in plasma samples of 25 AD patients and 27 age-matched controls revealed 92% sensitivity and 96% specificity, which was higher when compared to miR-34a from blood mononuclear cells from 78 AD patients and 85 age-matched normal elderly controls (sensitivity 84% and specificity 74%). Lower sensitivity and specificity of miR-34a when compared to miR-34c could be related to the 3-times larger patient cohort in the miR-34a study (Bhatnagar et al. 2014). This example clearly illustrates the need for better designed and larger studies, where biological variability is clearly reflected and not affected by small group statistics.

Expression of miR-125b was reported as increased in CSF in AD (Kiko et al. 2014; Dangla-Valls et al. 2017), and the levels were not influenced by blood contamination of CSF (Muller et al. 2016). In the serum of patients with AD the expression of miR125b was significantly lower in 3 different studies (Galimberti et al. 2014; Tan et al. 2014a; Jia and Liu 2016). Analysis of miR-125b alone reached specificity up to 68.3% and sensitivity of 80.8% (Tan et al. 2014a). Values of sensitivity and specificity were similar in the analysis of miR-125b with miR-181c, but diagnostic value of miR-125 alone was better. In pair of miR-125b with miR-9 the sensitivity was 80% and specificity 71.4% (Tan et al. 2014a). As of now, different studies on levels of miR-125b from more 200 AD patients have arrived on similar conclusion, suggesting that miR-125b can be considered as reliable peripheral miRNA biomarker for AD.

Interestingly, miR-128 over-expression was observed not only in blood monocytes, but also in lymphocytes of AD patients (Tiribuzi et al. 2014), suggesting common immunological alterations in peripheral blood cells in AD. Similar correlation was also identified for miR-455 with up-regulated levels in serum and blood B-lymphocytes of AD patients (Kumar et al. 2017; Kumar and Reddy 2018).

Unfortunately studies that perform analysis of patients suffering from various neurodegenerative diseases such as AD, multiple sclerosis, Parkinson disease, depression, bipolar disorder and schizophrenia are rare. In a particular study by Leidinger et al. (2013), the next-generation sequencing of miRNAs from blood samples of 48 AD patients and 22 controls was employed. Initially, 140 miRNAs were deregulated with 82 miRNAs found to be over-expressed and 58 miRNAs under-expressed in AD. Out of these, a panel of twelve miRNAs was selected for qRT-PCR validation using a larger cohort of 202 samples that included also patients with other CNS disorders. The cohort included 94 AD, 18 MCI, 16 multiple sclerosis, 9 Parkinson disease, 15 major depression, 15 bipolar disorder and 14 schizophrenia patients. miRNAs miR-112, miR-161, let-7d-3p, miR-5010-3p and miR-151a-3p were upregulated and miR-103a-3p, miR-107, miR-532-5p, miR-26b-5p, miR-26a-5p, miR-1285-5p and let-7f-5p were found to be downregulated. While 10 of the 12 miRNAs have already been annotated in the miRBase, two miRNAs, brain-miR-112 and brain-miR-161, were newly discovered. This 12-miRNAs panel was shown to differentiate AD and control subjects with a specificity of 95% and a sensitivity of 92%. The differentiation of AD from other neurological diseases is possible with accuracies between 74% and 78% (Leidinger et al. 2013).

Challenges in field of miRNAs in Alzheimer's disease

Currently, the most frequently used methods for detecting circulating cell-free miRNAs are next generation sequencing (NGS), hybridization methods and quantitative real time PCR (qPCR). Initially, candidate miRNA biomarkers are usually identified by using large scale screening such as, NGS or qPCR arrays. Using these methods expression level of several miRNAs can be analysed in a single experiment. miRNAs, previously found as dysregulated, are typically, but not always validated by individual qPCR assays. The rate of validation of NGS hits using qPCR is often poor. This phenomenon is present often due to the problem of finding a suitable endogenous control for normalization of miRNA qPCR data. Since there are no guidelines, the sample cohorts are being normalized to various amplified miRNAs, spike-in controls, amplification means or combinations of all mentioned before. This leads to controversies such as following: the studies found miR-125b up-regulated in AD CSF (Alexandrov et al. 2012; Muller et al. 2016; Dangla-Valls et al. 2017), however, two other studies identified the same miR-125b as downregulated in the same body fluid (Galimberti et al. 2014; Kiko et al. 2014). Another example of contradictory data comes from miR-9 that was described as up-regulated in AD CSF by two studies (Alexandrov et al. 2012; Lukiw et al. 2012), but also as non-detectable and was proposed as a marker for AD, since it is present only in healthy individuals (Riancho et al. 2017). This lack of reproducibility is linked to many variables in the study design and workflow. It can be due to limitation in size of population, different analytical methods used, different normalisation controls employed and characteristic of the study population such as age, ethnicity, stage of the disease and selection of adequate controls.

From a research point of view it is good to have broad robust data available, but for the development of diagnostic test there is an urgent need to standardize laboratory procedures for studies evaluating miRNA expression using different platforms and integrate the data into standardized meaningful outcomes. Many small-size cohort studies in the

Table 1 concluded particular miRNAs as potential biomarkers, however, in many cases these data were not confirmed by other research studies. Therefore, it should be noted that larger sample cohorts and well defined validation studies that repeatedly identified individual miRNAs or miRNA panels bring the most relevant and robust information on miRNAs to be considered as a diagnostic biomarkers for AD.

Recent publications enroll substantially increased number of patients in the research, and thus the informative value of such analyses is significantly higher. Studies on down-regulated miR-223 in serum with 84 AD and 62 controls (Jia and Liu 2016) or miR-639 in blood mononuclear cells with 45 AD and 41 control individuals (Ren et al. 2016) could serve as examples showing higher confidence.

Interestingly, more than 66% of reviewed research articles focused on analysis of miRNA in peripheral blood including serum, plasma and blood cells, representing the shift from the CSF as privileged source of biomarkers for CNS disorders. This is most probably due its sample accessibility. In some cases, the sensitivity and specificity reached very high "desired" values, but still there is no validated miRNA-based analytical method currently available for differential diagnostics of any neurodegenerative disease including AD.

Conclusion

miRNAs represent potential biomarkers for differential diagnosis of human diseases. Based on the current knowledge it is highly probable that diagnostics of AD using a single miRNA test will not be sensitive and specific enough. Therefore, a panel of multiple miRNAs will be more appropriate to provide precise information for clinical diagnosis. The "added value of miRNAs" is their direct link to the molecular mechanisms operating during the pathogenesis. This can be employed to solve a major challenge in the quest for early diagnostics and efficient treatment of AD: the existence of comorbidities in AD patients and combined neurodegenerative processes which could be developed in parallel. To solve this major task, we need to detect how miRNAs function is changed over the time in a cohort of cognitive healthy subjects and AD patients. To make sure that early changes are observed, such a study should start before the onset of clinical symptoms and finished by post-mortem analysis while molecular analyses of samples at different stages of disease should be evaluated in context with clinical symptoms.

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