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Characterization of circulating microRNAs as potential biomarkers for dementia

Dissertation

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TABLE OF CONTENTS

1 Synopsis

1.1 Alzheimer’s disease

1.1.1 Epidemiology and risk factors

In 1906, Alois Alzheimer, a Bavarian psychiatrist, described for the first time a neurological syndrome characterized by progressive memory impairment, disordered cognitive function and altered behaviour now known as Alzheimer's disease (AD) (1). Originally believed to be a rare form of dementia, AD has emerged as the most prevalent form of late-life mental failure in humans (2). While the causes of the disease remain unclear, the consequences for society are devastating. Current estimates suggest that 44 million people live with dementia worldwide, which is further predicted to triple by 2050 as the population ages (3). According to estimates of prevalence rates in Europe, nearly 1.6 million of the elderly suffered from dementia in Germany at the end of 2014, with about two thirds affected by AD (4). As the primary risk factor, age plays a decisive role in the onset of AD so that prevalence rates increase steeply with age while the number of patients doubles at intervals of about five years after age 65 (5). Family history (genetics) and environmental factors are further risk factors that play an important role. Here, mutations in the amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2) cause the familial, early-onset form of AD (6). In contrast, mutations of the apolipoprotein E-e4 (APOE4) gene increase the risk of developing the sporadic, late-onset form of AD (6). Finding the causes of the sporadic form of Alzheimer's disease is difficult. Since many influencing factors can play a role, risk factors are identified by means of epidemiological investigations, that is, by means of complex statistical procedures. Most prominent associations are a decreased

reserve capacity of the brain, including reduced brain size, low educational and occupational attainment, low mental ability in early life, and reduced mental and physical activity during late life (7). However, whether there is a causal relationship between the disease and these risk factors is often unclear.

1.1.2 Pathophysiology

There are a variety of different hypotheses describing the nerve cell death and the associated cognitive decline in AD. These include, for example, genetics, oxidative stress, dysfunctional calcium homeostasis, hormonal, inflammatory-immunological, vascular and cell-cycle dysregulation (8). However, the most proposed pathogenic mechanisms are the I) amyloid cascade hypothesis along with the II) tau hyperphosphorylation.

The amyloid hypothesis suggests that amyloid plaques are formed by aggregated amyloid-beta (Aβ) peptides that in turn are generated by proteolytic cleavage of APP (Fig. 1) (9). APP is a large type-1 transmembrane protein, which is constitutively expressed in many cell types (10) but at high levels in the brain and metabolized in a rapid and highly complex fashion by a series of sequential proteases, including the intramembranous γ-secretase complex (11). In the α-secretase pathway, α-secretase cleaves APP within the Aβ domain, releasing a large soluble APP fragment without production of Aβ. Instead, in the pathogenic condition, the β-secretase pathway is activated, causing the β-site APP cleaving enzyme (BACE1) to primarily cleave APP and to generate a membrane bound soluble C-terminal fragment (12). A subsequent cleavage of the C-terminal fragment by the γ-secretase activity further generates $A\beta_{40}$ and $A\beta_{42}$. Under normal conditions, brain Aβ is degraded by peptidases and cleared from the brain in a balanced process of in- and efflux (7). Under pathogenic conditions, these peptides aggregate into soluble oligomers and larger insoluble fibrils in plaques

outside the cell leading to inflammation, oxidative stress, synaptic dysfunction and neuronal cell death as depicted in Fig. 1a. However, the hypothesis is challenged by the fact that some older people may have amyloid deposits but display normal or near-normal cognitive function (13). In addition, APP constructs that have been introduced into the mouse genome lead to cognitive decline but most of the classic AD-associated pathologies do not develop. This relevant evidence suggests that amyloid alone may not be decisive for the disease.

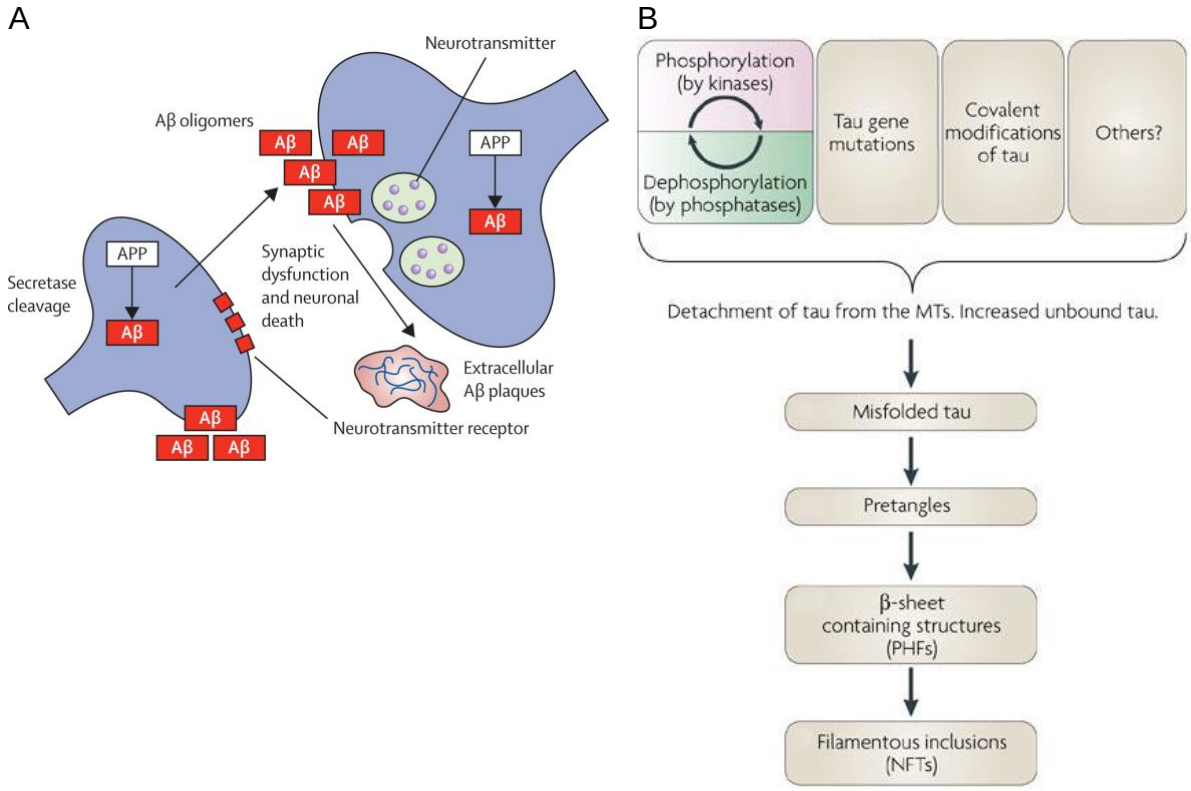

Fig. 1 Pathogenesis of Alzheimer's disease. A) Amyloid cascade hypothesis and **B)** Pathological aggregation of tau. Adapted from A) Ballard, C., et al. (2011). "Alzheimer's disease." Lancet 377(9770): 1019-1031 (14) and B) Ballatore, C., et al. (2007). "Tau-mediated neurodegeneration in Alzheimer's disease and related disorders." Nat Rev Neurosci 8(9): 663-672 (15).

In contrast, the tau hypothesis states that abnormal phosphorylation of the microtubule-associated protein (MAP) tau results in the transformation of normal adult tau to aggregated, paired helical filaments (PHFs), which manifest as neurofibrillary tangles (NFTs) in the neuronal soma (Fig. 1b) (8). The primary function of tau, which is particularly abundant in the axons of neurons, is to stabilize microtubules (15).

Microtubules are tubular filaments of proteins which, together with the intermediary filaments and the actin filaments, form the cytoskeleton of eukaryotic cells. In total, there are six major isoforms of tau expressed in the adult human brain, all of which are alternatively spliced from a single gene. In AD brain, all of the six tau isoforms are hyperphosphorylated and aggregated into PHFs (16). Tau hyperphosphorylation starts intracellularly and leads to sequestration of normal tau and other microtubule-associated proteins, which causes disassembly of microtubules and thus impaired axonal transport, compromising neuronal and synaptic function (7). However, the structural transitions from the native conformation of tau to its neurotoxic polymers remains to be elucidated.

In general, AD is considered a multifactorial disorder in which protein alterations, oxidative stress, neuroinflammation, immune deregulation, impairment of neuronal−glial communication, and neurotoxic agents trigger neuronal degeneration, and the balance among these factors may vary from patient to patient. Whether amyloid plaques and/or tau hyper phosphorylation are causes or consequence of AD is unknown. This, however, prompts researchers to seek alternative explanations for the cause(s) of human AD. One promising new field is epigenetics, which in addition to the traditional genetic basis for inheritance, deals with mechanisms that affect gene expression without modifying the genome. Being subject of this work, one prominent epigenetic process is RNA interference in which RNA molecules such as microRNAs (miRNAs) inhibit gene expression or translation by specifically targeting different mRNA molecules.

1.1.3 Neurochemical biomarkers

The term “biomarker”, an acronym for “biological marker”, refers to a broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly (17). The World Health Organization (WHO) in coordination with the United Nations and the International Labor Organization, has defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (18).

The core neurochemical biomarkers for AD are the proteins amyloid-beta$_{1–42}$ (A$\beta_{1–42}$), total-tau (t-tau), and phospho-tau$_{181}$ (p-tau), which are measured in cerebrospinal fluid (CSF) respectively (19). CSF is produced by the choroid plexus, a secretory tissue located in the ventricular system of the brain, and can be sampled during a lumbar puncture (20). A review by Blennow et al., 2010 showed that lower levels of A$\beta_{1–42}$ and higher levels of t-tau and p-tau, and especially a high ratio of t-tau/A$\beta_{1–42}$ or p-tau/A$\beta_{1–42}$, are found in patients with AD compared to patients with FTD or normal controls, with a sensitivity and specificity reaching 85–90% (21). However, many more studies have examined the diagnostic quality of these and other biomarkers. A recent meta-analysis by Olsson et al., 2016 proved the CSF core markers and CSF NfL (the light protein of neurofilament) as strongly associated with AD and mild cognitive impairment (MCI). MCI is considered an intermediate stage between the expected cognitive decline of normal aging and the more-serious decline of dementia (22). Due to their consistency, Olsson et al. hypothesised to use these markers in clinical practice and clinical research. However, they also tested blood-based biomarker and reported that plasma t-tau was the only blood biomarker that discriminates patients with Alzheimer’s disease from controls while this did not apply to plasma levels of A$\beta_{1–42}$ and A$\beta_{1–40}$. A

comment to that study pointed out that, nevertheless, CSF biomarkers have to be interpreted together with data from a full medical assessment of the patient. And the authors added that the overlap in pathology between Alzheimer's disease and other neurodegenerative disorders, and the high proportion of cognitively normal elderly patients with Alzheimer's disease-like changes may preclude CSF protein biomarkers from achieving a specificity of 100% (23).

Taken together, the comprehensive meta-analysis and other studies show that traditional CSF protein biomarker are far from being an ideal choice and that most blood-based (protein-) marker do not reach statistical difference. Consequently, alternative biomarkers in CSF and especially in the blood are of great interest to improve both the diagnostic performance for the classification of AD (and MCI) and to provide less invasive sampling strategies with respect to blood.

1.2 Frontotemporal lobar Degeneration

1.2.1 Epidemiology

Frontotemporal lobar degeneration (FTLD), also referred to as frontotemporal dementia (FTD), comprises a heterogeneous group of syndromes that is caused by progressive degeneration of the frontal lobes (24). The group consists of the behavioural variant (bvFTD) with patients suffering from disturbances in behaviour and changes in personality. And the primary progressive aphasia (PPA) including the non-fluent (nfvPPA), semantic (svPPA) and logopenic (lvPPA) variants that are associated with deficits in language (25). The corticobasal syndrome (CBS), progressive supranuclear palsy (PSP) and FTD with amyotrophic lateral sclerosis (FTD-ALS) extend the FTLD spectrum. The average age of onset is between 50 and 60 years, however, the range is very large (20 – 85 years). Clinically, bvFTD is the manifesting

syndrome of FTLD in 50–57% of autopsy-confirmed cases, whereas roughly 40% of autopsy-confirmed FTLD cases are PPA (26). The primary motor manifestations of FTLD (CBS, PSP and MND) are seen as the initial symptoms in less than 5% of cases. Compared to AD, FTLD is much rarer and it is estimated that about 3 - 9% of all dementia patients suffer from FTLD (for comparison: about 70% of all dementias are caused by Alzheimer's disease) (27). Reported prevalence rates for bvFTD and PPA range from 10-30 / 100,000 whereas PSP and CBS are even less frequent with 1-5 / 100,000 (28). In general, epidemiologic data suggest that FTLD is a common cause of early-onset (age <65 years) dementia, with an incidence and prevalence similar to Alzheimer's disease (29). Median survival in FTLD has been estimated at 6–11 years from symptom onset and 3–4 years from diagnosis (29). Overall, survival is shorter and cognitive and functional decline are more rapid than in Alzheimer's disease. Compared to AD, memory deficits develop late in FTLD whereas changes in behaviour and personality as well as language impairment occur early during the disease. Due to the increased prevalence, the focus of this work is on the behavioral variant of the FTLD spectrum.

1.2.2 Genetics and pathophysiology

Currently, there are no known non-genetic risk factors for FTLD. In fact, the FTLD spectrum exhibits a strong genetic component as demonstrated in Fig. 2a. Nearly 40% of cases are familial, while 10-15% are caused by an autosomal dominant pattern of inheritance (26). As demonstrated in Fig. 2a (lower part), these FTLD families can be explained predominantly by known mutations in C9 open reading frame 72 (*C9orf72*), the progranulin gene (*GRN*) and the gene for microtubule-associated protein Tau (*MAPT*) (26) as well as TANK-binding kinase 1 (*TBK1*), which, however, occurs less frequent (30).

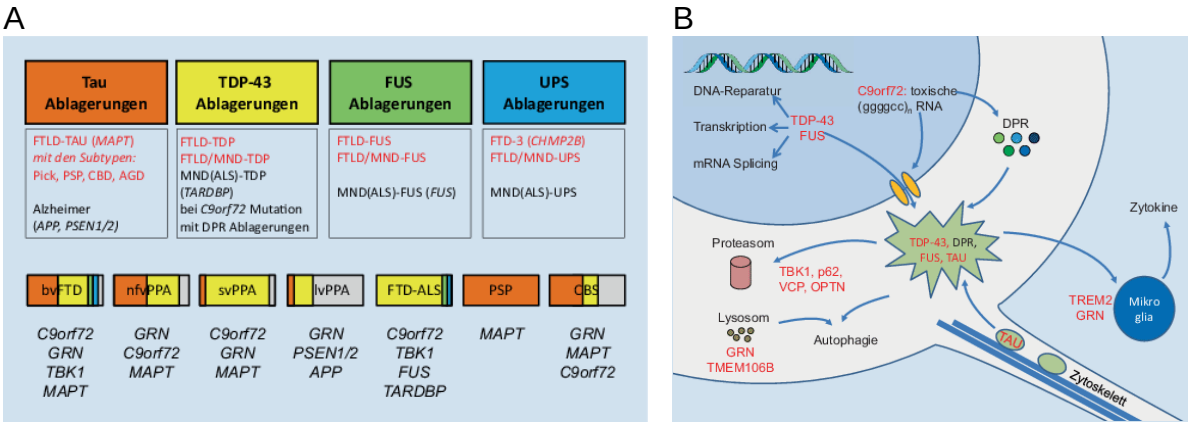

Fig. 2 Neuropathological classification of frontotemporal lobar degeneration (FTLD). A) Deposits of tau, TDP-43 or FUS are mostly found in the cytoplasm. In rare cases, deposits can detected with markers for the Ubiquitin Proteasome System (UPS). The frequency of tau, TDP-43, FUS and UPS deposition (color code see deposits) varies significantly according to the clinical diagnosis. Genetic causes of FTD subtypes are sorted by frequency. **B)** Molecular Mechanisms of FTLD. Scheme of a neuron with nucleus and axon. Adapted from Burger, K., et al. (2017). "Pathomechanisms and clinical aspects of frontotemporal lobar degeneration." Nervenarzt 88(2): 163-172 (28).

The neuropathology of FTLD is complex and the aggregation of proteins is a key feature that, due to recent advances in neuropathological studies, enables the classification of FTLD according to the type of protein found in the pathological aggregates in post-mortem brain tissue. These protein aggregates predominantly consist of Tau (FTLD-Tau), TDP-43 (FTLD-TDP), to a lesser extent of FUS (FTLD-FUS) or the very rare UPS (Fig. 2a upper part), and occur with different frequencies across the FTLD subtypes (Fig. 2a lower part).

Considering Tau, six isoforms exist as described earlier in section 1.1.2. However, three isoforms have three microtubule binding repeats (3R), and three of which have four repeats (4R) (31). This is especially important since Tauopathies are subclassified according to the predominant species of tau that accumulates. Approximately one-half of all patients with bvFTD have tau aggregates: 3R tau in Pick's disease, and 4R tau in PSP and CBD (32).

In contrast, TDP-43 protein is also found in approximately one-half of bvFTD cases on histological examination, and is seen in all cases of FTD-ALS (32). In addition, there are three major patterns of TDP-43 pathology: Type A, Type B, and Type C, which

correlate with different forms of FTD but won't be described in further detail. Importantly, mutations in the TDP-43 gene itself rarely cause FTD, usually with ALS. The two more common genetic mutations associated with TDP-43 pathology are *GRN* and *C9orf72.* In turn, a smaller proportion of patients with FTD have pathology without TDP-43 or tau aggregates (5%). The majority of these cases have *FUS* protein deposit (33).

In addition to the aggregation of proteins, other mechanisms that contribute to the pathogenesis of FTLD are described (Fig. 2c), since in most patients, proteins aggregate in the absence of a mutation. To give an example, genetic mutations affecting autophagy receptor proteins (ubiquilin-2, optineurin, SQSTM1/p62) and regulators (VCP) may impair clearance of autophagy substrates with pathological consequences (34). Furthermore, hexanucleotide expansion in the *C9orf72* gene was found to be implicated in many cases of familial and sporadic ALS and FTD (35). The extended repeat RNA found in *C9orf72* patients is highly concentrated in tiny foci in the nucleus, where it is likely to sequester many RNA-binding proteins, which in turn may impair neuronal RNA metabolism and gene expression (36).

1.2.3 Neurochemical biomarkers

The diagnosis of FTLD syndromes is currently only based on clinical symptoms and hampered by the great overlap of the clinical manifestation within the FTLD subtypes and with other types of dementia (e.g. AD) or movement disorders. It is even assumed that 10-30% of FTD patients are wrongly diagnosed and rather suffering from AD (37). As described earlier, changes in protein metabolism or function seem to play a pivotal role in the pathogenesis of FTLD, so that many studies have been performed to investigate proteomic alterations in CSF of FTLD patients compared with controls,

other neurodegenerative diseases or within FTLD subgroups (38). In fact, the AD biomarkers Tau, pTau and $A\beta_{1–42}$ are the most intense studied proteins in CSF of FTLD patients, usually in the context to test their selectivity for AD and differentiate AD from other dementias (39). Using different combinations of these biomarkers and including NfL, sensitivities of 61-86% and specificities of 92-100% have been reported to separate AD from FTLD (40). Other proteins such TDP-43, progranulin but also growth factors and neuropeptides have been investigated in CSF but the AD core biomarkers as well as NfL are still the most promising biomarker candidates for the differential diagnosis of FTLD as recently reviewed by Oeckl et al., 2016 (40). However, contradictory results have been reported (41) showing that protein-based biomarkers in the CSF can be problematic and require further analysis.

In contrast, investigations on blood-based biomarker for FTLD are limited and contradicting results exist as well. Using ELISA, it has been shown that total TDP-43 concentrations in blood plasma are increased in clinical FTLD patients compared to AD patients and healthy controls (42). Conversely, a different study has reported a significant decrease in plasma total TDP-43 of FTLD-TDP patients with genetic confirmation (43). The same lack of difference was found when comparing plasma levels of neuropathological confirmed FTLD patients with AD patients or in the comparison between FTLD pathological subtypes (44). Hence, more effort is needed to evaluate alternative non-protein biomarkers such as circulating miRNAs towards their diagnostic value and to shed light on the identification of the underlying proteinopathy that causes the various FTLD syndromes.

1.3 Role and properties of microRNAs

1.3.1 Biogenesis and function

MiRNAs are a diverse class of small (~ 22 nts), noncoding RNAs that selectively bind messenger RNA (mRNA) to regulate its activity at the post-transcriptional level (45). This function is considered an epigenetic mechanism that can sustainably influence the expression of genes in a cell and thus lead to alterations in the phenotype. The genomic organization (46) and transcriptional activity (47) of miRNAs is complex. For simplicity, only the canonical pathway of miRNA biogenesis will be described and the enzymatic steps explained that are required to produce a functional miRNA (Fig. 3). The initial transcription of miRNA genes is mediated by RNA polymerase II, which results in the expression of a primary miRNA (pri-miRNA) with several kilobases of length (48). As a consequence, the pri-miRNAs fold into hairpins, and consequently act as substrates for two members of the RNase III family of enzymes, Drosha and Dicer (49). Drosha and the double-stranded-RNA-binding protein, DGCR8, are part of the microprocessor complex (50) that initiates the maturation process by cropping the stem–loop of the pri-miRNA to release a small hairpin-shaped precursor RNA of ~65 nucleotides in length (pre-miRNA) (51). The pre-miRNA is then exported to the cytoplasm by Exportin 5 (XPO5) in a Ran-GTP-dependent manner (52). In the cytoplasm, the pre-miRNA is further processed by Dicer and its partner protein TRBP (53), producing a duplex RNA of ~22 nt with its 3′ ends having a two nucleotide overhang. While only the active strand (named the guide strand) is incorporated into the RNA induced silencing complex (RISC), the passenger strand (miRNA*) gets degraded. Thermodynamic features of the duplex appear to play an important role in this decision; the strand with the weakest binding at its 5′-end is more likely to become

the guide strand (54). The RISC consist of Dicer, TRBP, and Argonaute2 and identifies its targets based on complementarity between the miRNA guide strand and the 3'-untranslated region of the mRNA (55). The selectivity to bind and regulate only certain mRNAs is determined by the seed sequence defined by the nucleotides at position 2-8 at the 3'-end of the respective miRNA (56). Specific miRNA binding therefore either lead to endonucleolytic cleavage or translational repression and thus to a downregulation of the targeted mRNA and its protein levels, respectively.

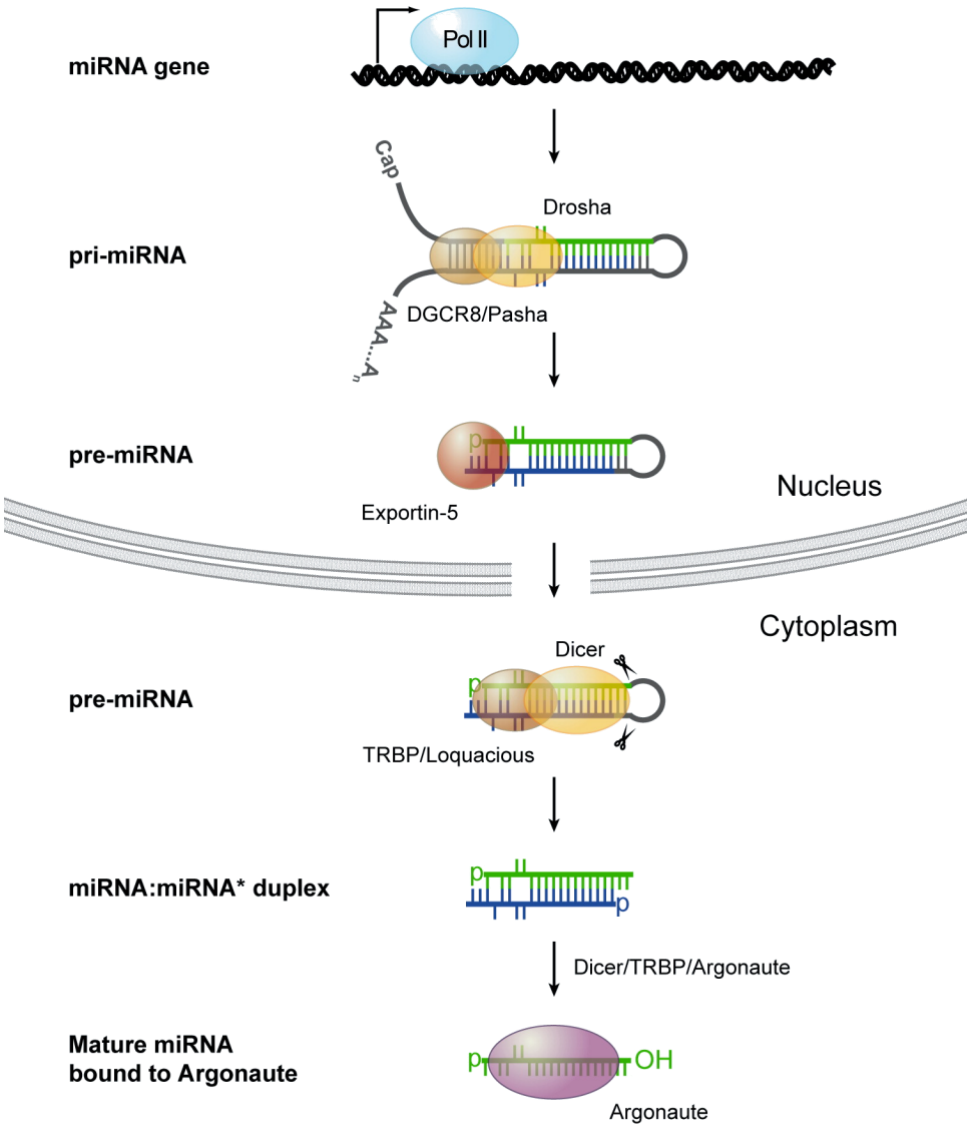

Fig. 3 Biogenesis of miRNA. Adapted from Bushati, N. and S. M. Cohen (2007). "microRNA functions." Annu Rev Cell Dev Biol 23: 175-205 (57)

1.3.2 microRNA characteristics

A uniform naming convention for miRNAs is not only necessary to characterize their diversity, genomic organization or sequence homologies but also important for a better interpretation of transcriptional expression data. Originally called the microRNA Registry (58), the current version of miRBase (http://www.mirbase.org/, version 21.0, accessed 31 January 2018) contains 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products in 223 species. As reviewed by Prichard et al., 2012 (59) the miRNA nomenclature is shown in Fig. 4 and defined as follows: Most miRNAs are named with a species prefix and a number that designates the specific miRNA. For example, hsa-miR-21 indicates Homo sapiens microRNA number 21, as hsa is the prefix for Homo sapiens. Prefixes may also be added to the name to convey information about mature (e.g. miR-16, note upper case "R") vs. miRNA primary transcript (e.g., pri-mir-16 and pre-mir-16). In addition, suffixes are sometimes added to designate whether the mature miRNA arose from the 3' or 5' arm of the pre-miRNA. For example, miR-142-5p designates a mature form based on the capitalization of the 'R' in 'miR' that arose from the 5 prime (i.e.,-5p) arm of the pre-miRNA hairpin. MiRNAs that comprise families that are related in sequence may have lower case letters following the name (e.g. miR-20a and miR-20b). In some cases, multiple transcriptional units at different loci in the genome encode miRNAs that are identical in sequence in their mature form; in this case a numerical suffix is appended by a dash to designate the different genomic origins (e.g. miR-16-1 and miR-16-2). The '*' (star) is appended to some miRNA names (e.g. miR-9*), especially in datasets generated using older miRNA profiling platforms. The designation of '*' is meant to indicate the "minor species" of the two mature miRNAs that are produced from the 3' and 5' arms of the pre-miRNA duplex (i.e. forming the miRNA:miRNA* duplex). It is

now recognized that both the dominant (non-star) and '*' forms can be functional and in some cases may be present at comparable concentrations in the cell, or the miRNA* form might even be at higher concentration depending on the precursor gene used and the cell, tissue, or species being examined. For these reasons, it has been recommended the miRNA/miRNA* nomenclature be dropped in favour of using '-3p' or '-5p' suffix in every case. This is especially important to avoid conflicting results as it may cause confusion when comparing old and new miRNA names. A useful tool that tracks und updates miRNA names is called miRBase Tracker (http://www.mirbasetracker.org/) and provides support towards this problem (60). However, the large amount of information from various RNA sequencing approaches and the associated problems of correct annotation has question the reliability of some signals in miRBase. As a consequence, other databases such as MirGeneDB (http://mirgenedb.org/) based on alternative annotation criteria have been established to minimize the amount of false positive signals (61).

Naming Convention	Meaning	Example(s)
3 letter prefix	Species identification	hsa (human) dme (fruit fly)
pri-mir or pre-mir (note lower case "r")	primary transcript (pri-mir) precursor transcript (pre-mir)	pri-mir-16 pre-mir-16
miR	mature miRNA	hsa-miR-16
-3p or -5p	miRNA originating from the 3' or 5' end of the pre-mir, respectively	hsa-miR-142-3p hsa-miR-142-5p
a or b	miRNAs closely related in sequence and evolutionary origin	hsa-miR-20a hsa-miR-20b
-1 or -2	Identical miRNA sequences that originate from different genomic loci	hsa-miR-16-1 hsa-miR-16-2
miR* (star)	'Passenger strand' (retired after miRBase v16)	hsa-miR-9*

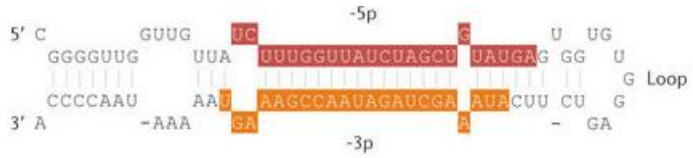

Fig. 4 MicroRNA Nomenclature. Shown is the original miRBase nomenclature and exemplarily a hairpin precursor of the canonical miR-9-5p and miR-9-3p sequences that are highlighted in red and orange, respectively. Adapted from Pritchard, C. C., et al. (2012). "MicroRNA profiling: approaches and considerations." Nat Rev Genet 13(5): 358-369 (59).

1.3.3 Role of microRNAs in dementia

A function of miRNAs during nervous system development in vivo has initially been shown using constitutive Dicer knockout zebrafish. As a consequence, these animals were unable to process pre-miRNAs into their mature counterparts and loss-of-function resulted in severe morphological malformations of the brain and spinal cord (62). In order to assess more specific functions of miRNAs, studies also performed expression profiling of miRNAs during the different stages of neural development. Signals such as let-7, miR-9 and miR-124 were identified as global regulators of neurogenesis (63). MiRNAs have also shown to control of the efficiency of synaptic transmission to several forms of plasticity. Schratt et al., 2006 showed that brain-specific miR-134 is localized to the synaptodendritic compartment of rat hippocampal neurons and negatively regulates the size of dendritic spines, postsynaptic sites of excitatory synaptic transmission, by mediating the inhibition of Limk1 that controls spine development (64). These early studies collectively showed that miRNAs operate in the regulation of neuronal signalling pathways related to the central nervous system.

So far, there have been a number of studies investigating various miRNAs and their possible role in the pathogenesis of AD as demonstrated in Fig. 5. Here, first causal evidence that linked impaired miRNA expression to AD came from a study by Hebert et al., 2008 (65). They identified the miR-29a/b-1 cluster as a potential major suppressor of BACE1 protein expression, where decreased miRNA levels in sporadic AD led to increased levels of BACE1 and consequently to an accumulation of amyloid-beta. Based on microarray analysis, another important study by Wang et al., 2008 demonstrated that levels of miR-107, which is strongly expressed in neurons, were decreased in human cerebral cortical gray matter early in the pathological progression of AD and correlated with increased levels of BACE1 mRNA (66). In turn, other studies

like Hebert et al., 2009 showed that miRNAs belonging to the miR-20a family (that is, miR-20a, miR-17-5p and miR-106b) can also directly regulate APP expression *in vitro* and at the endogenous level in neuronal cell lines (67). In addition, they found a tight correlation of these miRNAs with APP during brain development and in differentiating neurons. Another study by Lehmann et al., 2001 showed that extracellular let-7, a highly abundant regulator of gene expression in the CNS, activated the RNA-sensing Toll-like receptor (TLR) 7 and induced neurodegeneration through neuronal TLR7 (68). They also showed AD patients contained increased CSF levels of let-7b and that extracellular introduction of let-7b into the CSF of wild-type mice by intrathecal injection resulted in neurodegeneration.

There are now several studies that associate deregulated miRNAs with FTLD as well. Buratti et al., 2008 first reported TDP-43 as part of a group of 19 proteins that are specifically associated with the Drosha / DGCR8 microprocessor complex (see 1.3.1) and therefore to the biogenesis of miRNA (69). In a follow-up approach they found impaired expression levels of let-7b and miR-663 following TDP-43 knockdown in cultured cells (70). Another study by Jiao et al., 2010 reported that miR-29b levels led to decreased levels of human progranulin at both the mRNA and protein level and that a knockdown of endogenous miR-29b increased the production and secretion of progranulin in NIH3T3 cells (71). Since progranulin deficiency is thought to cause some forms of FTLD, they suggested miR-29b might be targeted therapeutically to increase progranulin levels in some FTLD patients. A more recent study by Gascon et al., 2014 showed that AMPA receptor (transmembrane receptor for glutamate that mediates fast synaptic transmission) subunits were regulated by the brain-enriched miR-124, whose abundance was markedly decreased in the superficial layers of the cerebral cortex of mice expressing the mutant CHMP2B (72). CHMP2B mutations are a rare cause of autosomal dominant FTLD. They demonstrated that ectopic miR-124 expression in the

medial prefrontal cortex of mutant mice decreased AMPA receptor levels and partially rescued behavioural deficits.

In summary, there is increasing evidence that miRNAs play important roles in the regulation of relevant genes that are directly associated with pathogenic mechanisms in AD and FTLD and may therefore represent important targets for therapeutic treatment options.

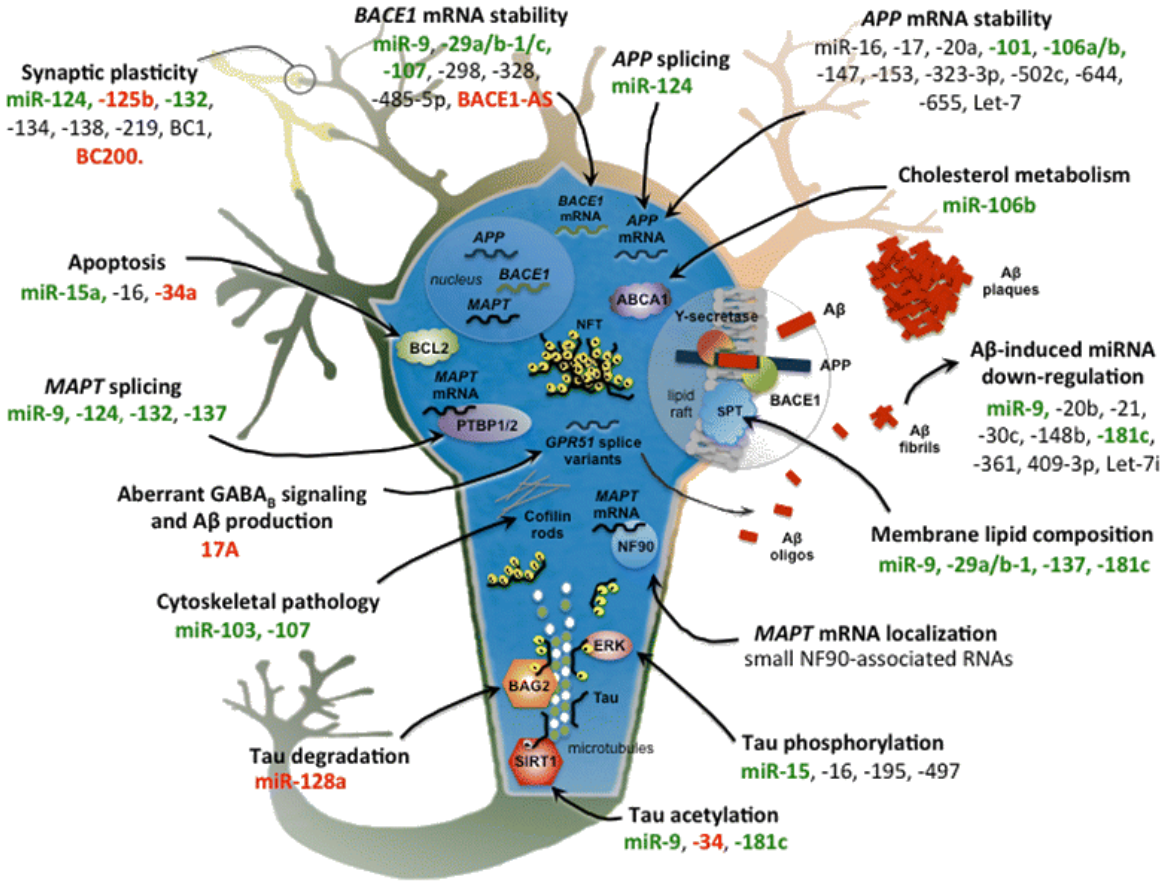

Fig. 5 microRNAs affecting neuronal pathways implicated in AD pathogenesis. Schematic representation of a neuron showing APP processing and amyloid-beta generation (enlarged). MicroRNAs regulating neuronal processes affected in AD are indicated, showing those that are either up-regulated (red) or down-regulated (green) in AD patients. Arrows point to the associated targets shown to be regulated by the specific microRNAs listed. Adapted from: Schonrock, N. and J. Gotz (2012). "Decoding the non-coding RNAs in Alzheimer's disease." Cellular and Molecular Life Sciences 69(21): 3543-3559 (73).

1.3.4 Detection of circulating microRNAs in body fluids

Circulating miRNAs are in many ways promising biomarkers. This is based on the fact that miRNAs 1) are released into circulation (74), 2) can reliably be detected by RT-qPCR (75), which can easily be integrated into a clinical laboratory, 3) show differential stability against physiological confounders such as pH (76), RNAse activity (77, 78) and temperature (freeze-thawing) (79) and 4) show differential expression levels in neurodegenerative diseases (80). A typical workflow for the detection and analysis of circulating miRNAs is illustrated in Fig. 6.

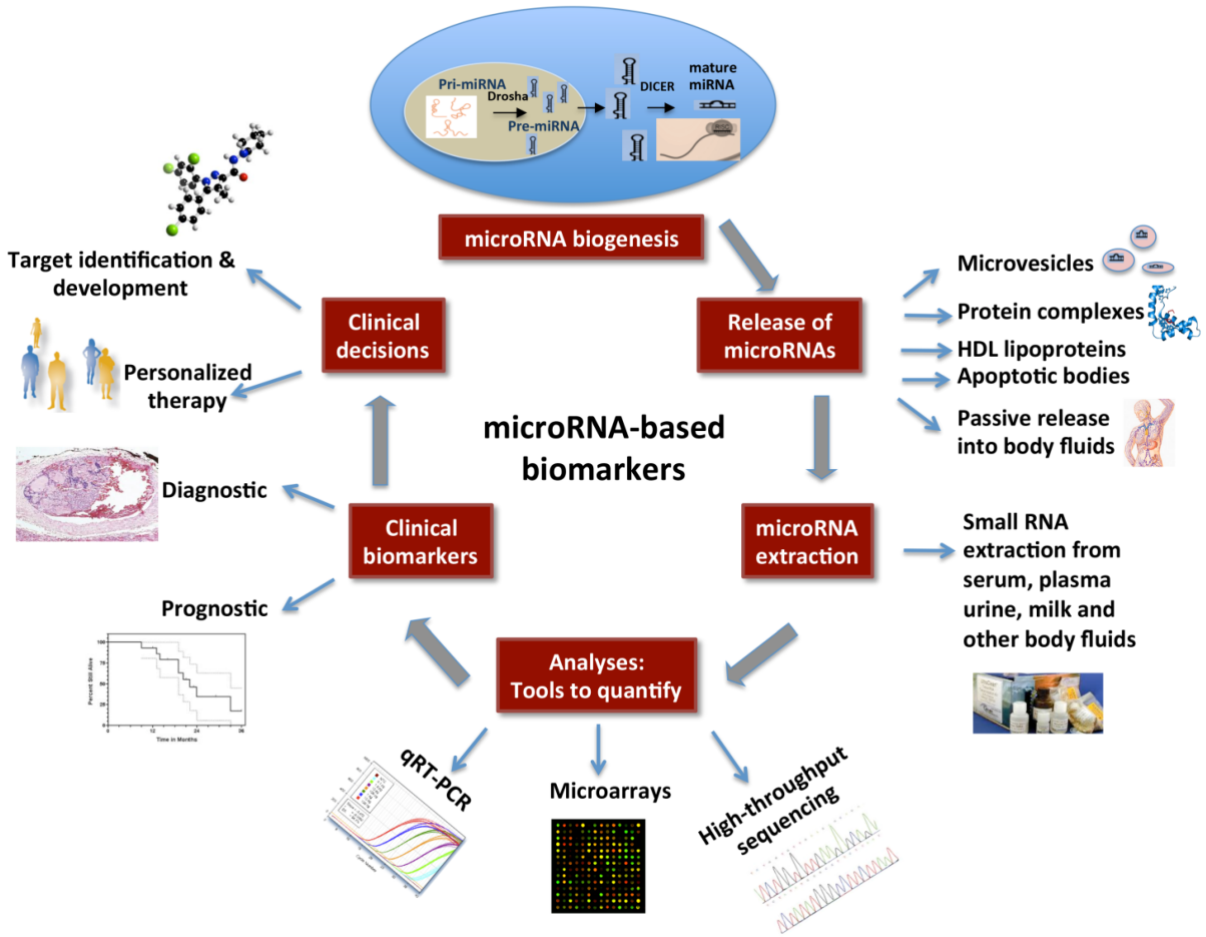

Fig. 6 Workflow to detect and analyse circulating miRNAs as biomarkers in body fluids. Adapted from Steer, C. J. and S. Subramanian (2012). "Circulating microRNAs as biomarkers: a new frontier in diagnostics." Liver Transpl 18(3): 265-269 (81).

As described earlier, the biogenesis of miRNAs results in the intracellular expression of a number of diverse miRNA signals that subsequently control the transcriptional activity of specifically targeted genes. However, there are several mechanisms by which miRNAs can enter the bloodstream. Either passive due to apoptotic or necrotic cells where the RNA transcripts form complexes with specific RNA-binding proteins (for example, miRNAs bind to the AGO2 protein) or active via secretion where the fusion of a multivesicular body with the plasma membrane leads to the release of exosomes (74). These circulating vesicles contain specific miRNAs and are thought to be involved in cell–cell communication (82). As a consequence, miRNAs are present in various body fluids such as blood including plasma and serum, CSF, urine or saliva (83). The first step in a protocol towards detection starts with the extraction of total RNA from these liquid biopsies. A bunch of commercial Kits that are mainly based on traditional single-step extraction using guanidinium thiocyanate-phenol-chloroform according to Chomczynski (84) are available on the market. These kits often include an initial cell lysis followed by a phenol-chloroform phase separation and a subsequent purification using ethanol. According to the literature, the miRNeasy Kit (Qiagen) has proven a reliable way to efficiently isolate RNA including miRNAs from body fluids compared to other methods (85). Towards quantification, many properties that are unique to miRNAs pose challenges to their accurate detection (59). The short sequence (~22 nts) and lack of a poly(A) tail of mature miRNAs preclude the annealing of traditional (universal) primers and a selective enrichment. MiRNAs within a family (for example, the let-7 family) can differ by as little as a single nucleotide, making the ability to discriminate between forms with single nucleotide differences important. In addition, there is a certain degree of sequence length heterogeneity that tends to vary among different miRNAs that are called “isomiRs” (86). However, the large majority of miRNAs typically show only modest length heterogeneity. Finally, the variance in

miRNA GC content leads to a wide variance in melting temperatures (Tm) for annealing reactions, creating miRNA-specific biases in high-throughput approaches. Despite these challenges, three major approaches (Fig. 6) are currently well-established: reverse transcription–qPCR (RT-qPCR), hybridization-based methods such as DNA microarrays and high-throughput sequencing i.e. RNA-seq. A recent study by Mestdagh et al., 2014 evaluated these miRNA profiling platforms in a large quality control (miRQC) study (87). They developed robust quality metrics to objectively assess platform performance in terms of reproducibility, sensitivity, accuracy, specificity and concordance of differential expression as demonstrated in Fig. 7. Overall, the authors stated that each platform has specific strengths and weaknesses. For example, sequencing provides very specific and reproducible results, but is less sensitive for low RNA input samples (e.g. CSF) whereas microarrays seem highly reproducible but generally less accurate and sensitive. In contrast, qPCR generally provides a good combination of all parameters, but with large differences within the individual methods and compared to samples with low RNA input. As a consequence, the TaqMan based OpenArray method (ABI) is a good choice for an initial screening study due to its high sensitivity. In contrast, the SYBR Green based miRCURY system (Exiqon) has performed well in terms of specificity and reproducibility (Fig. 7), parameters that provide reliability and that are of great importance to the area. There is certainly no doubt that next generation sequencing technology will become the leading methodology in miRNA research. Yet, the previously high cost, time and instrumentation requirement have prevented the establishment in laboratory routine. A potential biomarker, however, should be easy, fast, inexpensive and reliable to measure. Since qPCR provides superior sensitivity accompanied by high accuracy and the fact that body fluids generally contain rather small amounts of RNA, qPCR is still considered gold standard in this setting (88). Guidelines such as MIQE (89) have

already been established for qPCR to increase transparency, thus ensuring better comparability of qPCR-based studies. However, several studies do not adhere to MIQE, which complicates an interpretation and challenges the obtained results. Based on these considerations, RT-qPCR was used in accordance with the MIQE guidelines to quantify expression levels of circulating miRNAs in body fluids in this work.

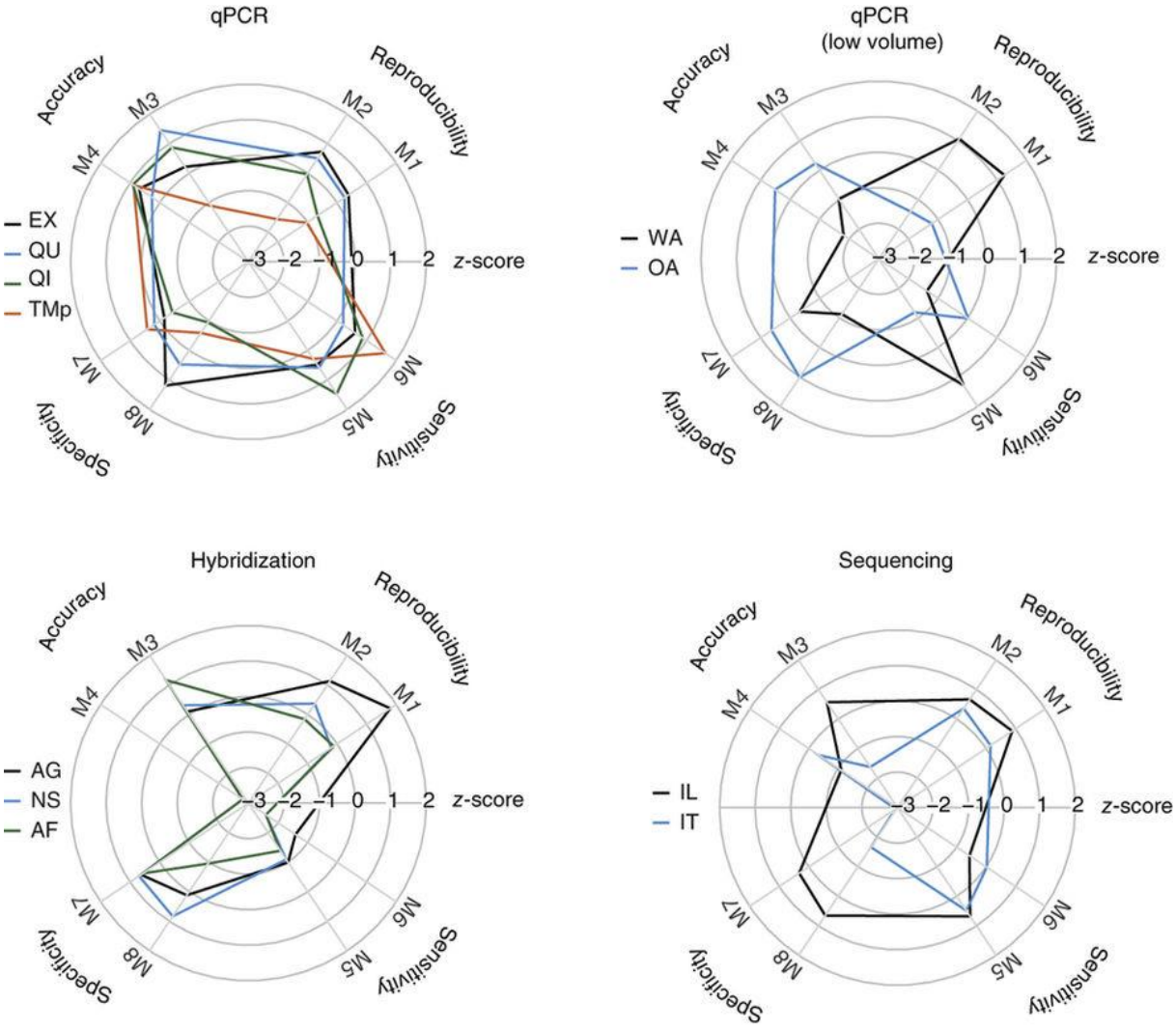

Fig. 7 Radial plot of performance metric *z*-scores from the miRQC study. Z-scores for eight metrics are shown. M1=reproducibility; M2=titration response; M3=accuracy; M4=accuracy low-input RNA; M5=sensitivity; M6=sensitivity low-input RNA; M7=specificity MS2 RNA; M8=assay cross-reactivity. Higher z-scores correspond to a better performance and each radial plot has an identical scale, which makes plots directly comparable. qPCR: EX=miRCury (Exiqon), OA=OpenArray (Life Technologies), TM=TaqMan Cards (Life Technologies), TMp=TaqMan Cards preAmp (Life Technologies), QI=miScript (Qiagen), QU= qScript (Quanta BioSciences), WA=SmartChip (WaferGen), microarray: AF=microarray (Affymetrix), AG=microarray (Agilent), NS=nCounter (Nanostring), sequencing: IL=TruSeq (Illumina), IT=Ion Torrent (Life Technologies). Adapted from Mestdagh, P., et al. (2014). "Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study." Nat Methods 11(8): 809-815 (87).

1.4 Objective

The accurate diagnosis of dementia such as Alzheimer's disease or frontotemporal dementia currently requires a careful assessment of clinical history, cognitive testing, neurological examination, and structural brain MRI. Traditional protein biomarker exist, however, there are certain circumstances related to their detection such as standard operating procedures, harmonization, quality control, establishment of reference material and methods, and comparison of analytical platforms. As matter of fact, validated AD biomarkers are currently detected only in the CSF, which limits their usage in screening or monitoring patients.

Hence, there is currently no single biomarker that meets the high requirements that are necessary to make an easy, fast, cheap and accurate diagnosis. MiRNAs are in many respects a promising alternative, but so far contradictory results, caused by methodological diversity, have prevented progress of the field.

The overarching aim of this work was to measure expression levels of circulating miRNAs in body fluids by using RT-qPCR and subsequently to assess their value as potential biomarkers for neurodegenerative disease such as AD and FTLD. The work consists of two consecutive studies that differ mainly in the number and composition of samples and miRNAs as well as the method used.

The first study focused on a small cohort of Alzheimer patients compared to a heterogeneous disease control group and was considered an initial screening study based on a high-throughput approach using RT-qPCR OpenArray technology that encompassed the detection of >1000 miRNAs to 1) gain an overview of the number of miRNAs detectable in CSF, 2) assess the quality of expression strength and frequency, 3) identify differentially expressed miRNAs, 4) investigate the miRNA biomarker

potential in comparison to classical protein biomarker amyloid-beta, tau and p-tau, and 4) consider the general applicability of the method.

The second study was considered a candidate approach that compared to the first study, based on a medium-throughput RT-qPCR method using the SYBR Green miRCURY system with a focus on 1) reliability of miRNA expression data using appropriate quality controls, 2) samples collected from multiple clinical centers across Germany, 2) homogenous patient groups including bvFTD, AD and cognitively healthy control cases, III) miRNA expression levels detected in both serum and CSF samples and IV) a limited set of miRNAs (n=96). As in the first study, the goal was to examine and evaluate the measured miRNAs for their use as potential biomarkers under these aspects.

1.5 Results

1.5.1 Publication 1 “MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer`s Disease” (Denk et al., 2015)

In this preliminary screening study we profiled the expression of in total 1266 circulating miRNAs (miRBase version 14) in CSF of 22 AD patients and 28 disease controls (i.e. n=5 cognitively healthy controls, n=2 patients with normal pressure hydrocephalus (NPH), n=9 patients with FTLD and n=12 patients with cognitive impairment due to affective disorders or vascular disease) by using the TaqMan OpenArray platform including pre-amplification. One goal was to gain an overview of the number, level of expression and frequency of the analyzed miRNAs. Applying an expression cutoff of Cq ≤ 34 resulted in 411 positively detected miRNAs, which suggested that ~37% are actively expressed in the brain. In total, 729 signals were consequently either not detected or did not meet the criteria. Considering only those showing an expression

frequency n ≥ 3, i.e. the number of positive miRNAs in each group that passed the Cq cutoff, further reduced the number to n=204 miRNAs. Of these, expression levels and frequencies were in the ranges as illustrated in Fig. 8a-b. The results show that only a small proportion of the circulating miRNAs measured in CSF (~n=60) is strongly expressed (Cq < 26) correspondingly with high frequency (> 84%) and that basically expression levels of miRNAs were observed to also negatively correlate with their frequencies (R^2=40%) as shown in Fig. 8c.

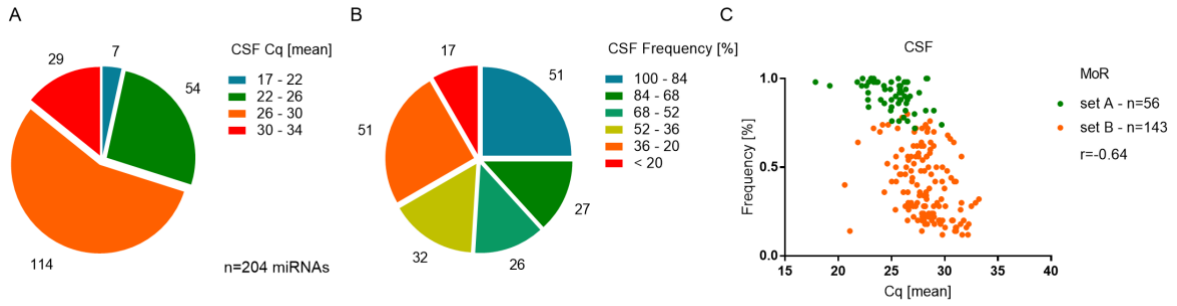

Fig. 8 Quality of CSF expression data obtained by TaqMan OpenArray with pre-amplification. Depicted are the n=204 most abundant and frequently expressed miRNAs and their respective, **A)** ranges of expression levels, **B)** expression frequencies based on n=50 CSF samples and **C)** correlation of expression levels vs expression frequencies and classification into MoR set A (mean Cq 25.06) and set B (mean Cq 28.2). FOC=Frequency of occurrence, MoR = Measure of Relevance.

Since we were also interested in the extent to which significantly deregulated miRNAs were present in the pool we compared expression levels of AD and controls cases. Using a simple t-test, we observed 11 miRNAs with significantly different expression levels, however, in total we identified 74 miRNAs as down- and 74 as differentially upregulated each with a fold change ≥ |1.5| (Fig.1 Denk et al., 2015). In order to consider also the non-significant miRNAs as potential biomarker candidates, we used an explorative statistical non-hypothesis based method called “Measure of Relevance” (MoR) (90). Due to the differences in data quality, we classified the n=204 miRNAs into a set A and set B including higher and more frequently expressed as well as lower and less frequently expressed signals, respectively (Fig. 8c). Initially, we analyzed set A using MoR followed by a reliability investigation, which included a repetitive random

subsampling to improve the results. As a consequence, only miRNAs identified by MoR in ≥80% of cases after repetitive subsampling were considered reliable biomarkers to discriminate AD from control cases. The reliability investigation identified miR-4449, miR-1274a, miR-4674 and miR-106a without and miR-4449, miR-1274a, miR-146a, miR-335 and miR-100 after substitution of missing values as reliable biomarker candidates (Fig 2a-b, Denk et al., 2015). Set A also included CSF protein biomarker levels of total tau, p-tau and $A\beta_{1-42}$. In this case, both, total tau as well as p-tau scored with 100%, confirming the functionality the MoR approach as well as the high discriminatory value of these signals. Interestingly, $A\beta_{1-42}$ was not identified as a reliable biomarker, which was probably due to the fact that $A\beta_{1-42}$ levels vary across various dementia forms, again displaying that its degree of information as a single biomarker may not suffice in clinical routine diagnostics due to its low specificity. We also used MANCOVA and confirmed the reliable biomarkers miR-1274A, miR-100 and miR-146a at Bonferroni corrected significance. The covariates sex and age did not exert significant effects on the considered miRNAs.

We subsequently analyzed set B using MoR. However, set B covered only moderately expressed and less frequent miRNAs so that we couldn't perform a reliability investigation. The MoR-method identified miR-505-5p, miR-4467, miR-766, miR-375, miR-708, miR-3622b-3p, miR-296, miR-219 and miR-103 as informative signals, which was further confirmed by MANCOVA for each individual signal (Fig. 3, Denk et al., 2015). Next, we were interested to see to what extent the potential biomarkers were able to classify candidate AD cases. Using discriminant analyses, the combination of miR-146a (AUC=0.64) and p-tau (AUC=0.79) allowed a correct classification with 86.4% accuracy (S5 Dataset, Denk et al., 2015). Another discriminant analysis performed on the most reliable biomarker miR-100 from set A (Fig 2B and 2C and S3 Dataset, Denk et al., 2015) and the most abundant miR-103 and miR-375 from set B

(S2 Dataset and S3 Dataset, Denk et al., 2015) revealed for the two test groups a total correct classification rate of 96% after substitution of missing values, positively classifying controls and AD cases with 96.4% and 95.5% accuracy, respectively. ROC curve analysis showed an AUC of 0.72 (miR-100), an AUC of 0.87 (miR-103) and an AUC of 0.99 (miR-375) for this combination (S5 Dataset, Denk et al., 2015). In addition, a complex correlation pattern was identified in which the biomarker candidate miR-146a correlated negatively with the protein levels tau and Aβ42 of the cerebrospinal fluid exclusively in the Alzheimer group (R^2~30%).

In summary, the results from the discovery study showed that 1) only small subset of miRNAs is detectable in CSF, 2) an even smaller portion appears to circulate in CSF with increased expression levels and frequency, 3) the measurable miRNAs show differential expression levels equally up- and downregulated in AD compared to disease controls, 4) a subset of de-regulated miRNAs were identified as biomarker candidates for AD by demonstrating moderate to superior classification performance compared to disease controls and scored as good as protein biomarker tau and p-tau and even better than levels of $A\beta_{1-42}$, and 5) that complex correlation pattern of miRNAs with classical protein biomarker further support its role in the regulation of genes in AD pathogenesis.

1.5.2 Publication 2 “Specific serum and CSF microRNA profiles distinguish sporadic behavioural variant of frontotemporal dementia compared with Alzheimer patients and cognitively healthy controls” (Denk et al., 2018)

In this study, we changed from a screening to a more candidate oriented approach by switching from TaqMan OpenArray to SYBR Green based miRCURY technology with a focus on reliability (as discussed in 1.3.4). Hence, we further increased the number of samples to n=48 bvFTD, n=48 AD and n=44 cognitively healthy controls and extended our investigation towards serum. Overall, we concentrated on two almost identical miRNA panels (each n=96 miRNAs) - one measured in CSF and the other measured in serum.

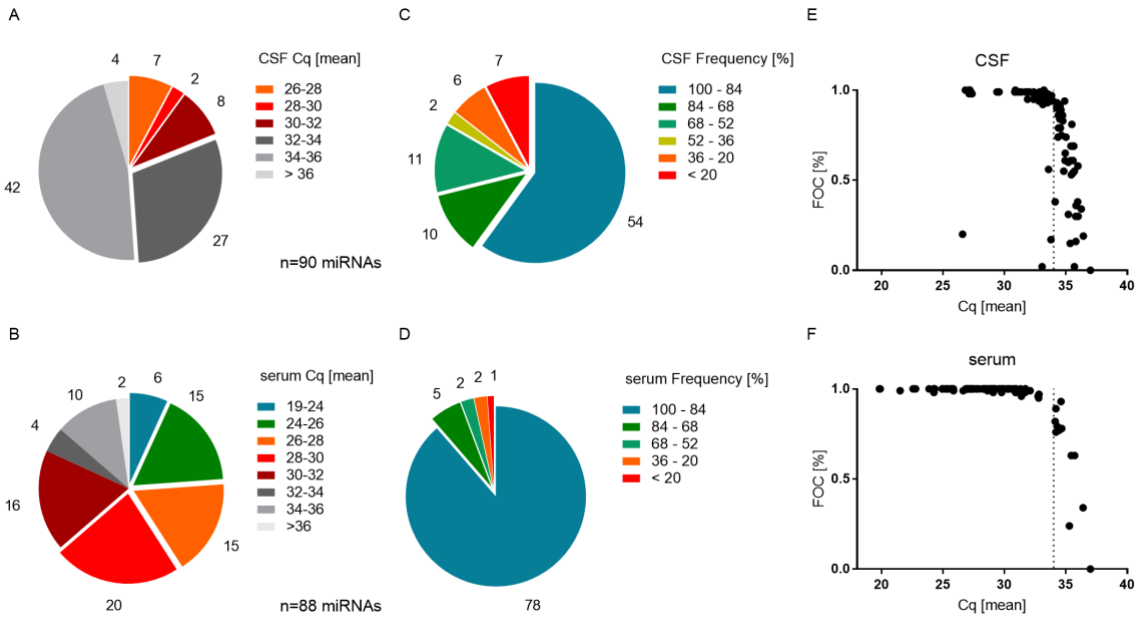

Fig. 9 Quality of expression data obtained by miRCURY assays without pre-amplification. Depicted are miRNAs of CSF (mean Cq 33.6) and serum (mean Cq 28.9) panel (without controls) and their respective **A–B)** ranges of expression levels, **C–D)** expression frequencies based on n=140 (CSF) and n=131 (serum) samples and **E–F)** correlation of expression levels vs expression frequencies.

One objective was to evaluate our protocol towards data reliability. Looking at the quality of expression data, CSF yielded much lower and less frequently expressed

signals compared to serum (Fig. 9a-d). Most CSF miRNAs are in the range Cq 32-36 (Fig. 9a) with ~60% signals showing frequencies > 84% (Fig. 9c), whereas serum miRNAs appear more heterogeneous being predominantly in a higher range of Cq 24-32 (Fig. 9b) with ~90% signals showing frequencies > 84% (Fig. 9d). Both, CSF and serum miRNAs are measured at a constant high frequency until Cq 34, then expression levels and frequencies show a strong negative correlation (Fig. 9e-f). Using artificial and endogenous quality control miRNAs, the protocol also demonstrated 1) constant extraction efficiency with low intra-assay variation, 2) constant efficiency of the reverse transcription step, 3) no hemolysis of serum samples and 4) reduced technical variation after normalizing CSF and serum data to a subset of miRNAs identified by GeNorm and NormFinder (S2 Figure, Denk et al., 2018). Data reliability was also demonstrated by constant PCR efficiencies (E = 0.95-0.98) for a subset of assays and high day-day reproducibility for serum (R^2=0.99) and to a lesser extent for CSF (R^2=0.97) (S3-S4 Figure, Denk et al., 2018). In summary, the protocol generated reliable data, however, with miRNAs measured in serum having an overall higher quality compared to miRNAs detected in CSF and that appear more variable at lower expression levels. Another objective was to analyse any associations of miRNA expression levels in CSF and serum. However, a global correlation of miRNA expression levels between serum and CSF was not observed. Rather, a large number of inter-correlations in the serum dataset were observed. As a consequence, cluster analysis revealed that miRNA expression pattern in serum reflect, in part, their affiliation to a specific miRNA family or genomic cluster, which was specifically altered in bvFTD, AD, and control groups. It was concluded that the profile of circulating miRNAs is more similar to the intracellular profile than expected. Accordingly, deregulated miRNAs identified outside the cell may contain information to detect alterations of intracellular origin - a valuable property of a circulating biomarker. Due to the high correlative nature in the serum data, we further

applied factor analysis to identify potential biomarker pattern. Applying factor analysis we identified a 3-factor model characterized by a 29-miRNA-signature that explained 80% of the variance classifying healthy controls with 97%, bvFTD with 77% and AD with 72% accuracy. Using statistical validation, total classification performance ranged between 60 – 84%. Interestingly, some of the miRNAs that correlated with the individual factors of the model could be assigned to different miRNA families and clusters. Most of these miRNAs were also confirmed by MANOVA as deregulated between AD, bvFTD and control cases with BH corrected significance. Almost all of these signals were regulated in the same direction e.g. miR-320a was down-regulated in AD and bvFTD compared to healthy controls - opposite effects could not be observed. Compared to CSF, serum miRNAs generally showed superior classification performance to discriminate AD and bvFTD cases from healthy controls by displaying AUC values of up to 0.97 and specificities and sensitivities ranging from 92 – 96%, respectively. CSF miRNAs performed more moderately but indicated a trend towards classifying bvFTD from AD cases with AUC values of 0.73 that demonstrated 78% sensitivity and 68% specificity, respectively. Interestingly, several serum miRNAs from our 3-factor-model also correlated with CSF levels of amyloid-beta and pNfH either in the control- or bvFTD group, which further supports their role as potential biomarkers to detect the progressive neuronal decay observed in dementias such as AD or bvFTD.

1.6 Discussion

It has been about 10 years since the first reports of altered miRNA abundance and speciation: (i) in anatomical regions of the brain targeted by the AD process after post-mortem examination, (ii) in blood serum, and (iii) in CSF (91-93). Since then an in depth overview of the peer-reviewed literature has provided no general consensus of what miRNAs are up- or down regulated in any tissue or biofluid compartment in thousands of AD patients (94) as reviewed by Wu et al., 2016 (95) and Kumar et al., 2016 (96). Various sources of (technical) variability have led to inconsistencies in the miRNA profiling data for these studies (97) and prevented the assessment of whether circulating miRNAs in body fluids can act as biomarkers for neurological disorders such as AD.

Instead of comparing up- or down-regulated miRNAs, it is more practical at this point to review previous studies for their data reliability. This would provide an overview of reliable studies, identify potential vulnerabilities and improve future studies to drive the field forward. To this regard, I established a “study reliability score” (SRS), which considers key confounders to rate studies whether they are technically sound or not. Each included item in the SRS is assigned points based on its potential impact on the study reliability as listed in Tab. 1. The items and its corresponding weights are then summarized into a so called “study reliability index” (SRI), which allows to directly compare and evaluate studies based their reliability. The SR score includes the following items: I) sample size, II) normalization strategy, III) fold change (effect size), IV) hemolysis, V) multicenter study and VI) MIQE compliance. As there are too few studies on CSF to make a meaningful comparison, I have evaluated only the studies on serum miRNA biomarkers in AD compared to healthy controls. As a consequence,

only biomarker identified in Denk et al., 2018 were evaluated towards classification performance and reliability.

Tab. 1 Possible factors that challenge the reproducibility of miRNA profiling data from body fluids and that can be used to assess their reliability. The study reliability index SRI consists of different items, whose consideration and extent are summarized into a study reliability score called SRS to evaluate individual studies according to their reliability. The reference column provides valuable literature including tools and methods with respect to each confounding factor that support the reader to improve individual protocols. FC = fold change.

Confounder	Reference	SRS [items]	SRS [points]
Sample collection	(97)	-	-
CSF	(98)	-	-
Serum / Plasma	(99, 100)	-	-
Hemolysis	(75, 101)	Yes	2 = yes 0 = no
Storage	(102)	-	-
Cohort size	(103, 104)	Yes	0.05 / sample (max 5 / group)
Multicenter		Yes	2 = yes 0 = no
RNA isolation	(85, 105-108)	-	-
Platform	(59, 87, 109-112)	-	-
RT-qPCR[1]	(106)	-	-
NGS[2]	(113-117)	-	-
Microarray[3]	(118-120)	-	-
Normalisation	(121-125)	Yes	0.5 / spike and/or miRNA (max 3)
MIQE (RT-qPCR)[4]	(89, 126)	Yes	2 = yes 0 = no
isomiRs	(86, 127, 128)	-	-
Fold change	(129)	Yes	0.5 = FC < 1.5 1.5 = 1.5 ≤ FC ≤ 2 3.0 = FC ≥ 2
Total		6	23

Related confounders are [1] RNA input, PCR inhibitors, reverse transcription and PCR step [2] library preparation, alignment, ribosomal RNA, bioinformatic data processing [3] labelling and cross-hybridization, [4] especially parameters such as no template controls, melting curve analysis, PCR efficiency, intra- and inter-assay variation, limit of detection and basic information that haven't been assessed and reported in related studies.

In total, 12 studies qualified for a comparison because they reported diagnostic sensitivities and specificities of miRNA biomarker candidates for AD in serum. To increase transparency, only miRNAs (n=13) which displayed differential expression levels in AD compared to healthy controls and above a fold change of |1.5| at Bonferroni corrected significance were selected from the study Denk et al., 2018. These serum miRNA biomarkers scored mediocre in comparison to the other studies with an average diagnostic sensitivity of 83% as shown Fig. 10a. Nevertheless, they were slightly above the combined sensitivity of 81%. By contrast and with an average specificity of 86%, the n=13 miRNA candidates were among the top four studies as displayed in Fig. 10b. However, most studies and their biomarker signature(s) performed poorly in terms of reliability as demonstrated by the SR index in Fig. 10c and its respective items included in the SR score displayed in Fig. 10d.

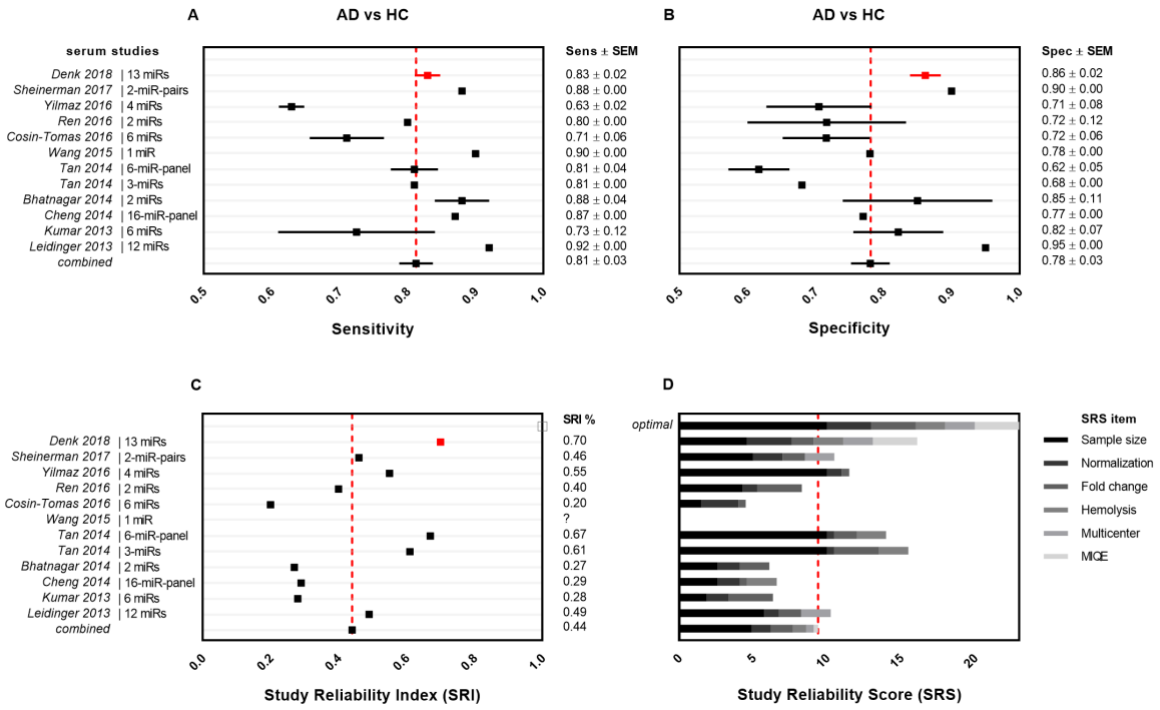

Fig. 10 Classification performance of potential serum miRNA biomarkers for AD compared to the extent of their reliability. I compared the classification performance of different serum biomarker signatures in AD compared to controls from several studies and evaluated these with regard to their reliability by means of the SRI and SRS. Depicted are **A)** the diagnostic sensitivity, **B)** the diagnostic specificity, **C)** the reliability of each study by means of the SRI and **D)** the SRS and its contributing items. The most reliable (optimal) study would indicate a SRI = 1.0, which equals a SRS with 23 points. In total,

n=13 miRNAs from Denk et al., 2018 that displayed different expression levels ≥ |1.5| in AD compared to healthy controls at Bonferroni corrected significance were included. Sens = sensitivity, Spec = specificity.

In summary, this means that 1) blood-based miRNA signals indicate promising biomarker for AD with acceptable classification performance, 2) that classification performance of serum biomarkers in AD does not necessarily correlate with the reliability of the study they have been identified by and 3) that there is room for improvement with regard to the items included in the SR index. One shortcoming associated with the SR index is that not all parameters have been taken into consideration. IsomiRs for example are miRNA variants that differ from the sequence on their 5′- and/or -3′ end, that also circulate in body fluids (127) and that show differential expression in AD (130). However, common RT-qPCR based methods are currently not able to distinguish isomiR variants (128). This bias is of great importance and cannot be controlled until RT-qPCR detection chemistry progress. Secondly, a systematic basis for evaluation towards the SRI items needs to be established in order to rule out an arbitrary award of points. And third, SRI items such as sample size may outweigh due to their redundancy. Nevertheless, the SRI reveals that the criteria and quality controls of the studies investigating circulating miRNAs in body fluids for AD are in need of improvement. General guidelines for qPCR like MIQE (89) already exist but due to methodological variety and the small amount of RNA and microRNA in body fluids, the items in Tab. 1 should be given more focus and greater consideration. Additional literature including tools and methods for avoiding pitfalls are also listed. In addition, inter- and intra-platform differences further contribute to variability (87). Hence, basic parameters such as limit of detection, linear dynamic range, PCR efficiency and intra-assay variation are critical to define an expression cutoff and to discard off-scale data. This can be assessed by absolute quantification by means of standard curves (131, 132). Each study should provide such information to prove the

reliability of microRNA profiling data of the individual protocol and to qualify for a direct comparison of expression levels of circulating miRNAs and thus allowing the field to proceed more rapidly. With regard to miRNA biomarker in CSF, information are still very limited, which complicates a comparison of signals. With regard to miRNA biomarker for bvFTD, miRNA expression data is limited as well. However, in Denk et al., 2018, we generally observe similar de-regulated expression patterns in bvFTD as in AD, which points to a common basis of impairment. However, FTLD and AD are heterogeneous diseases and our studies looked at a limited panel of miRNAs in a cross-sectional setting. As diagnosis and detection towards proteinopathies advance, stratification will improve thus increasing the chance to detect other differentially expressed miRNAs better classifying related dementia types. From an economic point of view, the classical CSF protein biomarker tests are expensive. On the other hand, a biomarker test based on an 8-miRNA signature and the method used in Denk et al., 2018 would cost ~30 € per sample, which makes the identification and establishment of a microRNA-based biomarker by using RT-qPCR economically attractive.

In summary, at the present time it cannot be conclusively assessed whether circulating microRNA are suitable as a biomarker for Alzheimer or frontotemporal dementia. As with the classical CSF protein biomarker - hundreds of studies and standardization efforts will be necessary to establish specific miRNA signatures for the stratification, monitoring or diagnosis of AD or FTLD. However, new advances in both sequencing and qPCR research, including careful optimization and standardization of techniques and protocols as demonstrated in Denk et al., 2018 will certainly foster progress toward highly specific miRNA based biomarker signatures in neurodegenerative diseases such as Alzheimer’s disease and frontotemporal dementia.

2 List of abbreviations

AD	Alzheimer's disease
AGO2	argonaute-2
APOE4	apolipoprotein E-e4
APP	amyloid precursor protein
Aβ	amyloid-beta
BACE1	β-site APP cleaving enzyme
bvFTD	behavioural variant of frontotemporal dementia
C9orf72	chromosome 9 open reading frame 72
CBS	corticobasal syndrome
CHMP2b	charged multivesicular body protein 2B
CNS	central nervous system
FC	fold change
Fig	figure
FTD-ALS	frontotemporal dementia with amyotrophic lateral sclerosis
FTLD	frontotemporal lobar degeneration
FTLD-TDP	frontotemporal dementia TAR DNA-binding protein-43
FUS	fused in sarcoma
GC	guanine-cytosine content
GRN	progranulin
hsa	homo sapiens
lvPPA	logopenic variant primary progressive aphasia
MANCOVA	multiple analysis of covariance
MAPT	microtubule-associated protein tau
MCI	mild cognitive impairment
MIQE	Minimum information for publication of quantitative real-time PCR experiments
miR	microRNA
miRNA	microRNA
MND	motor neuron disease
MoR	measure of relevance
mRNA	messenger RNA
NfL	neurofilament light chain

nfvPPA	nonfluent-agrammatic primary progressive aphasia
NGS	next generation sequencing
NPH	normal pressure hydrocephalus
NTF	neurofibrillary tangles
PHF	paired helical filaments
PPA	primary progressive aphasia
pre-miR	precursor microRNA
pri-miR	primary microRNA
PS-1	presenilin-1
PS-2	presenilin-2
PSP	progressive supranuclear palsy
p-tau	phosphorylated tau
qPCR	quantitative real-time polymerase chain reaction
RISC	RNA induced silencing complex
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
SRI	study reliability index
SRS	study reliability score
svPPA	semantic variant primary progressive aphasia
Tab	table
TBK1	serine/threonine-protein kinase
TDP-43	TAR DNA-binding protein 43
TRBP	transactivation response RNA binding protein
t-tau	total tau

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4 Reprints of original publications

4.1 Denk et al., 2015

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RESEARCH ARTICLE

MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer`s Disease

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Abstract

The miRBase-21 database currently lists 1881 microRNA (miRNA) precursors and 2585 unique mature human miRNAs. Since their discovery, miRNAs have proved to present a new level of epigenetic post-transcriptional control of protein synthesis. Initial results point to a possible involvement of miRNA in Alzheimer's disease (AD). We applied OpenArray technology to profile the expression of 1178 unique miRNAs in cerebrospinal fluid (CSF) samples of AD patients (n = 22) and controls (n = 28). Using a Cq of 34 as cut-off, we identified positive signals for 441 miRNAs, while 729 miRNAs could not be detected, indicating that at least 37% of miRNAs are present in the brain. We found 74 miRNAs being down- and 74 miRNAs being up-regulated in AD using a 1.5 fold change threshold. By applying the new explorative "Measure of relevance" method, 6 *reliable* and 9 *informative* biomarkers were identified. Confirmatory MANCOVA revealed *reliable* miR-100, miR-146a and miR-1274a as differentially expressed in AD reaching Bonferroni corrected significance. MANCOVA also confirmed differential expression of *informative* miR-103, miR-375, miR-505#, miR-708, miR-4467, miR-219, miR-296, miR-766 and miR-3622b-3p. Discrimination analysis using a combination of miR-100, miR-103 and miR-375 was able to detect AD in CSF by positively classifying controls and AD cases with 96.4% and 95.5% accuracy, respectively. Referring to the Ingenuity database we could identify a set of AD associated genes that are targeted by these miRNAs. Highly predicted targets included genes involved in the regulation of tau and amyloid pathways in AD like MAPT, BACE1 and mTOR.

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Introduction

Alzheimer`s disease (AD) is a neurodegenerative, progressive disorder, which primarily affects people over the age of 65 [1]. Individuals suffer from memory deficiencies and other cognitive impairments as a result of synaptic dysfunction and neuronal decay. The development of preventive or curative therapeutic options as well as the establishment of favorable clinical biomarkers for (early) diagnosis and treatment efficacy is a permanent issue [2]. In spite of well-established diagnostic criteria such as traditional guidelines from the National Institute of

Neurological Disorders and Stroke−Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), sensitivity and specificity of AD diagnosis is still lower than desirable. Amyloid-β ($A\beta_{1-42}$), total-tau (tau) and phospho-tau (p-tau) are currently used as suitable cerebrospinal fluid (CSF) biomarkers to detect AD [3]. These proteins are components of the pathogenic hallmarks of AD, amyloid plaques and neurofibrillary tangles, and can be reliably measured in CSF if meticulous collection procedures are applied [4]. Another issue is the relatively less conclusive diagnosis of AD in contrast to related dementia pathologies such as vascular dementia, frontotemporal lobe dementia (FTLD), or Lewy body dementia.

It is commonly accepted that the late onset form of AD, which occurs after the age of 65, and accounts for about 90% of all AD cases, develops within complex interactions of multiple risk factors including genetic components, environmental influences and epigenetic mechanisms, making the identification of novel and informative biomarkers a challenging task [5, 6]. In the last decade, it has become increasingly clear that epigenetic mechanisms, such as DNA methylation, RNA editing or RNA interference considerably contribute to the development and course of AD pathophysiology [7]. RNA interference, especially, may offer potential for new diagnostic and therapeutic options for treatment of AD [8].

Central to this epigenetic process are miRNAs, a subclass of small noncoding RNAs, which are transcribed from either intra- or intergenic regions modulating gene expression post-transcriptionally by targeting mRNAs for cleavage or translational repression via base complementarity [9]. Their significant role in the proliferation, differentiation, function and maintenance of neuronal cells has already been demonstrated in several experimental systems [10]. Moreover, they are specifically expressed in neurons where they are suggested to function in synapse formation [11], synapse plasticity [12] and the differentiation of neurites [13]. The potential role that differentially expressed miRNAs may play in AD pathophysiology was first demonstrated by Lukiw (2007) [14] in hippocampal tissue and by Cogswell *et al*. (2008) [15], who studied miRNA expression changes in CSF and regions of the brain most affected by AD pathology. Furthermore, Hébert *et al*. (2008) showed that a loss of the miR-29a/b-1 cluster correlates with increased beta-secretase (BACE1) activity in Alzheimer's disease pointing to a potential causative association [16]. Pathogenetically, it is suggested that elevated levels of BACE1 expression and activity might initiate or accelerate AD pathophysiology contributing to accumulated amyloid peptides [17]. In addition, Wang *et al*. (2008) reported that a change in neuronal miR-107 expression, which also targets BACE1, could contribute to the pathogenesis of AD [18]. Liu *et al*. (2012) provided strong evidence in AD SAMP8 (senescence-accelerated mouse prone 8) mice models, which have age-related learning and memory deficits, that miR-16 can regulate amyloid-precursor protein (APP) in vivo and that abnormally low expression of miR-16 levels potentially lead to APP accumulation [19].

Hence, miRNAs may provide valuable insight into the cellular mechanisms by which AD related genes are expressed or inhibited, thus improving the current understanding of cause or consequence of the disease progression at molecular level. They are considered as extremely stable [20, 21] and, owing to their function as regulators of gene expression, as well as their presence as circulating molecules in various body fluids [22], may arguably carry promise as biomarkers [23−25]. When measuring circulating miRNAs in neurodegenerative diseases such as AD, CSF is the best material, beside brain tissue, for pathological assessment and the identification of informative signals. Low RNA content in CSF, limited sample size and methodological problems accompanied by low detection limits have led to the production of conflicting results [26, 27]. Nevertheless, recent advances in technology and the development of guidelines may now facilitate research in this field. In our case, the entire qPCR protocol was performed on the basis of the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [28] to reduce technical variability and to provide sufficient

Table 1. Summary of demographic data.

Variable	Control group	AD group	p
Number	28	22	
Gender (f/m)	14/14	13/9	ns
Age	61.0 ± 12.7	72.1 ± 8.5	0.0009
total tau [pg/ml]	308.9 ± 227.7	708.5 ± 282.9	< 0.0001
p-tau [pg/ml]	52.6 ± 28.5	92 ± 93.3	0.0003
Aβ1–42 [pg/ml]	719.9 ± 406.7	446.7 ± 164.1	0.0025
total RNA [ng/μl]	6.9 ± 3.4	6.7 ± 2.6	ns
purity [260/280 nm]	2.3 ± 0.7	2.3 ± 0.6	ns

Mean ± SD. Demographic data for the control- and AD group samples including cognitively healthy controls, NPH, FTLD as well as controls with cognitive impairment due to affective disorders or vascular disease and patients with probable AD and MCI due to AD. Given are numbers for each group and gender, the averages for age, total tau, p-tau, $A\beta_{1-42}$, total RNA and RNA purity.

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experimental detail to increase data transparency and validity. We profiled the expression of 1178 unique mature miRNAs (miRBase, version 14) in a patient cohort comprised of AD cases (n = 22) and a set of disease controls (n = 28) in human CSF drawn in a naturalistic approach from patients presenting to our memory clinic. Testing against disease controls instead against healthy probands is in our view a better way to differentiate towards AD specific changes in the miRNA signature.

Materials and Methods

Patient data and CSF

We measured the expression of miRNAs in CSF samples of a total of 50 probands by OpenArray RT-qPCR. The study cohort consisted of a naturalistic control group (n = 28) including cognitively healthy test subjects (n = 5), patients with normal pressure hydrocephalus (NPH) (n = 2), patients with FTLD (n = 9) and patients with cognitive impairment due to affective disorders or vascular disease (n = 12). This control group was compared to a group of AD cases (n = 22) composed of patients with probable AD (mild late onset AD) (n = 19) and mild cognitive impairment due to AD (n = 3). The groups were stratified for gender. Patients selected in this study referred to the memory clinic of the University Hospital Hamburg-Eppendorf. All patients underwent a diagnostic work-up and were diagnosed according to ICD-10 [29] and NINCDS-ADRDA criteria [30] to identify patients with AD involving new criteria and guidelines to diagnose AD supplanting the previous guidelines first published in 1984 [31–35]. Vascular dementia was diagnosed accordingly, FTLD (combining frontotemporal dementia and progressive non-fluent aphasia) according to the New Diagnostic Criteria for the Behavioural Variant of Frontotemporal Dementia [36, 37]. MCI diagnoses were made according to the criteria of Petersen [38]. Patients with mixed dementia etiologies were excluded. The present demographic data is summarized in Table 1.

CSF was obtained by lumbar puncture in a sitting position according to standard procedures [39]. 4 ml CSF was collected into a polypropylene test tube for routine diagnosis as well as for further studies. CSF was free of blood contaminations and tested for hemoglobin. The sample was centrifuged (1600 g, 4°C, 15 min) and frozen within 30–40 min after the puncture and stored at -80°C until use. The CSF was at no time thawed/refrozen.

Ethics Statement

Procedures were approved by the local ethics-committee of the Ärztekammer Hamburg. All patients and/or their relatives gave written informed consent. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki and have been carried out according to the international Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) standards.

Immunochemistry

The CSF levels of $A\beta_{1-42}$, total tau, and $phospho_{181}$-tau were measured using commercial ELISAs (Innogenetics, Ghent, Belgium) according to the manufacturer's protocol. Cut-off values for AD suspicious biomarker concentrations were > 540 pg/ml for total tau, > 61 pg/ml for p-tau and < 240 + 1.186 x total tau pg/ml for $A\beta_{1-42}$ values [3].

RNA extraction, reverse transcription–qPCR and miRNA quantification

All qPCR experiments were designed and performed in compliance with the MIQE guidelines [28, 40]. We included a checklist to provide experimental detail related to each MIQE item (S1 Dataset).

Total RNA including small RNA was isolated using the mirVana PARIS Kit (Ambion, PN AM1556) following the manufacturer's recommendations. In brief, the samples were homogenized in a denaturing lysis solution, spiked with kshv-miR-K12-1-5p (artificial miRNA) and subjected to an acid-phenol:chloroform extraction. After first separation of the two phases, an additional spiking with ath-miR159a cDNA was performed. Hereafter, the samples were purified on a glass-fiber filter and quantified using a Bioanalyzer 2100 (Agilent Technologies). Concentration and purity were measured using the Nanodrop ND1000 (Peqlab).

Total RNA was converted to cDNA using Megaplex stem-loop RT primer (Life Technologies, PN 4444750) for Human Pool A and B and custom RT primer for Pool C and D in combination with the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, PN 4366596). This allowed simultaneous cDNA synthesis of 377 unique miRNAs for each Pool A and B and 212 unique miRNAs for each Pool C and D. In brief, 3 μl of total RNA was supplemented with RT primer mix (10x), dNTPs with dTTP (100 mM), Multiscribe Reverse Transcriptase (50 U/μl), RT buffer (10x), $MgCl_2$ (25 mM), and RNase inhibitor (20 U/μl) in a total reaction volume of 7.5 μl. Thermal-cycling conditions were as follows: 40 cycles at 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second, followed by reverse transcriptase inactivation at 85°C for 5 minutes.

The RT product (7.5 μl) was preamplified by using the TaqMan PreAmp Master Mix (Life Technologies, PN 4391128) and preamplification primers (Life Technologies, PN 4444750) in a 40 μl PCR reaction. For each pool of stem-looped RT primers in the cDNA reaction, a different pool of PreAmp Primers (Human Pool A and B resp. custom PreAmp primers Pool C and D) was used. Thermal cycling conditions were as follows: 95°C for 10 minutes, 55°C for 2 minutes, and 72°C for 2 minutes, followed by 16 cycles of 95°C for 15 seconds and 60°C for 4 minutes. 4 μl PreAmp product was diluted in 156 μl 0.1x TE-Buffer.

The performance of RNA extraction, RT-qPCR and preamplification was checked by running single quantitative PCRs including the assays for U6 snRNA, ath-miR-159a, and kshv-miR-K12-1-5p on a 7900HT Fast Real-Time PCR System (Life Technologies, Darmstadt, Germany). In brief, 1 μl of the diluted preamplified product was supplemented with 10 μl TaqMan Universal PCR Master Mix, No AmpErase UNG(2x), 1 μl individual TaqMan Assay (20x) and 8 μl aqua dest. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

miRNA quantification was performed with the TaqMan OpenArray Human MicroRNA Panel according to the recommended protocol (TaqMan OpenArray MicroRNA Panels, PN 4461306) for the reactions A and B (Life Technologies, PN 4461104) on one array with in total 818 TaqMan assays and two custom OpenArray plates for reaction C and D (Life Technologies, PN 4461104) separately, each on an individual array with 212 miRNA assays. For each reaction A and B, 45 μl of PCR reaction mix containing 22.5 μl of TaqMan OpenArray Real-Time PCR Master Mix (Life Technologies, PN 4462159) and 22.5 μl 1:40 prediluted preamplified product were prepared. For each reaction C and D, 25 μl of PCR reaction mix containing 12.5 μl of TaqMan OpenArray Real-Time PCR Master Mix and 12.5 μl 1:40 prediluted preamplified product were prepared. 5 μl of each prepared master mix were loaded in one well of a 384-well plate several times to obtain a usable format for automatic pipetting. TaqMan OpenArray Human MicroRNA Panels and custom OpenArray plates were then automatically loaded using the AccuFill System (AccuFill System User Guide, PN 4456986). Up to 3 resp. 12 samples per OpenArray plate were cycled simultaneously on a Biotrove OpenArray NT Cycler (Life Technologies) using OpenArray Real-Time qPCR Analysis Software (v1.0.4) with a pre-assigned cycling program to calculate quantification cycle (Cq) defined as the number of cycles at which the fluorescence signal is significantly above the threshold.

The NormFinder algorithm was applied using GenEx software version 5.4.3 (MultiD) to identify reference genes. The arithmetic mean of their Cq values was calculated for normalization and was subtracted from all miRNAs of each pool to yield ΔCq values. Relative miRNA expression levels between test groups were calculated by using the $2^{(-\Delta\Delta Ct)}$ method [41]. Relative expression levels of individual miRNAs were presented as $2^{(-\Delta Ct)}$ in log2 scale.

miRNA target predictions

MiRNA targets were predicted *in silico* by using the microRNA target filter tool implemented in Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com). Prediction confidence was set to experimentally observed and highly predicted targets. Disease filter was limited to "neurological" and "psychological", i.e. psychiatric disorders. Species was set to human and only tissues and primary cell lines of the central nervous system and CNS cell lines were considered for filtering.

Statistical analysis

We set Cq ≤ 34 as cut-off to define a miRNA as positive or as actively expressed. From all positive miRNAs in the sample population only those showing an occurrence frequency (FOC) of at least 3 in each of the considered two groups (n = 199 miRNAs and Tau, p-tau and $A\beta_{1-42}$) were considered for statistical analysis. The subset was further divided into a set A of 59 abundant markers (FOC≥19 in the control- and ≥17 in the AD group) and a set B including 143 less abundant markers (FOC≤18 in the control- and ≤16 in the AD group). Finally, 202 potential markers were statistically evaluated:

We first applied the explorative 'Measure of Relevance' (MoR) method (Yassouridis *et al*., 2012) to the 202 potential markers in order to identify the most *informative* miRNAs (further called as "*informative* biomarkers"), i.e. miRNAs that can differentiate well between AD and control groups [42]. The explorative MoR method, which is based on a measure containing relevant information of the distribution form, location and dispersion parameters of the samples, enables reasonable reduction of data dimensionality without the need for test decisions, corrections of significance levels and other presumptions. Especially for our two samples that possess relative small sizes (28 and 22) compared to the large number (202) of potential markers, inferential statistics applied to all of them are not advisable, not only because of the commenced

power weakness to detect significances after correction of the level of significance but also because the considered miRNAs (e.g. for miRNA families) will likely display dependencies to each other. Therefore, we decided to apply first the aforementioned explorative analysis for identifying the most *informative* miRNAs and thereafter to perform confirmatory statistical analysis only to them. This method works as follows:

In a two-sample problem each of the considered variables—irrespective of their abundance—is provided after suitable transformations and rank allocations with a positive number (measure of relevance) which is proportional to the capability degree of the variable to discriminate between the samples. The higher the discrimination capability of a variable, the more informative it is towards the two-sample problem. All attached MoR-values are then sorted in an ascending order (information chain) along which a critical value by means of a suitable algorithm (stop criterion) has to be determined. After the determination of the critical value all variables with corresponding MoR-values bigger than the critical value of the information chain are declared as *informative* variables. If the critical value is higher than all MoR-values, none of the variables is considered *informative*. We chose at least 0.57 as critical value as it corresponds to a medium to large effect size with effect sizes higher in set A compared to set B.

To further improve the results of the explorative analysis an additional reliability investigation by applying repeatedly the MoR method to randomly chosen smaller and different subgroups of the considered groups was performed. For receiving a sufficient number of such subgroups only those 59 potential markers with at least 17 positive signals in each group (set A) were considered for the reliability analysis. For the other 143 miRNAs (set B) the explorative analysis was restricted to a unique application of the MoR method. For the reliability analysis 800 different sub-samples were used with 15 probands randomly chosen from each group. Variables among the 800 repetitions proven to be *informative* with a relative frequency (RF) over 0.8 were also declared as *reliable* biomarker candidates (further called as "*reliable* biomarkers") because they are able to distinguish very well between AD and control group at the exploratory level. The reliability investigation was performed twice: once without substitution and once by substitution of missing values with group mean. After identifying the *reliable* biomarker candidates of set A and the most *informative* variables of set B, inferential statistics followed by applying multivariate analyses of covariance (MANCOVA) with sex and age as covariates. Those miRNAs among the biomarker candidates, which revealed significant differences between the AD and control group after Bonferroni adjustments on the confirmatory level, were designated as significant biomarkers. To explore the discrimination power of the *informative* miRNAs from set A and set B some of their combinations were additionally subjected to a discriminant analysis. For testing associations between miRNA markers and predicted mRNA targets in amyloid and tau pathways according to IPA's database, Pearson's correlation coefficients were calculated and proved about significance. For testing significance in some demographic variables with metrical or non-metrical data structure, two-sided student t-tests and x^2-test were applied, respectively. As nominal level of significance $\alpha = 0.05$ was accepted and corrected according to the Bonferroni procedure, whenever post-hoc multiple tests have to be performed.

Results

CSF miRNA expression profile in the sample population

After profiling the expression of in total 1266 (1178 w/o controls) miRNAs (miRBase version 14) in CSF of 22 AD patients and 28 disease controls, 441 (380 in control- 359 in AD group) miRNAs were positively detected in our sample cohort (S2 Dataset). For 729 miRNAs we did not find detectable traces in CSF. This is an indication that at least 37% of the investigated miRNAs appear in CSF and are potentially active in the brain corresponding well with the fact, that

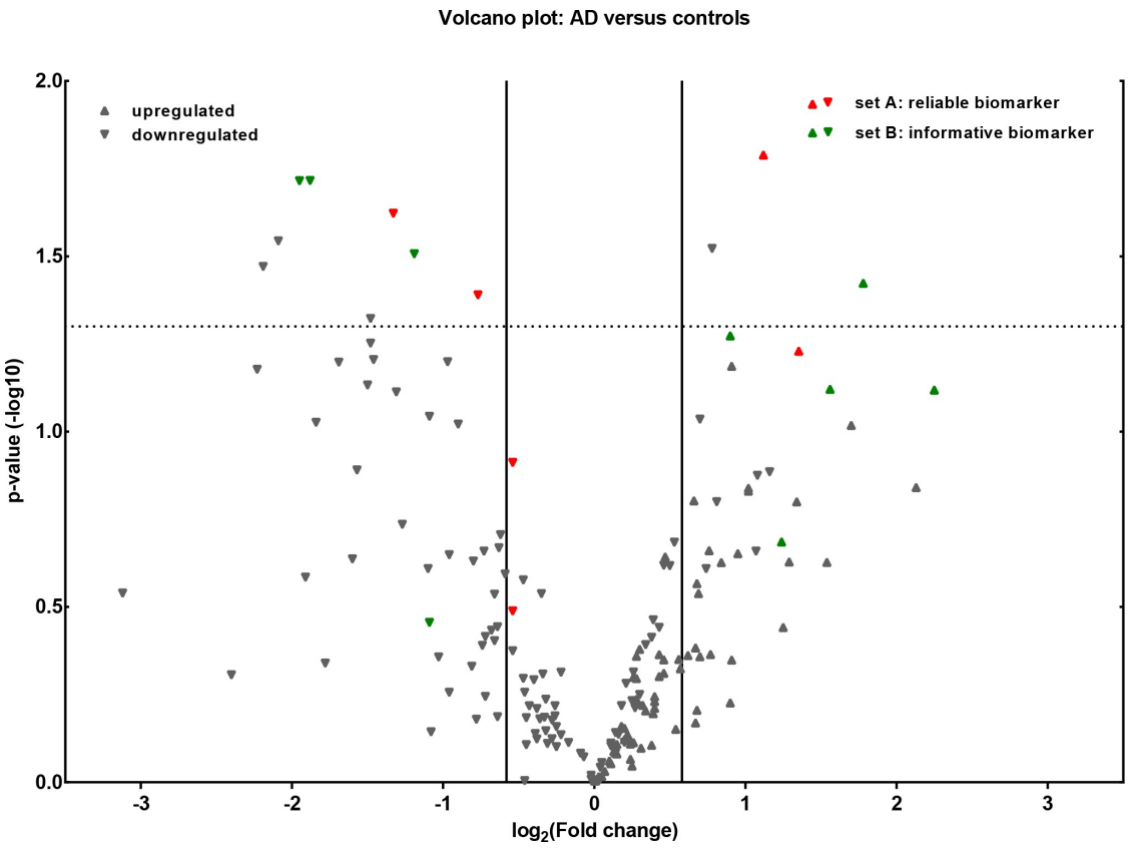

Fig 1. Volcano plot of group comparisons. Comparisons of 199 miRNAs assessed in OpenArray analysis of smallRNA isolated from CSF of patients with AD (n = 22) and controls (n = 28). The volcano plot displays the relationship between fold change and significance between the two groups, applying a student's t-test. The y-axis depicts the negative log10 of p-values of the t-tests (the horizontal slider at 1.3 corresponds to a p-value of 0.05, a higher value indicates greater significance) and the x-axis is the difference in expression between the two experimental groups as log2 fold changes (vertical sliders indicate miRNAs as either up- or down regulated above a fold change of 1.5). Highlighted in green are the *most reliable* (6 abundant miRNAs from set A with RF2:0.8) and in red the *most informative* (9 less abundant miRNAs from set B above the critical MoR value d = 0.57) biomarkers according to the MoR-method (see Figs 2 and 3).

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about a third of the approximately 20.000 different genes that make up the human genome are active in adult brain [43]. Fig 1 displays only those miRNAs from set A (n = 56) and set B (n = 143) to illustrate only corresponding fold changes of potential markers that have been detected in both groups with an FOC of at least 2: 3. All remaining miRNAs were not included in statistical analysis either due to low expression levels or low occurrence frequencies. When comparing AD cases with controls, 74 miRNAs were identified as down-regulated and 74 miRNAs up-regulated, using a fold change threshold 2: 1.5. (Fig 1). Moreover, Fig 1 highlights 15 miRNAs that were identified as *reliable* or *informative* biomarkers by using the MoR-method.

A problem in miRNA studies is often the lack of suitable normalization procedures. For CSF no consensus exists. We identified mir-21, miR-24, miR-328, miR-99b, miR-let-7c and miR-1274B as not regulated between groups, and as potential reference genes for normalization of miRNA expression levels in CSF. The application of the explorative MoR method based on standardized differences of ranks works almost independent of data structures. However,

normalization procedures are also necessary on the confirmatory level, i.e. in our case the subsequent MANCOVA analyses.

The difference in mean age between the two groups reached statistical significance (t-test, $p<0.05$) (Table 1), prompting us to define age as a covariate, although we did not find associations of miRNAs expression with age. Regarding sex, a possible second covariate, the two groups were stratified and therefore statistically significant differences between groups (x^2-test, p = ns, Table 1) should not arise. However, similarly to some studies already reporting gender effects on some miRNAs for e.g. human brain tissue [44], we found gender specific differences for miR-106a, miR-17 and miR-320 in set A and miR-19a, miR-221, miR-532, miR-95 in set B. Therefore, we decided to control the results towards age and sex by considering these variables as covariates in the confirmatory MANCOVAs.

Identification of potential biomarkers applying the "Measure of Relevance" method

Reliability analysis in set A. As already reported for obtaining robust and better results in the explorative analysis a reliability investigation based on the MoR-method was performed on set A. This included 59 potential markers with n = 56 highly expressed (mean Cq 25.06) miRNA species with elevated FOC and CSF markers tau, p-tau and $A\beta_{1-42}$. Without substitution of the missing values the reliability investigation identified among the 59 biomarker candidates in set A, miR-4449, miR-1274a, miR-4674 and miR-106a as *reliable* biomarker candidates with RF 2' 0.8 threshold (Fig 2A). After substitution of missing values by group mean, miR-4449, miR-1274a, miR-146a, miR-335 and miR-100 were found as *reliable* candidates with RF 2' 0.8 (Fig 2B). Interestingly, miR-106a that proved to be a *reliable* biomarker candidate without substitution after missing-values substitution lost this property. A possible explanation would be that after missing values substitution, which generally reduces the pooled variance between groups, the MoR-values of other miRNAs will be somewhat higher than of miR-106a and push the position of miR-106a below the critical MoR-value of the information chain. Classical CSF biomarkers total tau, p-tau and $A\beta_{1-42}$ were also subjected to the reliability analysis as internal controls to validate the MoR algorithm of correctly identifying *reliable* biomarker candidates and to compare relative frequencies with miRNAs from set A. In this case, both, total tau as well as p-tau scored with RF = 1.0, confirming functionality of the MoR approach (Fig 2A and 2B). Interestingly, $A\beta_{1-42}$ was not identified as a *reliable* biomarker (Fig 2A and 2B). This is probably due to the fact that $A\beta_{1-42}$ protein levels vary widely across various dementia forms, again displaying that its degree of information as a single biomarker may not suffice in clinical routine diagnostics due to its low specificity [45]. The 6 *reliable* miRNA biomarker candidates from set A, tau and p-tau were subsequently subjected to MANCOVA after substitution of missing values by the corresponding group mean with age and sex as covariates in order to prove by inferential means the capability of these miRNAs to distinguish between the AD and control group. MANCOVA revealed a significant group effect [Wilks multivariate test of significance; $F(8,39) = 8.79$, sig of $F < 0.00001$]. Bonferroni correction pointed to a significant differential expression of miR-1274A, $F(1, 46) = 16.58$, $p = 0.000$, miR-100 [$F(1, 46) = 7.85$, $p = 0.007$], miR-146a [$F(1, 46) = 4.78$, $p = 0.034$] and naturally tau [$F(1, 46) = 22.67$, $p = 0.000$] and p-tau [$F(1, 46) = 13.96$, $p = 0.001$] between groups (Fig 2C). In this case, miR-1274A, miR-100 and miR-146a (Fig 2A and 2C and S3 Dataset) were confirmed as *reliable* and significant biomarkers. The covariates sex and age did not seem to exert significant effects on the considered miRNAs [Wilks multivariate test of significance; $F(16,78) = 0.85$, sig of $F = 0.629$]. IPA analyses predicted GRIN2A (miR-4449); IRAK3 (miR-4674); MAPT (miR-

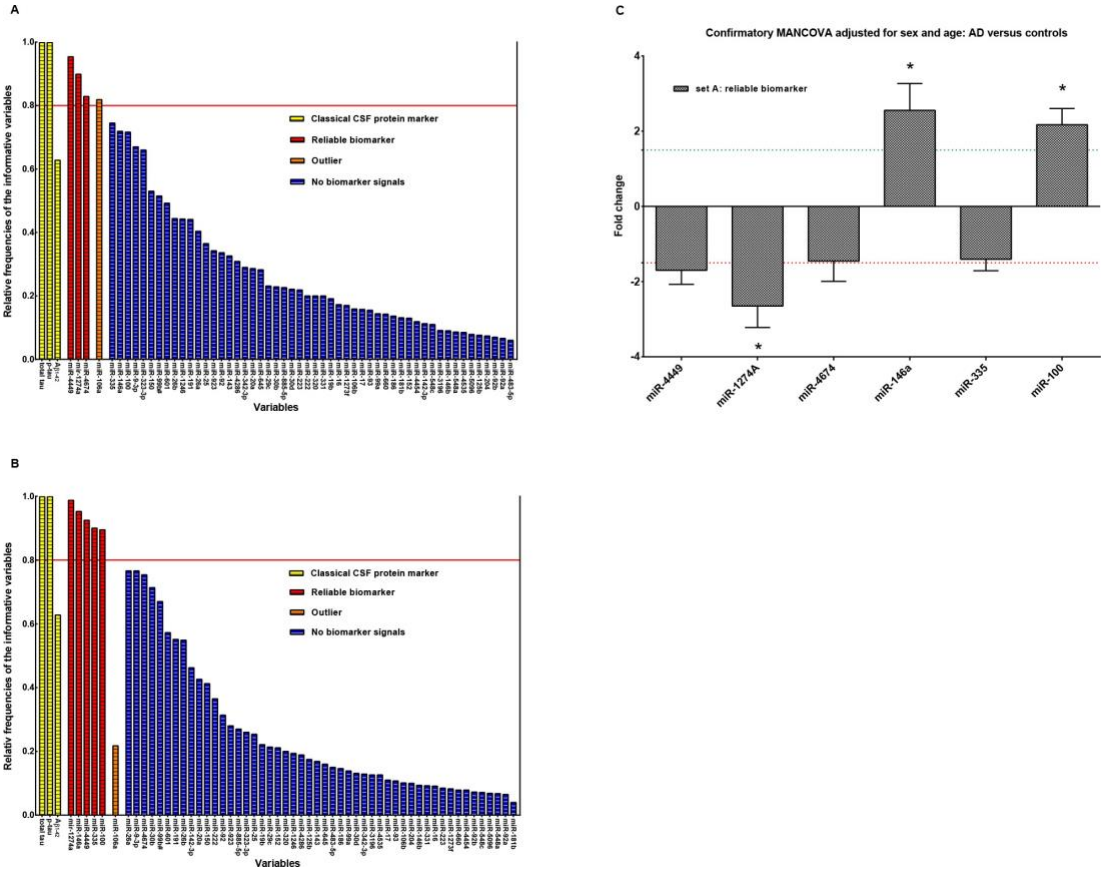

Fig 2. Reliability investigation. Plot of relative frequencies denoting for a miRNA how often among 800 MoR repeats with random subsamples of the original groups it has been crystallized as *informative* (original groups: controls n = 28, AD n = 22 subjects). (A) Relative frequencies of set A miRNAs with reliable biomarker candidates over the solid red line (RF = 0.8); (B) Relative frequencies of set A miRNAs with reliable biomarker candidates over the solid red line (RF = 0.8) after substitution by corresponding group means; (C) Bar diagram of the reliable biomarker signals of set A. Stars (*) over the bars point to significant p-values (MANCOVA, $p < \alpha^*$, where α^* is Bonferroni corrected $\alpha = 0.05$) and therewith to significant biomarkers.

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146a); ADAM19, BDNF (miR-335) and mTOR, TARDPB (miR-100) as targets of our deregulated miRNAs from set A (S4 Dataset).

Informative miRNAs in set B. Set B covered all moderately expressed (mean Cq 28.2) miRNAs (n = 143) with lower FOC and was exclusively subjected to a unique MoR analysis. Applying the MoR approach 9 out of the 143 potential miRNA biomarker candidates were identified as *informative* (Fig 3). The MoR plot illustrates the 9 *most informative* miRNAs, hsa-miR-505-5p, hsa-miR-4467, hsa-miR-766, hsa-miR-375, hsa-miR-708, hsa-miR-3622b-3p, hsa-miR-296, hsa-miR-219 and hsa-miR-103, each reaching a MoR value :2 0.57 (critical MoR value on the information chain). The 9 *informative* miRNAs were subsequently subjected to MANCOVA again with sex and age as covariates. After substitution of missing values by the corresponding group mean, MANCOVA revealed a significant group effect [Wilks multivariate test of significance; $F(9,38) = 90.79$, sig of $F < 0.00001$] for all *informative* miRNAs identified in set B. This effect was further shown to be highly significant for each individual marker

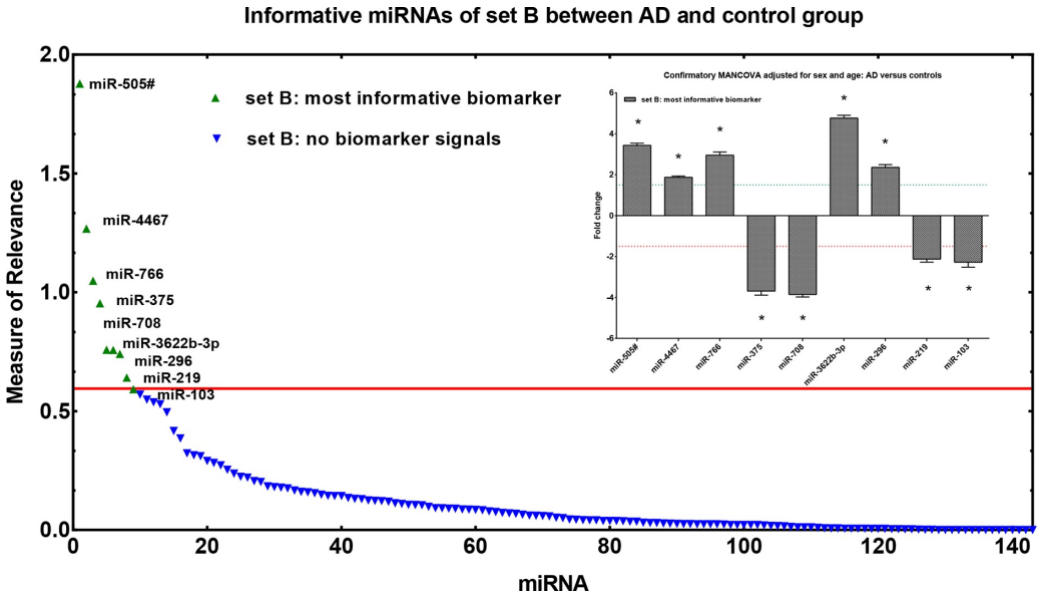

Fig 3. Measure of Relevance for miRNA expression data in CSF. Application of the MoR approach to miRNAs of set B for identifying relevant expression differences between controls and AD cases. In the control group n = 28 and in the AD group n = 22 subjects were examined. MoR-values over the red line are to be declared as *informative* (critical MoR-value for the *informative* designation d = 0.57). Bar diagram of the most *informative* biomarker signals of set B. Stars (*) over the bars point to significant p-values in MANCOVA (MANCOVA, $p < \alpha^*$, where α^* is Bonferroni corrected $\alpha = 0.05$).

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by reaching Bonferroni corrected significance (Fig 3; univariate F-tests, $p < 0.000$). Furthermore, the covariates sex and age did not show a significant association with the miRNAs. Most relevant gene targets identified by IPA were BACE1, REST for miR-103, MAPT for miR-219 and CDK5R1 for miR-375 (S4 Dataset).

miR-146a expression levels implicated in tau pathomechanism

Increased levels of tau protein and its phosphorylated derivate as well as decreased levels of extracellular $A\beta_{1-42}$ peptides have been proven as markers to detect AD in CSF [1]. It remains still unclear to what extent these proteins contribute to AD pathogenesis and whether the expression of one protein explains the toxic effect of the other. We investigated correlations of our significant miRNA signals with these classical biomarkers. According to Ingenuity's database, miR-146a is highly predicted to target the MAPT gene. MiR-146a expression levels were significantly upregulated in CSF of AD patients (Fig 2C) and showed a significantly inverse correlation with tau and $A\beta_{1-42}$. Lower miR-146a expression levels were accompanied by higher levels of tau (AD cases: $r = -0.5142$, $p = 0.0171$) and $A\beta_{1-42}$ (AD cases: $r = -0.5364$, $p = 0.01$), and vice versa in our AD group (Fig 4A and 4B). No significant correlation with concentrations of p-tau was observed in the AD group (Fig 4C). In the control group no significant correlations with miR-146a emerged. We also found significant correlations between miR-103 targeting BACE1 and both tau for the whole study sample ($r = -0.4223$, $p = 0.045$) and $A\beta_{1-42}$ ($r = 0.5980$, $p = 0.024$) for the control group. MiR-375, which is thought to downregulate CDK5R1, was downregulated in our AD cases and correlated significantly with $A\beta_{1-42}$ ($r = 0.7481$, $p = 0.002$).

Classification

The combination of miR-146a and p-tau as biomarkers already allowed correct classification in 86.4% of all cases performing discriminant analysis. Using ROC curve analysis the combination showed an AUC of 0.64 for miR-146a and an AUC of 0.79 for p-tau (S5 Dataset). Another discriminant analysis performed on the most *reliable* biomarker miR-100 from set A (Fig 2B and 2C and S3 Dataset) and the most abundant miR-103 and miR-375 from set B (S2 Dataset and S3 Dataset) revealed for the two test groups a total correct classification rate of 96% after substitution of missing values, positively classifying controls and AD cases with 96.4% and 95.5% accuracy, respectively. ROC curve analysis showed an AUC of 0.72 (miR-100), an AUC of 0.87 (miR-103) and an AUC of 0.99 (miR-375) for this combination (S5 Dataset).

Discussion

Due to its direct and intimate relationship with brain tissue we consider CSF a more suitable and informative material for the potential monitoring of neurophysiological changes in AD.

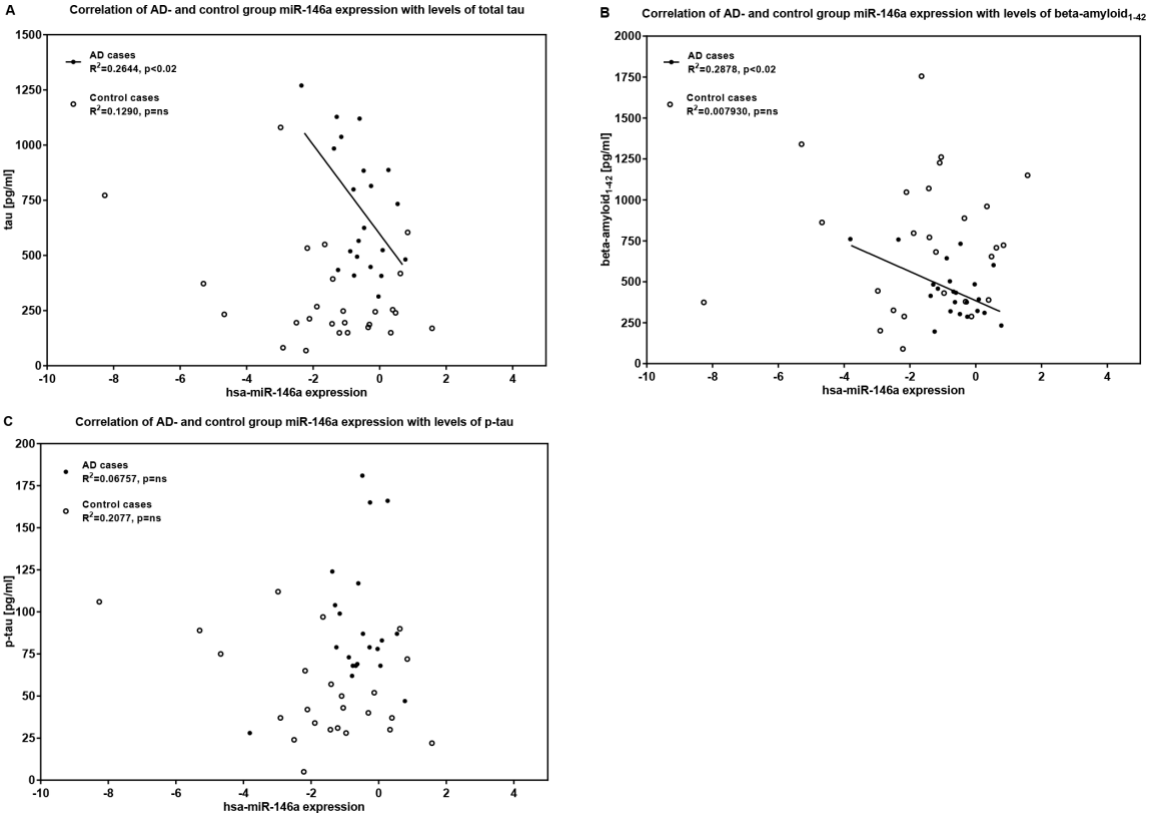

Fig 4. Scatter plot: Correlation of CSF miR-146a expression and levels of total tau, p-tau and $A\beta_{1-42}$. Correlation significances were proven by Pearson correlation coefficients. Data were analysed applying Cq 32 as cut-off for miR-146a expression levels. Expression levels of miR-146a (2^-dCt log2) are inversely correlated with concentrations of total tau and $A\beta_{1-42}$ in the AD group: (A) miR-146a expression levels vs. tau, AD cases: r = -0.5142, 98% CI -0.8065 to -0.01993, p = 0.0171; (B) miR-146a expression levels vs. $A\beta_{1-42}$, AD cases: r = -0.5364, 98% CI -0.8121 to -0.06519, p = 0.0101. (C) No significant correlations were observed for concentrations of p-tau and miR-146a expression levels in the AD- and control group. $A\beta_{1-42}$ = b-amyloid 42; CI = confidence interval; p-tau = phosphorylated tau.

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To the best of our knowledge, we have profiled the largest number of unique miRNAs (n = 1178) in CSF from the largest AD/control (n = 22/28) sample cohort. We reported here that at least 37% of miRNAs are expressed in the human brain, i.e. 441 out of 1178 investigated miRNAs showed detectable traces pointing to transcriptional activity in CSF. This observation corresponds well with the fact that the highest expression of tissue specific miRNAs is also found in the brain [46]. Consistent with results of the Cogswell study [15], which looked at a smaller set of miRNAs, we also observed an even distribution of under- and overexpressed microRNAs in CSF of patients with AD compared to a control group. The number of detected miRNAs in our study was considerably higher than those reported in a recent miRNA profiling study by Frigerio *et al*. [47]. This is probably due to the different platform used and the inclusion of a preamplification step in our protocol. We applied OpenArray technology including a preamplification step on a larger set of patients (n = 50) to overcome some obstacles that may have caused contradicting results [48]. The preamplification step improves sensitivity and increases the number of detectable miRNAs without introducing a systemic bias in the estimation of miRNA expression [49]. However, we observed a great overlap of detected miRNAs in our dataset with those reported by Frigerio *et al*. [47].

For the first time, we applied a sophisticated and exploratory statistical approach (Measure of Relevance) to analyse miRNA expression data in order to identify potential biomarkers. The Measure of Relevance algorithm detected 15 *informative* miRNA markers in our CSF samples. Of those 15 candidates, 3 of 6 miRNAs from set A, on which a reliability analysis could be performed, were also inferentially confirmed at Bonferroni corrected significance by MANCOVA. Thus, miR-100, miR-1274a and miR-146a might be strong candidates for new AD biomarkers.

Beside its biomarker potential miR-100 could also be an interesting target for therapeutic interventions. Due to a high seed pairing stability and its CG dinucleotide rich seed site, miR-100 is supposed to have only few mRNA targets, among them mTOR (mammalian target of rapamycin) and TARDPB [50]. Recently, it was shown that reducing mTOR signalling increases lifespan. There is an association between mTOR and tau, which is linked to GSK3β and autophagy function. A reduction of mTOR signalling might alleviate pathologically increased tau phosphorylation [51]. While Caccamo *et al*. provided preclinical data indicating that reducing mTOR signalling may be a valid therapeutic approach for tauopathies, our results suggest that this salvage pathway may already be active in AD patients by up-regulation of miR-100 (fold change 2.17). Interestingly, we found on a trend-level a negative correlation of CSF miR-100 concentrations with CSF p-tau in our controls ($r = -0.42$, $p = 0.065$) but not in our AD samples ($r = -0.0188$, $p = 0.941$). This might point to a ceiling effect. Furthermore, miR-100 is up-regulated in the medial frontal gyrus of AD patients but not in hippocampus in analogy to the expected tau progression in AD, which could explain elevated CSF concentrations of miR-100 due to the release during atrophic processes [15].

An unexpected result was the identification of miR-1274a, which resembles a t-RNA and probably not a real miRNA, as significantly deregulated in AD, whereas miR-1274b was identified as a reference gene at the same time, demonstrating stable expression levels across our study population. According to annotated miRbase.org, the mature sequences of miR-1274 are considered as fragments of a Lys tRNA and are proposed to be endogenous retroviral elements [52]. It is reported that genes from human endogenous retroviruses have been detected as transcripts and proteins in the central nervous system, frequently in the context of neuro-inflammation. These elements have also been implicated in multiple sclerosis and other neurological diseases and should, according to our findings, be subject of further investigation [53].

MiR-146a is a brain-specific miRNA that is also associated with neuro-inflammation [54]. It is suggested that pro-inflammatory and innate immune system-associated factors play a role in pathways that drive the pathological AD process [55]. In line with results of Alexandrov

(2012) we also observed significant increases of miR-146a expression in AD and proved miR-146a to be abundant in CSF [56]. Another study reported that miR-146a expression is induced by NF-kB and considered to downregulate complement factor h, an important repressor of the inflammatory immune response of the brain, which could explain differential expression in AD brain and relate neuro-inflammation to AD pathogenesis [57]. Analysing correlations of miRNA expression levels with clinical biomarkers (tau, p-tau and $A\beta_{1-42}$) yielded a complex correlation pattern for miR-146a, which is also predicted to target MAPT in-silico (S4 Dataset). We found high miR-146a expression in our AD patients (fold change 1.81) and significant negative correlations of miR-146a with tau and $A\beta_{1-42}$ levels, pointing to a possible inhibitory mechanism of miR-146a on tau production. Changes of miR-146a concentrations in CSF explained 26% of tau and 29% of $A\beta_{1-42}$ variation in the AD group. The similar impact of miR-146a on these biomarkers possibly suggests a further nexus between $A\beta_{1-42}$ and tau pathologies in AD. Another study has found elevated expression levels of miR-146a in CSF and brain regions affected by AD and also in mouse models implicating a role of miR-146a in AD pathogenesis [58, 59]. Furthermore, we did not see any significant correlations of miR-146a with tau, p-tau or $A\beta_{1-42}$ concentrations in the control group. Hence, these findings may be specific for our AD patients.

Frigerio *et al*. reported miR-27a-3p to be significantly reduced in CSF of AD compared to controls and to correlate with tau, p-tau and $A\beta_{1-42}$ [47]. We could not replicate the reported correlations with high tau and low $A\beta_{1-42}$ CSF concentrations and did not observe a downregulation of miR-27a-3p in our AD samples.

Interestingly, we observed that in addition to the 6 miRNAs from set A, also potential markers such as miR-9, which did not exceed the 0.8 threshold, scored substantially higher than amyloid-beta after the reliability analysis as indicated in Fig 2A and 2B. MiR-9 is specifically enriched in the brain [60] and suggested part of a network, that indirectly regulates the APP processing, Aβ production and accumulation [61].

Moreover, we could also confirm the 9 *informative* miRNAs (miR-505-5p, miR-4467, miR-766, miR-375, miR-708, miR-3622b-3p, miR-296, miR-219 and miR-103) from set B as significant biomarkers by MANCOVA all reaching Bonferroni corrected significance. However, the low FOC in set B did not only prevent a reliability analysis but may also have reduced the property of these miRNAs to be robust biomarkers as a direct consequence. By performing discriminant analysis including candidate miRNAs of both subsets as well as in combination with CSF protein marker, we could, irrespective of FOC, demonstrate overall classification rates of 96% (miR-100, miR-375 and miR-103) and 86.4% (miR-146a and p-tau). This clearly demonstrated that already a limited number of miRNAs may be sufficient to detect AD in CSF and support our hypothesis that miRNAs could be promising and robust biomarkers for the diagnosis of neurodegenerative diseases like AD. Comparing results with those found in other body fluids, overall classification rates observed with CSF-based miRNAs are substantially higher [62].

The few biomarker screening studies [15, 47, 56, 63–65] who investigated CSF miRNA expression levels in AD did not lead to the unequivocal identification of biomarkers (S6 Dataset), in part due to problems with replicability [26, 48, 65]. Several miRNA profiling protocols for the detection of miRNAs, as reviewed in Pritchard *et al*. (2012), exist [27]. Mestdagh *et al*. (2014) suggested that differences in these protocols may explain some of the divergent results [66]. Recently, our RT-qPCR approach has been validated by Carre *et al* (2014) by using plasma samples [67]. Validation results showed that this customized method is not only sensitive and highly specific but also repeatable and accurate to detect circulating miRNAs in body fluids. According to established guidelines in the field, we favour—at least to allow a better comparability and transparency—to adhere to the MIQE guidelines or report the extent to which these guidelines were applied (S1 Dataset).

The experiments reported here demonstrate that differentially expressed miRNAs in CSF present *informative* markers that are able to detect AD compared to heterogeneous controls. However, developing microRNAs into accurate and useful tools for diagnosis of AD, will require an extensive phase of validation with multiple replication studies. This compares to the intensive work that was required to establish and approve the use of the classical protein markers tau, p-tau and beta-amyloid species in clinical routine diagnosis. These traditional CSF markers are in use in the field of dementia diagnosis for over two decades now and are far from being implemented as an easy standardized laboratory method due to pre-analytical and analytical problems that are still unsolved [68]. One can assume that this will also be an issue in miRNA based diagnostic procedures. Another negative aspect is that only a limited number of miRNAs appear to abundantly circulate in CSF. We suggest future investigations to focus on those miRNAs, like in our set A, which demonstrate high occurrence frequencies and high expression levels. A further limitation is the currently observed inter-platform variability and diversity of different experimental procedures to measure miRNA expression levels that led to inconsistencies among comparable studies (S6 Dataset) [69]. More work is required to increase data transparency (e.g. adherence to MIQE if usinq RT-qPCR) and to allow better comparisons of miRNA expression data. This is an important prerequisite on the way to establish the clinical utility of circulating miRNAs in CSF in AD diagnosis. In addition to these pre-analytical considerations, it is important that further pilot screening or candidate approach studies are based on larger patient cohorts than those reported thus far. This limitation needs to be addressed to compensate for technical and confounding variation when looking at circulating miRNAs with low expression levels [70].

However, it is advantageous that miRNAs are robust and stable in CSF and very resistant to RNAse activities that cause many problems with e.g. mRNA measurements [21]. The stability of miRNAs may greatly facilitate the standardization of sampling and detection procedures solving an issue that currently hampers the use of beta-amyloid as a biomarker for AD, which requires stringent pre-analytic sample procedures to deliver reliable results [71].

In summary, we have found putative new AD biomarkers, which display some promising attributes and face validity with view to their targets, and which, if developed into diagnostic markers, could prove to be an advantageous opportunity in clinical routines for neurodegenerative diseases such as AD. Another upcoming field is the development of miRNA treatment strategies. The identification of dysregulated miRNAs is a first step to this endeavor.

Supporting Information

S1 Dataset. MIQE checklist. E = essential, D = desired, MP = manufacturer's protocol, N/A = not applicable.
(XLSX)

S2 Dataset. MiRNAs detected in ante-mortem CSF of AD- and control group patients. N/A = not applicable, ND = not detected (Cq > 34).
(XLSX)

S3 Dataset. Scatter plots of differentially regulated miRNAs in CSF of AD patients. Log2--transformed miRNA expression ratios obtained from RT-qPCR analysis are plotted for the *most reliable* (RF ≥ 0.8) miRNAs from set A: (A) miR-100, (B) miR-146a, (C) miR-1274B and the *most informative* (MoR-value ⊄0.57) miRNAs from set B: (D) miR-505*, (E) miR-375, and (F) miR-103. All miRNAs were statistically confirmed by MANCOVA at Bonferroni corrected significance α = 0.05). Each data point represents one sample. For each sample, fold

change in miRNA expression is calculated over its mean expression in the control group.
(TIF)

S4 Dataset. In-silico predicted mRNA targets of miRNA biomarker from set A and B. MiRNA targets were predicted in silico by using the microRNA target filter tool implemented in Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com). Set = array set, target gene = predicted target, confidence = prediction confidence, pathway = related biological pathway.
(XLSX)

S5 Dataset. ROC curve analysis. ROC curves for the combination of (A) miR-146a and p-tau, and (B) miR-100, miR-103 and miR-375 to separate 28 control- from 22 AD cases.
(TIF)

S6 Dataset. Differentially expressed CSF miRNAs in AD. Listed are CSF miRNAs from comparable studies that were identified as significantly deregulated in AD compared to controls. MiRNAs in green indicate replicated markers and in bold novel markers that were identified in our study according to the MoR method.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JD HJ. Performed the experiments: JD CS HJ KB SA. Analyzed the data: JD HJ DL CS. Contributed reagents/materials/analysis tools: KB SA HJ CS DL. Wrote the paper: JD HJ.

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4.2 Denk et al., 2018

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RESEARCH ARTICLE

Specific serum and CSF microRNA profiles distinguish sporadic behavioural variant of frontotemporal dementia compared with Alzheimer patients and cognitively healthy controls

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Abstract

Information on circulating miRNAs in frontotemporal lobar degeneration is very limited and conflicting results have complicated an interpretation in Alzheimer's disease thus far. In the present study we I) collected samples from multiple clinical centers across Germany, II) defined 3 homogenous patient groups with high sample sizes (bvFTD n = 48, AD n = 48 and cognitively healthy controls n = 44), III) compared expression levels in both CSF and serum samples and IV) detected a limited set of miRNAs by using a MIQE compliant protocol based on SYBR-green miRCURY assays that have proven reliable to generate reproducible results. We included several quality controls that identified and reduced technical variation to increase the reliability of our data. We showed that the expression levels of circulating miRNAs measured in CSF did not correlate with levels in serum. Using cluster analysis we found expression pattern in serum that, in part, reflects the genomic organization and affiliation to a specific miRNA family and that were specifically altered in bvFTD, AD, and control groups. Applying factor analysis we identified a 3-factor model characterized by a miRNA signature that explained 80% of the variance classifying healthy controls with 97%, bvFTD with 77% and AD with 72% accuracy. MANOVA confirmed signals like miR-320a and miR-26b-5p at BH corrected significance that contributed most to discriminate bvFTD cases with 96% sensitivity and 90% specificity and AD cases with 89% sensitivity and specificity

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compared to healthy controls, respectively. Correlation analysis revealed that miRNAs from the 3-factor model also correlated with levels of protein biomarker amyloid-beta$_{1-42}$ and phosphorylated neurofilament heavy chain, indicating their potential role in the monitoring of progressive neuronal degeneration. Our data show that miRNAs can be reproducibly measured in serum and CSF without pre-amplification and that serum includes higher expressed signals that demonstrate an overall better ability to classify bvFTD, AD and healthy controls compared to signals detected in CSF.

Introduction

The role of microRNAs (miRNAs) in neurodegenerative disorders has gained growing interest in the field [1]. This is due to matured technologies, which now enable the reliable detection of miRNAs in body fluids [2]. MiRNAs are small non-coding RNA that selectively bind different messengerRNAs (mRNA) to downregulate its translation into proteins. These ~22 nucleotides long molecules control fundamental biological processes such as neurogenesis, immune responses and aging and are critical to cellular expression homeostasis [3]. In addition, the transport of miRNAs in extracellular vesicles such as exosomes secreted by neurons and glia plays a key role in intercellular communication and neuroinflammation [4]. The fact that miRNAs are released as circulating miRNAs into the bloodstream not only enables detection, but deregulated signals may reflect neurodegenerative conditions that occur in AD [1, 5]. Thus, miRNAs are easily accessible in minimally invasive body fluids such as serum and cerebrospinal fluid (CSF) [6] and are also known to remain largely stable towards freeze-thawing cycles and RNAse activity [7, 8]. Detection by RT-qPCR is accurate and inexpensive and potentially easier to integrate into clinical routine than assays detecting proteins. A deeper investigation of the role of miRNAs may also foster our understanding of neurodegenerative diseases and lead to new therapeutic approaches.

In contrast to AD, frontotemporal lobar degeneration (FTLD) is a neurodegenerative disorder for which information on circulating miRNAs is very limited. Our knowledge on the miRNome is still scarce. FTLD is characterized by a progressive degeneration of the frontal and anterior temporal lobes causing pathological changes in behaviour and language. About 10–30% of FTLD cases have a known genetic predisposition. Mutations in genes like *C9orf72*, *GRN* (progranulin), *MAPT* (microtubule associated protein tau) or *TBK-1* (encoding TANK-binding kinase 1) can cause FTLD [9–12]. Autosomal dominant inherited cases often occur clustered in families [13]. The large majority of sporadic FTLD cases is, however, of unknown etiology, albeit genetic alterations are to be expected there too [14]. The behavioural variant (bvFTD) accounts for more than half of the cases [15] and is histopathologically described by distinct inclusion bodies either comprised of Tau (FTLD-TAU) [16] or ubiquitinated TDP-43 (FTLD-TDP) [17]. Despite considerable efforts, in-vivo biomarkers are not yet available for FTLD.

We hypothesize that miRNAs in serum and CSF may serve as biomarkers differentiating patients with bvFTD, AD and cognitively healthy controls. Hence, we designed a study following guidelines such as the "minimum information for publication of quantitative real-time PCR experiments" (MIQE) [18] by using SYBR-green based LNA assays (Exiqon A/S), which have proven reliable to measure miRNAs in body fluids compared to other technologies. We profiled two customized miRNA panels each including n = 96 comparable assays in 48 bvFTD, 48 AD and 44 control CSF and serum samples to identify possible biomarker pattern.

Methods

Ethics statement

Collection and analysis of samples were approved by the local Ethics Committees of Departments of Neurology and Psychiatry participating in the German FTLD Consortium, a quality-controlled, monitored, multicenter initiative (Ulm approval number 20/10) [19]. All participants of the registry gave their written informed consent for all investigations and their data were fully anonymized prior to any entries in the database. The data we worked with were fully anonymized. All investigations were carried out according to international Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) standard.

Patient data

Our study population included n = 140 CSF and n = 131 serum samples of bvFTD (48 CSF / 48 serum), AD (48 CSF / 47 serum) and cognitively healthy control cases (HC) (44 CSF / 38 serum). For each case a pair of CSF and serum material was available except for n = 9 serum samples. All samples were provided by the German consortium for frontotemporal lobar degeneration (FTLDc), which were collected from 10 academic centers across Germany [19]. All bvFTD patients met standard diagnostic criteria according to Rascovsky et al. (2011) [20]. Alzheimer's disease (AD) was diagnosed according to criteria from the National Institute of Neurological and Communicative Diseases and Stroke (NINCDS)–Alzheimer's Disease and Related Disorders Association [21]. Participants were assessed with extensive diagnostic tools including physical and neurological examination, clinical laboratory testing and analysis of CSF, genetic screening of *C9orf72*, *GRN*, *MAPT* for pathogenic mutations. CSF was obtained by lumbar puncture in a sitting position according to standard procedures [22]. 4 ml CSF was collected into a polypropylene test tube for routine diagnosis as well as for further studies. CSF was free of blood contaminations and tested for hemoglobin. CSF was centrifuged (1600 g, RT, 10 min) and frozen within 30 min after the puncture at -80˚C until use. Blood was drawn in 7.5 ml S-Monovette Serum Gel Z tubes (Sarstedt, Germany), incubated for 10 min at RT, centrifuged (2000 g, 4˚C, 10 min) and frozen within 30–40 min after blood sampling at -80˚C until use. The CSF and serum samples were at no time thawed/refrozen.

Genetic analyses

DNA was available from 61 participants. Screening of *C9orf72* for pathogenic repeat expansion was performed by amplicon length analysis and repeat-primed PCR was conducted in 61 samples. In case of a suspected expansion, southern blot analysis was performed for confirmation (Akimoto et al., 2014) [23]. In samples from participants with a familial history of a neurodegenerative disease, all exons and flanking intronic regions in *MAPT* and *GRN* were screened by Sanger sequencing (details available upon request). The sequencing results were compared to reference sequences (GenBank entry NM_005910, NM_002087) using the SEQUENCE pilot software (JSI medical systems).

Laboratory markers

The concentration of albumin, IgG, IgA and IgM in CSF and sera was determined as described earlier (Jesse et al., 2011) [24]. Samples were analysed in the neurochemical lab at the department of Neurology in Ulm. Commercially available ELISA kits were used for Neurofilament light chain (NfL) (IBL, Hamburg, Germany), phosphorylated Neurofilament heavy chain (pNfH) (Biovendor, Heidelberg, Germany), Progranulin (Biovendor, Heidelberg, Germany),

Tau, pTau and amyloid-beta$_{1-42}$ (Fujirebio, Hannover, Germany) according to the manufacturer's instructions.

RNA isolation

Total RNA, including miRNA, was purified from 250 μl cell-free CSF and 200 μl serum samples using the miRNeasy Serum/Plasma Kit (Qiagen, P/N 217184). The protocol was applied according to the manufacturer's recommendations with the following modifications. QIAzol Lysis Reagent mixture containing 1.25 μg/ml MS2 bacteriophage RNA (Roche Applied Science) and 1 μl RNA spike-in template mixture containing synthetic UniSp2, UniSp4, and UniSp5 (Exiqon, P/N 203203) was added to all samples. The total RNA was not treated with DNase and eluted with 22 μl RNAse-free water and stored at -80˚C until use.

RT-qPCR

Total RNA was reverse transcribed using the Universal cDNA synthesis kit II, (Exiqon, P/N 203301). Briefly, cDNA was synthesized using 8 μl of total RNA isolated from CSF or serum including 1 μl of a spike-in mix containing synthetic UniSp6 and cel-39-3p (Exiqon, P/N 203203) using a poly-T primer with a 3' degenerate anchor and a 5' universal tag in a 20 μl reaction. Reverse transcription was performed at 42˚C for 60 min and 95˚C for 5 min on a qTower 2.2 (Jena Analytik). 10 μl cDNA was diluted 50x with RNase-free water and added to 500 μl 2x PCR Master mix according to the protocol for the miRCURY LNA Universal RT microRNA PCR System (Exiqon A/S). 10 μl of each sample was assayed on a custom Pick-&-Mix microRNA PCR panel containing pre-spotted LNA primers (S1 Table). Both the CSF and the serum panel each consisted of n = 96 abundant and highly expressed miRNAs. N = 76 miRNAs were identical on both panels, but n = 20 different. All miRNAs were selected based on a preliminary pilot study [1] and a comparable study by Mooney et al. [25], that both used OpenArray technology and pre-amplification. A no-template control (NTC) of RNase-free water was co-purified and profiled like the samples to measure background. Amplification was performed on a qTower 2.2 (Jena Analytik) using the following thermal cycling protocol: 95˚C for 10 min, 45 amplification cycles at 95˚C for 10 s and at 60˚C for 60 s (fluorescent reading), followed by a melting curve analysis.

Pre-processing, quality control and normalisation

We adapted our RT-qPCR protocol to the MIQE guidelines in order to provide sufficient experimental details to increase the reliability of our data (S2 Table) [18]. Raw unprocessed fluorescence values were exported from the qTower 2.2 and analysed with the "qpcR" package [26]. Cq values were calculated by using the second derivative maximum after fitting a 5-parameter sigmoidal model to the fluorescence data [27]. In addition, qPCR runs with kinetic outliers were identified and removed [28]. Furthermore, all signals showing $<$ 80% valid data after applying the following QC criteria were excluded from analysis: (1) Signals with Cq $\geq$ 37 and dCq $\leq$ 3 (Ct_{NTC}–$Ct_{miR\text{-}x}$) were considered background, (2) all assays were inspected for distinct melting curves and (3) serum samples with a dCq $\geq$ 7 ($Cq_{miR\text{-}23a}$ –$_{miR\text{-}451}$) were considered at high risk of hemolysis [29].

NormFinder and GeNorm were used to identify potential reference miRNAs using GenEx [30, 31]. CSF samples were normalised to miR-101-3p, miR-320a and miR-99a-5p. Serum samples were normalised to let-7i-5p, miR-23a-3p, miR-23b-3p and miR-30e-5p. Relative expression was calculated as follows: $dCt = Ct\ (Ct_{mean\ RefmiR} - Ct_{miR})$ and $ddCt = mean\ dCt_{AD\ or\ bvFTD} - mean\ dCt_{HC}$. Higher values indicate higher expression. Only signals with a ddCt $\geq$ |0.58|, which corresponds to a fold change of $\geq$ |1.5|, were considered as differentially regulated.

Absolute quantification

Synthetic oligos that match mature miRNA sequences (S3 Table) were ordered from IDT (Integrated DNA Technologies) to prepare an initial pool of oligos each with a concentration of 33 nM. Standard curves were generated by preparing 4-fold serial dilutions (n = 7) each at a volume of 80 μl containing 0.8 μg/μl MS2 carrier RNA (Roche) following RT-qPCR as described earlier. Standards were measured as cDNA duplicates and PCR triplicates and assayed on a custom Pick-&-Mix microRNA PCR Panel containing n = 96 pre-spotted LNA primers (S3 Table). Background was measured with a no template control including water. Standard curves were obtained by fitting a regression line to the samples with known concentrations.

Statistics

Two-tailed unpaired Mann-Whitney t-tests at a significance level of 5% were used to determine statistical differences between two groups and chi square tests for dichotomous variables to examine demographic group differences. One-way MANOVA was applied to identify statistical differences between more than two groups, respectively. $P < 0.05$ (two sided) was considered as statistically significant. The false discovery rate was controlled by using the Benjamini–Hochberg procedure when conducting multiple comparisons. Correlations were determined using Pearson r for normally distributed data or the Spearman test when data were not normally distributed at a 5% significance level. To evaluate diagnostic accuracy we used receiver operating curves and discriminant analysis. Unsupervised hierarchical clustering and differential correlations were calculated by using the DiffCorr v0.4.1 package for R [32].

Exploratory Factor Analysis (EFA) permits examination of how unmeasured latent variables (factors) summarize patterns of correlations found in the measured relationships between miRNA expression levels. The following criteria for the factorability of a correlation were used: 1) correlation of all variables with at least 0.3 with at least one other variable, 2) the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy for each variable must be ≥ 7.5 and Bartlett's test of sphericity should be significant, 3) the diagonals of the anti-image correlation should be above 0.5, 4) the communalities above 0.3 and 5) only miRNAs based on factor loadings $\geq |0.5|$ were considered significant in contributing to the respective factor. A scree plot assisted of how many factors should be extracted. The eigenvalues indicate how much of the variance is explained by each factor. Calculation and graphics were done using XLStat (v19.4), GraphPad (v7.03) and SPSS (v24) software and the R 3.4.2 statistical programming language (R Development Core 2008).

Results

Characteristics of study population

As summarized in **Table 1**, no significant difference was observed in the distribution of age and gender. The bvFTD group had higher MMSE scores compared to the AD group ($p<0.05$). Classical protein biomarkers Tau and pTau were increased in CSF in the AD patients compared to the bvFTD and control cases ($p<0.0001$). Conversely, amyloid-beta$_{1-42}$ levels were decreased in both the AD and bvFTD group compared to the controls ($p<0.0001$). We also measured levels of the neurofilament light and the phosphorylated heavy chain but detected large variation in CSF. NfL levels were observed significantly higher ($p<0.05$) in bvFTD cases and pNFH levels were higher ($p<0.05$) in AD cases compared to controls. Since we were primarily interested in the sporadic, non-genetic bvFTD, we examined our samples for mutations in the genes *C9orf72*, *GRN* and *MAPT*. A total of 41 of the 48 bvFTD and 20 of the 48 AD cases were tested negative for the most prominent gene *C9orf72*. Further, no mutations in the genes

Table 1. Demographic and descriptive parameters for study population.

Characteristic[1] / samples	HC (CSF n = 44) (serum n = 38)[2]	bvFTD (CSF n = 48) (serum n = 48)	AD (CSF n = 48) (serum n = 47)[2]	p-value (ANOVA / x^2)		
				AD vs HC	AD vs bvFTD	bvFTD vs HC
Age (years)	64 ± 11.3	65 ± 9.2	65 ± 9.3	ns	ns	ns
Sex (male/female)	20/24	30/18	22/26	ns	ns	ns
MMSE	nm	24 ± 4	21 ± 5.3	na	< .05	na
Tau [pg/ml]	317.6 ± 118.8	433.4 ± 414.7	738 ± 288.9	< .0001	< .0001	ns
pTau [pg/ml]	35.1 ± 6.7	59.4 ± 37.2	96 ± 38.2	< .0001	< .0001	< .05
$A\beta_{1-42}$ [pg/ml]	1031.5 ± 272.2	872.1 ± 338	513 ± 160.6	< .0001	< .0001	< .05
NfL [pg/ml]	1449.1 ± 940.2	2706.4 ± 1816.2	2157.6 ± 1026.4	ns	ns	< .05
pNfH [pg/ml]	307.6 ± 151.8	464.56 ± 253.85	540.33 ± 244.96	< .05	ns	ns
Tested / not tested for mutation in *C9orf72, MAPT, GRN* (n)	na	41 / 7 11 / 37 11 / 37	20 / 28 11 / 37 11 / 37	na	na	na
Tested negative for mutation in *C9orf72/MAPT/GRN*	nm	41 11 11	20 11 11	na	na	na

[1] Data represent mean ± SD analysed by 1-way ANOVA (age and CSF biomarker) and t-test (MMSE). Gender ratio was analysed by $\chi 2$.

[2] Reduced number of serum samples did not lead to a significant change in demographical data. AD = Alzheimer's disease, bvFTD = behavioural variant frontotemporal dementia, HC = healthy controls, na = not applicable, nm = not measured.

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MAPT and *GRN* were identified in the tested AD (n = 11) and bvFTD (n = 11) cases. To address possible center-effects, we compared the baseline miRNA expression data of the study sites (S1 Fig).

Quality controls indicate low technical variation and consistent detection of miRNA levels in CSF and serum

With a mean Cq of 33.61 ± 2.2, the expression of circulating miRNAs in CSF was > 26-fold lower compared to serum with a mean Cq of 28.89 ± 3.66. This was also reflected by the increased number of positive signals detected for each miRNA in our serum data. In total, 77 miRNAs displayed ≥ 80% positive signals per miRNA in serum while this only applied to 55 miRNAs in CSF. RNA isolation was monitored by using a subset of synthetic miRNAs that simulate high (UniSp2), medium (UniSp4) and low (UniSp5) expressed signals to measure and compare extraction efficiency [29]. cDNA synthesis was controlled by UniSp6 and cel-miR-39. Our results demonstrate constant extraction efficiency across all samples with acceptable intra-assay variation as well as constant efficiency of the reverse transcription step with no signs of inhibition (S2A Fig). However, two serum samples were removed due to suspicious expression of spikes. We also checked the degree of hemolysis in our serum samples. The obtained data were not affected by erythrocyte miRNA contamination. Only a minority of serum samples indicated a possible but no high risk of hemolysis (S2B Fig). Using Normfinder and GeNorm, we were able to identify a subset of miRNAs for CSF and serum that considerably reduced technical variation compared to un-normalized data (S2C and S2D Fig). Furthermore, standard curves based on synthetic miRNA oligos were generated for a subset of assays to assess PCR efficiency and to estimate copy numbers. For the selected set of miRNA assays, PCR efficiencies were within an acceptable range of E = 0.95–0.98, which demonstrated low variation and good assay performance (S3 Fig). We also measured CSF and serum samples isolated at different days to test the inter-assay variance of our protocol. SYBR-green miRNA

assays displayed consistent expression with low inter-assay variance for serum (R^2 = 0.99, F(1,70) = 11078, p < .0001) and CSF (R^2 = 0.97, F(1,74) = 2215, p < .0001) showing a trend towards increasing day-day variation at higher expression levels as seen in the CSF data (S4 Fig).

Expression levels of circulating miRNAs in serum do not correlate with levels in CSF

Following quality control and the removal of low expressed signals, we were able to compare 34 of the 76 identical CSF and serum miRNA assays in 131 samples. Taking into account all subjects, an average correlation of r = 0.077 (p = ns) indicated no association of miRNA expression levels in serum compared to expression levels in CSF. If we considered expression levels of individual miRNAs, we observed that some of these correlated significantly between serum and CSF, however, only with weak (r<0.3) associations. This finding applied to all three subgroups. Taking into account the mean values of the respective subgroups, the control (r = 0.051, p = ns), bvFTD (r = 0.077, p = ns) and AD (r = 0.057, p = ns) group displayed no significant associations. However, expression levels of a few miRNAs showed significant correlations of moderate extent in the respective subgroups. This applied to both miR-19b-3p (r = 0.37, p = 0.023) and miR-25-3p (r = 0.45, p = 0.011) in the control group. Significant positive correlations of miRNA expression levels in serum with expression levels in CSF were also found for miR-143-3p (r = 0.34, p = 0.023), miR-29b-3p (r = 0.33, p = 0.025) and miR-29c-3p (r = 0.44, p = 0.002) in the bvFTD group. Interestingly, miR-24-3p showed a significant negative correlation, r = -0.33, p = 0.026. In the AD group, serum expression levels of let-7f-5p (r = 0.32, p = 0.032), miR-100 (r = 0.36, p = 0.017) and miR-143-3p (r = 0.32, p = 0.047) correlated positive with levels in CSF. In turn, we observed a significant negative correlation of miR-197-3p (r = -0.42, p = 0.005) and miR-30a-5p (r = -0.38, p = 0.012).

Cluster analysis of serum expression levels reveals miRNA families and genomic clusters altered in bvFTD and AD samples

As a first approach to understand the interaction of miRNAs, hierarchical clustering was applied to the serum dataset containing 131 samples measured for the expression of 96 miRNAs. To reduce the noise, we excluded miRNAs with low expression values (detailed in Material and Methods). Using average linkage bottom up clustering a total of 7 clusters in the control, 6 clusters in the bvFTD and 7 clusters in the AD group were detected (**Fig 1**). These clusters each contain 2–24 miRNAs (**Fig 1**). Notably, many cluster contained miRNAs with similar sequences (miRNA families) as well as miRNAs of a polycistronic unit that share a common promotor (referred to as genomic cluster, http://www.mirbase.org/, <10 kb). Genomic clusters containing only members of a particular family (homo-clusters) as well as clusters containing miRNAs with different seed sequences (hetero-clusters) were present. A good example for co-expression related to similar sequences is Cluster 7 in the control group that contains hsa-miR-27a and hsa-miR-27b or hsa-miR-148a and hsa-miR-148b in Cluster 4. In contrast, the genomic cluster let-7e/miR-99b/125a in Cluster 3 is an example of a hetero-cluster. In addition, we identified at least three Modules (dotted lines) in each condition. Modules are clusters of highly interconnected miRNAs that are characterized by dense interactions. Interestingly, the modules differed not only in terms of number and composition of miRNAs between the conditions but Modules 2 and 3 were also identified as densely connected groups in the bvFTD and AD group, which was not observed in the control group. As expected, we identified several pair-wise differential correlations in each condition with a trend of increased correlations in the bvFTD group. This was supported by the fact that some families such as

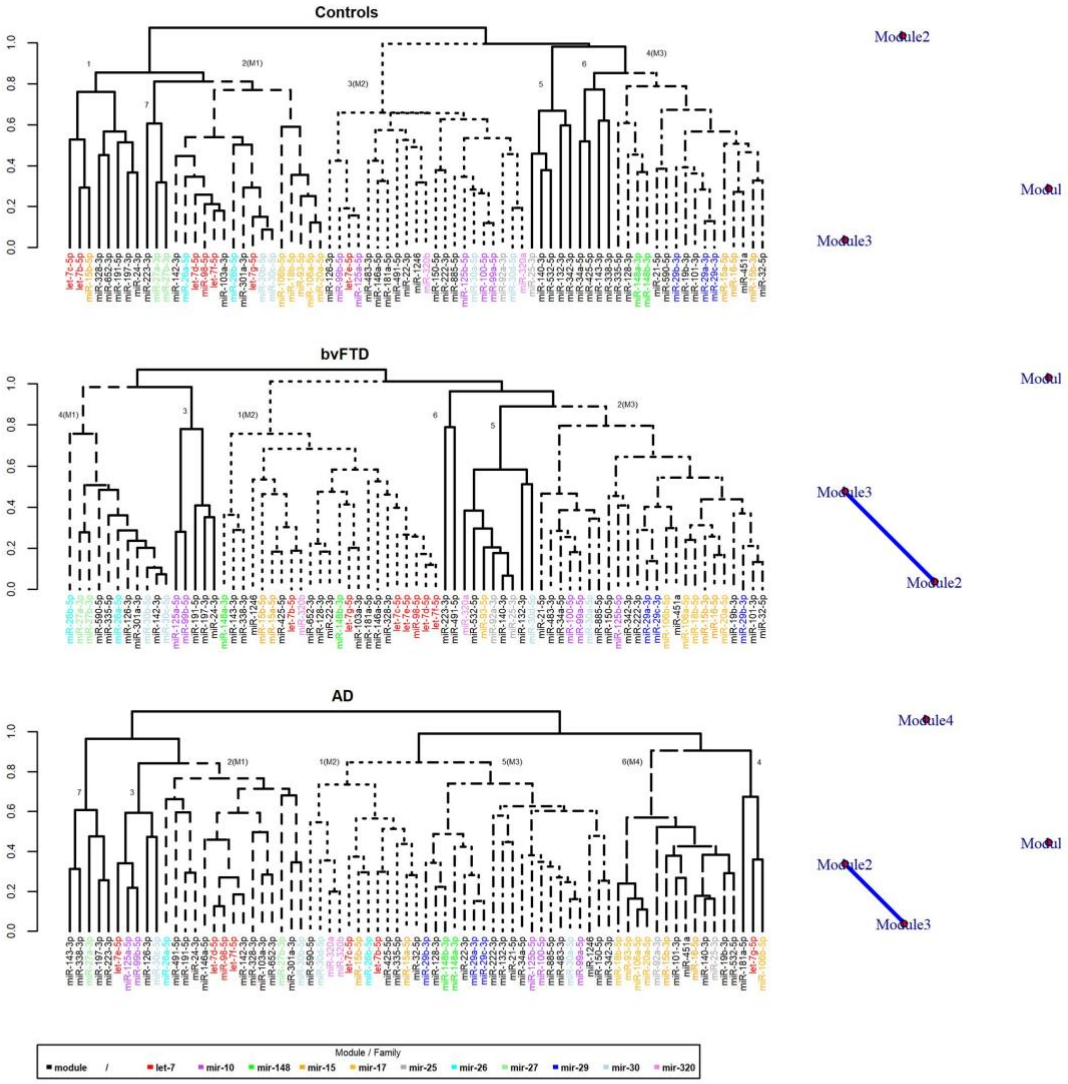

Fig 1. Unsupervised hierarchal clustering of miRNA levels measured in serum of healthy control, bvFTD and AD samples. Using the DiffCorr package, the genes were grouped according to their expression patterns in each subtypes (cognitively healthy controls, bvFTD and AD) using the cluster.molecule function. We used (1 − correlation coefficient) as a distance measure (the cutoff value was a coefficient of 0.6) based on the cutree function. We then visualized the module network using the get.eigen.molecule and get.eigen.molecule.graph functions. MiRNAs that share similar seed sequences (miRNA families) are coloured. MiRNAs that are co-transcribed as a polycistronic unit (http://www.mirbase.org/, < 10 kb) are listed in S5 Table. AD = Alzheimer's disease, bvFTD = behavioural variant frontotemporal dementia, M = Module.

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mir-17 or let-7 or clusters such as miR-29b/29a and miR-29b/29c appeared more intermixed in the bvFTD condition. S4 Table shows the top 20 significantly differential co-expressed miRNAs (FDR<0.05) in bvFTD cases compared to cognitively healthy controls.

Factor analysis reveals miRNA structure in serum, which allows the classification of bvFTD, AD and control cases

The variety of differential correlations in the individual conditions made an interpretation difficult. This led us to consider all samples simultaneously using factor analysis to reduce the number of manifest variables to a few hypothetical variables that could be associated with the diagnoses of our patient samples. The goal of factor analysis is to detect a small set of factors that elucidate as much of the variance of the output variables as possible.

The factorability of 73 miRNAs in serum was initially examined. Applying several well-recognised criteria [33–35], 29 miRNAs were tested, which led to an acceptable subject-to-item ratio of ~ 1:5. A total of 44 variables were eliminated because they either failed to meet the minimum criteria or did not contribute to a simple factor structure by primary factor loadings of <|0.5|. All items in this analysis had primary loadings over .5. Some of the variables showed cross-loadings, but most showed strong primary loadings and were therefore not excluded. Looking at the scree plot of the serum data, eigenvalues showed that in each case 34.97%, 19.56%, 12.73% and accordingly a total of 67.26% of the variation were explained by the first three factors (S5 Fig). The fourth factor also had an eigenvalue > 1, but explained only a small part of the total variability with 4.72%. As a result, the three-factor solution was preferred to the four-factor model as depicted in **Fig 2**. **Fig 2A** shows how the initial miRNAs are correlated with the three factors. We could observe that Factor 1 (green) positively correlated with hsa-let-7g-5p, -miR-101-3p, -106a-5p, -106b-5p, -18b-5p, -20a-5p, -26b-5p, -29b-3p, -301a-3p, -30b-5p and -27a-3p and negatively correlated with hsa-miR-1246, -146a-5p, -30d-5p, -miR-320a and -320b. In contrast. Factor 2 (red) positively correlated with hsa-let-7d-5p, -let-7f-5p and -miR-98-5p and negatively correlated with hsa-miR-15b-3p, -16-5p, -32-5p, -451a, -532-5p and -19b-3p whereas Factor 3 (brown) negatively correlated with hsa-let-7c-5p, -let-7e-5p, -miR-22-3p and miR-29c-3p. The factor loading matrix for this final solution is presented in S6 Table. Interestingly, the use of the coordinates of the observations of the two-factor model already resulted in a significant discrimination of cognitively healthy controls from the bvFTD and AD group (**Fig 2B**). More interestingly, the structure also visually indicated a trend towards a grouping of AD and bvFTD cases, however, with a certain number of false positive signals, respectively (**Fig 2B**). To improve the differentiation of the samples, the miRNAs of the third factor, which also contributed with 12.73% to the total variance, were taken into account in a subsequent discriminant analysis as shown in **Fig 2C**. After the data was split into a training- (n = 78) and validation (n = 53) set, 84.62% and respectively 60% of the total cases were successfully classified based on the analysis (**Fig 2C**). If the individual subgroups were considered, 100% of the controls, 70% of the bvFTD and 86% of the AD cases of the training set were correctly classified (**Fig 2C**). On the other hand, the validation set showed that 71% of controls, 67% of bvFTD and 44% of AD cases were correctly assigned (**Fig 2C**). Considering the entire data set (n = 131 serum samples), the controls were correctly predicted with 97%, the bvFTD cases with 77% and the AD cases with 72% accuracy (**Fig 2D**).

Looking at the CSF data, n = 15 miRNAs were suitable for factor analysis. This resulted in an acceptable subject-to-item ratio of ~ 1:9. The scree plot indicated that the first three factors had eigenvalues >1, which explained 26.33%, 24.25% and 9.29% of the variance, respectively (S5 Fig). Since we did not observe a trend towards a valuable separation of our samples using the CSF miRNA factor model, we further concentrated on our serum data.

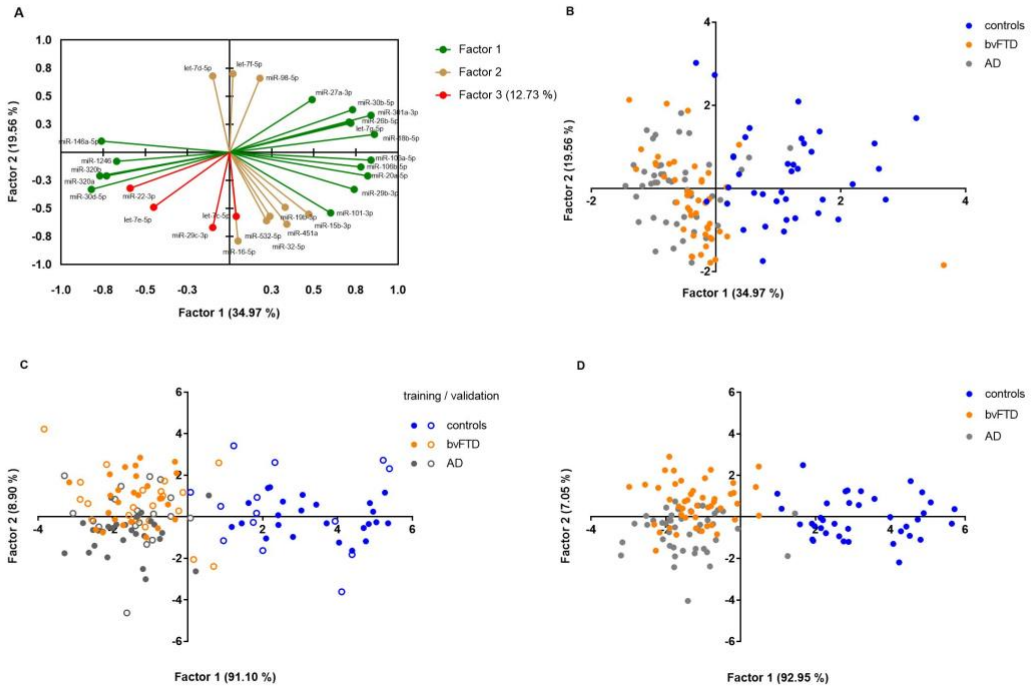

Fig 2. Factor and discriminant analysis of miRNA signals detected in serum. We have used factor and discriminant analysis to identify the variables that can best characterize and classify the bvFTD, AD and healthy control samples in our serum cohort. The following results are shown: (A) factor model of n = 29 serum miRNAs that load on the Factors 1–3 with factor loadings ≥ |0.5|, (B) observation plot showing coordinates of the observations resulting from the two-factor model (Factors 1–2), and result of the discriminant analysis using a re-factoring 2-factor model based on the n = 29 miRNAs from the original 3-factor model (**Fig 2A**) using (C) a training (n = 78) and validation (n = 53) set and (D) all cases (n = 131).

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Expression analysis identifies significantly de-regulated miRNAs between bvFTD, AD and control samples

Next, we tested whether the miRNAs of the 3-factor model as well as other miRNAs in our CSF and serum data displayed significantly different expression levels and to what extent these signals contributed to the classification of our samples by calculating corresponding AUC values. Using MANOVA, we identified a number of miRNAs, all of which were differentially expressed over a ddCt of |0.58| (S7 Table). Except for miR-30d-5p and miR-101-3p, all signals of Factor 1 were significantly de-regulated. In contrast, only miR-22-3p of Factor 3 was found as differentially expressed but none of the signals from Factor 2.

In our CSF data, we identified a total of 10 miRNAs with significantly different expression levels (Fig 3A). The best classifier was miR-125a-5p that discriminated bvFTD cases with 72% sensitivity and 81% specificity as well as AD cases with 74% sensitivity and 82% specificity from our controls (Fig 3B). In contrast, we also observed miRNAs with significantly different expression levels between AD and bvFTD patients (Fig 3A), which showed more moderate classification performance. With an AUC of 0.73 (95% CI, 0.623–0.832), miR-30a-5p yielded

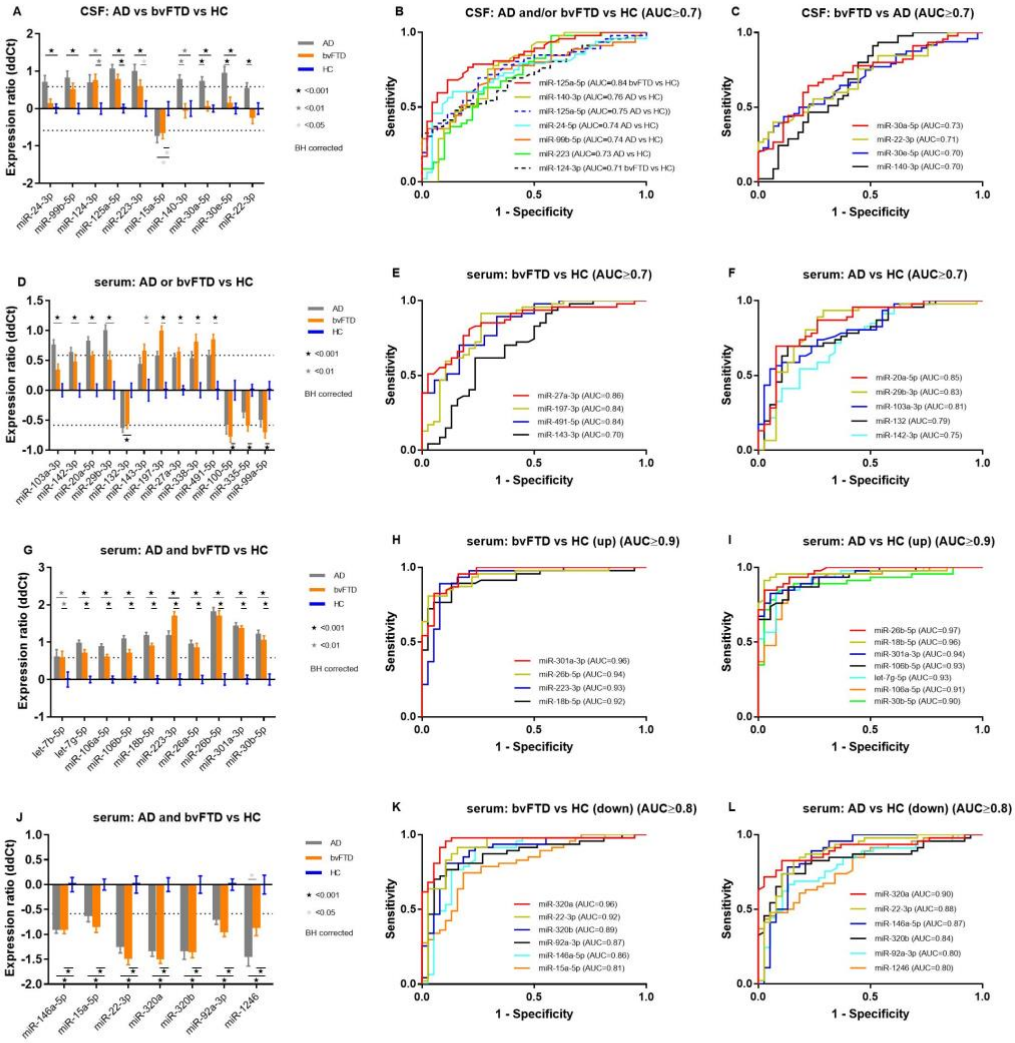

Fig 3. Differentially expressed miRNAs in bvFTD, AD and cognitively healthy control cases detected in CSF and serum. Expression levels of n = 96 circulating miRNAs were measured in CSF (n = 140) and serum (n = 131) samples from bvFTD (n = 48/48) and AD patients (n = 48/47) as well as healthy controls (n = 44/38) and compared using MANOVA and ROC curves. Displayed are signals with significantly different expression levels after multiple comparisons detected in (A) CSF: group comparisons of AD, bvFTD and healthy controls with (B-C) corresponding ROC curves and (D) serum: group comparisons of AD, bvFTD and healthy controls (up- and downregulated miRNAs) with (E-F) corresponding ROC curves, (G) serum: group comparisons of AD, bvFTD and healthy controls (only upregulated miRNAs) with (H-I) corresponding ROC curves and (J) serum: group comparisons of AD, bvFTD and healthy controls (only downregulated miRNAs) with (K-L) corresponding ROC curves. Expression ratio: $ddCt = mean\ dCt_{AD\ or\ bvFTD} - mean\ dCt_{HC}$. Dotted lines indicate ddCt cut-off of |0.58|. Error bars indicate mean ± SEM. BH = Benjamini-Hochberg.

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the best classification by separating bvFTD from AD cases with 78% sensitivity and 68% specificity (Fig 3C).

In our serum data, a total of n = 31 miRNAs were identified with significantly different expression levels between our subgroups. Two basic expression patterns could be observed. One was characterized by signals that were differentially regulated in the bvFTD or AD group compared to the controls (Fig 3D). Here, bvFTD cases separated best from controls with 77% sensitivity and 72% specificity by miR-27a with an AUC of 0.86 (95% CI, 0.775–0.935) (Fig 3E). In contrast, miR-20a-5p demonstrated an AUC of 0.85 (95% CI, 0.768–0.938) and the highest specificity of 92%, whereas miR-29b-3p displayed an AUC of 0.83 (95% CI, 0.738–0.931) by separating AD cases from controls with 93% sensitivity (Fig 3F). In the second expression pattern, miRNAs were either significantly up- or downregulated both in bvFTD and AD patients compared to controls (Fig 3G and 3J). For example, miR-301a-3p demonstrated an AUC of 0.96 (95% CI, 0.918–0.996) and classified bvFTD cases with high sensitivity (96%) and specificity (84%), whereas miR-26b-5p showed and AUC of 0.97 (95% CI, 0.940–0.999) and classified AD cases with 89% sensitivity and specificity with respect to our control group (Fig 3H and 3I). Accordingly, miR-320a was the best classifier against controls observed as collectively down regulated in bvFTD and AD. ROC analysis resulted in an AUC of 0.96 (95% CI, 0.909–1.003), which classified bvFTD cases with 96% sensitivity and 90% specificity and an AUC of 0.90 (95% CI, 0.835–0.969) to predict AD cases with 83% sensitivity and 90% specificity (Fig 3K and 3I).

We also examined the respective groups on a gender-specific basis. As expected, most signals showed the same differentially expressed levels in both men and women. Interestingly, in our serum data, we identified signals with increased classification performance to classify bvFTD from AD in the male but not in the female cohort compared to the original analysis that considered both males and females. Signals miR-103a-3p (AUC = 0.80), miR-106a-5p (AUC = 0.80) and miR-1246 (AUC = 0.85) demonstrated increased sensitivities (70–77%) and specificities (75–85%) to classify bvFTD from AD cases. This trend was also overserved in our CSF data but less significant in terms of classification performance.

MiRNAs from factor model in serum correlate with levels of amyloid-beta$_{1-42}$ and neurofilaments light chain detected in CSF

As described previously, we were unable to observe a global relationship between miRNA expression levels in serum and CSF. Instead, only a weak trend towards associations of single signals was observed. However, we were also interested in whether the expression levels of miRNAs correlated with those of classical protein biomarkers in the CSF. As a result, we found a large number of significant correlations of our serum miRNAs with these biomarkers. The most interesting finding was that individual miRNAs that correlated with Factor 1 predominantly correlated with amyloid-beta$_{1-42}$ (Fig 4A) whereas miRNAs that correlated with Factor 2 primarily correlated with pNfH (Fig 4B). For example, miR-320a ($r = 0.54$, $p = 0.028$) showed a significantly positive correlation, whereas miR-18b-5p ($r = -0.42$, $p = 0.042$) displayed a significantly negative correlation with CSF amyloid-beta$_{1-42}$ levels. Both signals also positively correlated with Factor 1 (Fig 2A). Another example are let-7d ($r = -0.52$, $p<0.001$), let-7f ($r = -0.48$, $p<0.001$) and miR-98-5p ($r = -0.44$, $p = 0.005$), all of which show significantly negative correlations with CSF pNFH levels (Fig 4B). These signals were also found to positively correlate with Factor 2 as identified by factor analysis (Fig 2A).

Discussion

In the present study we I) collected samples from multiple clinical centers across Germany, II) defined 3 homogenous patient groups with increased sample sizes (bvFTD n = 48, AD n = 48

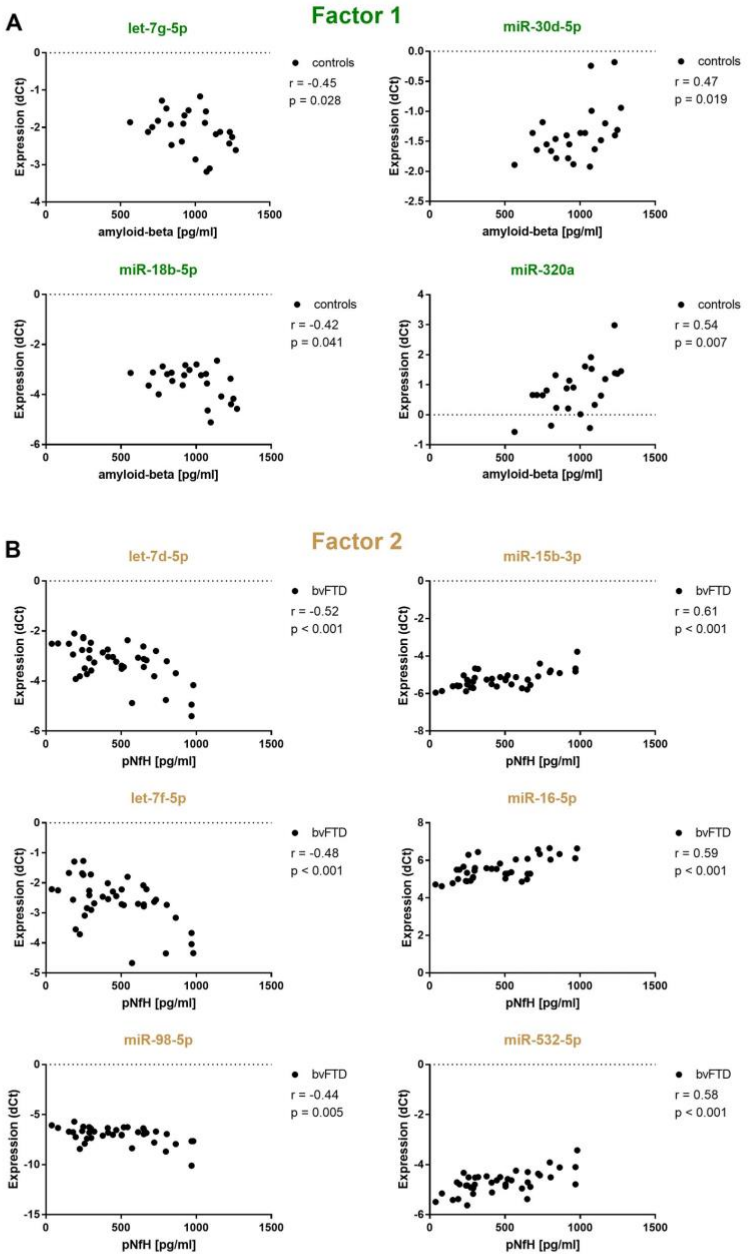
A
Factor 1
let-7g-5p
controls
r = -0.45
p = 0.028
Expression (dCt)
amyloid-beta [pg/ml]
miR-30d-5p
controls
r = 0.47
p = 0.019
miR-18b-5p
controls
r = -0.42
p = 0.041
miR-320a
controls
r = 0.54
p = 0.007
B
Factor 2
let-7d-5p
bvFTD
r = -0.52
p < 0.001
pNfH [pg/ml]
miR-15b-3p
bvFTD
r = 0.61
p < 0.001
let-7f-5p
bvFTD
r = -0.48
p < 0.001
miR-16-5p
bvFTD
r = 0.59
p < 0.001
miR-98-5p
bvFTD
r = -0.44
p = 0.005
miR-532-5p
bvFTD
r = 0.58
p < 0.001

Fig 4. Correlations of miRNA expression levels in serum with CSF protein biomarkers. Depicted are normalized expression levels $dCt = Ct\ (Ct_{mean\ RefmiR} - Ct_{miR})$ of (A) miRNAs from the original 3-factor model that correlated with Factor 1 vs CSF levels of amylod-beta$_{1-42}$ in the control group and (B) miRNAs from the original 3-factor model that correlated with Factor 2 vs CSF levels of pNfH in the bvFTD group. pNfH = phosphorylated neurofilament heavy chain.

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and cognitively healthy controls n = 44), III) compared expression levels in both CSF and serum samples and IV) focused on a limited set of miRNAs. Many studies use RT-qPCR to search for circulating miRNA biomarkers but do not consider the MIQE guidelines, do not control for hemolysis in serum or plasma samples, or use an inappropriate normalization method. We included appropriate quality control procedures that identified and reduced known (pre-) analytical sources of variation (S1–S4 Figs) and adapted our protocol to the MIQE guidelines (S2 Table) to increase the experimental transparency and reliability of our data.

One major finding was that we did not observe a strong association of miRNA expression levels throughout the cohort or subgroups between our CSF and serum samples. This is consistent with results from a comparable study by Wang et al. and Freischmidt et al., who also observed a poor association of miRNA levels in CSF and serum [36, 37]. Wang et al., however, showed an increased correlation of CSF miRNA levels with miRNAs from the Choroid Plexus [37]. In our case single miRNAs such as miR-29b and miR-29c indicated a small trend for a correlation (R^2 = 10–20%) between CSF and serum. However, these miRNAs are known to control fibrinogen production, which is a basic pathway both in the brain and the periphery [38]. In summary, the results point to autonomous networks that may independently respond to neurodegenerative processes and show little similarity in cognitively healthy controls. However, one has to keep in mind that low CSF levels point to low input into CSF and that levels might dilute out during circulation and after blood-brain-barrier transport. This might be another reason why potential changes in CSF cannot necessarily be seen in serum.

The main goal was to identify miRNA signatures to classify our bvFTD, AD and cognitively healthy control cases using multivariate statistics. To increase reliability, we focused on the serum data, which contained higher quality signals compared to the CSF data. One approach towards this aim was to use co-expression analysis. The first step included unsupervised cluster analysis to analyse possible interactions within each subgroup. An interesting observation was that the cluster analysis, which based on cell-free serum expression levels, grouped many signals according to their affiliation with a polycistronic unit (e.g. miR-106a/-b cluster) or a particular miRNA family (e.g. mir-17, let-7, mir-15 or mir-320 family). These (co-)expression patterns are known to occur within tissue cells [39, 40], different blood cell types [41] or whole blood [42]. The result therefore suggested that the profile of circulating miRNAs in serum appears to be more similar to the cell than expected but also point to a more unspecific release into the circulation. Interestingly, Leidinger and colleagues identified similar miRNA clusters and families as specifically enriched in blood cell populations positive for different CD markers, which supports this idea [41]. However, the extent to which members of a miRNA family (independent transcription units) displayed similar expression patterns in serum was unexpected because the mechanisms responsible for interchromosomally-coordinated co-expression are not yet fully understood and are the subject of current research [43]. In the second step we calculated differential correlations to better compare the individual profiles between our subgroups. However, the increased number of differential correlations made the interpretation difficult and prompted us to use factor analysis, which considered samples of all subgroups. The initially 73 serum miRNAs were reduced by ~40% to 29 signals, which in turn resulted in a three-factor miRNA model.

Factor 1 correlated with miR-106b-5p and miR-20a-5p, which in turn belong to the mir-106b~25 and mir-17 cluster. Both were found as significantly upregulated in bvFTD and AD, which is in line with results from Cheng et al, who investigated and validated miRNA levels isolated from serum exosomes using RNA seq [44]. In addition, both signals demonstrated a good to excellent diagnostic accuracy to distinguish AD cases from controls. Interestingly, these miRNAs were also shown to regulate APP *in vitro* and in neuronal cell lines [45]. In addition, we identified a differential correlation pattern for the mir-30b/30d cluster. We observed that miR-30b-5p was positively and miR-30d-5p negatively correlated with Factor 1 and we further confirmed significantly upregulated expression levels in bvFTD and AD compared to our controls. Notably, miR-30b-5p was found to correlate with amyloid plaque density by a study of Burgos et al. [5]. In addition, we found miR-30d-5p (R^2 = 22%) to positively correlate with CSF amyloid-beta$_{1-42}$ levels in our cognitively healthy controls. This also applied to miR-320a (R^2 = 30%), which is predicted to downregulate APP [46], whereas its family member miR-320b was found as a possible regulator of human-specific neural development [47]. We could also confirm both signals as significantly down-regulated in bvFTD and AD compared to our cognitively healthy controls. Overall, miR-320a and miR-320b showed a good to excellent diagnostic accuracy to correctly separate bvFTD and AD cases from controls, with miR-30b-5p ranking only AD cases correctly.

Factor 2 correlated positively with the let-7f/7d and let-7f/mir-98 clusters, whose members belong to the let-7 family, but we could not observe significantly different expression levels. However, let-7d and miR-98 were found as significantly down-regulated in AD compared to controls by Burgos et al. [5]. Interestingly, miR-98-5p was also found to act as a target for AD by regulating the production of beta-amyloid through modulating SNX6 Expression [48]. Another study showed, that inhibition of miR-98 in N2a/APP cells up-regulated the IGF-1 protein level and suppressed Aβ production [49]. Interestingly, we found all members of the let-7f/7d (R^2 = 23–27%) and let-7f/mir-98 (R^2 = 19%) cluster to negatively correlate with CSF pNfH levels in the bvFTD group. This also applied to miR-532 (R^2 = 34%) and the mir-15 family members' miR-15b-3p (R^2 = 37%) and miR-16-5p (R^2 = 37%) that negatively correlated with Factor 2. miR-15b-3p displayed significantly upregulated expression in AD and miR-16-5p significantly downregulated expression levels in bvFTD and AD compared to controls, however, each below a ddCt of .58. In addition, both miRNAs correlated significantly positively with CSF pNfH levels in the bvFTD group. Neurofilaments are major proteins of neurons and are particularly concentrated in axons and detection in CSF provides information about the degree of axonal injury [50] and was found a biomarker for genetic frontotemporal dementia [51, 52]. Notably, Burgos et al. identified miR-16-5p to negatively correlate with Braak stage [5], which supports the idea of miR-16-5p as a potential marker of neuronal injury. Furthermore, the miR-15 family has also been shown to modulate Tau phosphorylation through ERK1 leading to neuronal death in Neuro2a cells and primary cortical neurones [53]. However, we did not observe significant correlations with CSF Tau or pTau levels in our groups.

Factor 3 contained no more than 2 members of a specific miRNA family. With the exception of the miR-29 family. In this case, miR-29c-3p correlated negatively with Factor 3, with the family member miR-29b-3p positively correlating with Factor 1. In contrast, miR-29a-3p did not correlate with any of the three factors but was found to negatively correlate with CSF Tau (r = -0.56) and pTau (r = -69) levels (data not shown). However, we only found expression levels of miR-29b-3p as significantly upregulated in AD compared to our controls. Interestingly, Hebert et al. identified the mir-29a/b-1 as significantly decreased in AD brain as well as its regulation of amyloid-beta levels by upregulated levels of BACE1 [54]. miRNAs correlating

with Factor 2 and Factor 3 showed an overall lower diagnostic potential compared to those correlated with Factor 1. Except miR-29b-3p (AUC = 0.83) for the classification of AD cases.

In summary, all signals from the three-factor model explained in total > 67% of the variance. We therefore calculated a subsequent discriminant analysis to evaluate the performance of this model to classify our subgroups. As expected from the structure, the miRNA signature was able to identify cognitively healthy controls with 97% accuracy. The result outperforms other blood-based assays such as the detection of amyloid levels [55] and performs at least as well [56–58], if not better [59–61] compared to miRNAs in blood that have been identified in other studies. More interestingly, the same signature was able to classify bvFTD cases with 77% and AD cases with 72% accuracy and can at least in part compete with results from traditional protein based tests in CSF [62]. This is mostly due to the fact that the majority of miRNAs that were either up- or downregulated in AD compared to our cognitively healthy controls displayed a similar expression pattern in bvFTD patients. We believe that these rather unspecific signals are mainly due to the underlying neurodegeneration observed in AD and bvFTD. There were basically no signals on our panel that showed an opposing expression except for those that have been identified in our gender specific analysis as mentioned earlier. However, this should be the focus of further studies as specific signals would improve diagnosis by helping to exclude other dementias.

Concerning our CSF data, we identified individual miRNAs that showed significantly different expression levels and displayed diagnostic potential. For example miR-125a-5p that discriminated cognitively healthy controls from AD with good (AUC = 0.84) or miR-30a-5p that classified bvFTD from AD cases with moderate (AUC = 0.73) accuracy. This is in line with Cogswell, who also identified these signals as significantly upregulated in AD [63]. However, due to quality control we lost some signals in our CSF data and factor analysis did not reveal a miRNA signature with high discriminatory value.

In summary, our data show that our circulating miRNA profile in CSF was not comparable to that in serum and that serum miRNAs were better detectable compared to those in CSF. In addition, circulating miRNAs in serum show a strong tendency to form clusters, either because of their genomic organization or because of homologies in their sequences. Co-expression analysis displayed differently co-expressed miRNAs between our subgroups. However, the identified associations were complex and difficult to interpret. In addition, possible associations of other miRNAs could not be demonstrated, since we did not include related signals on our serum panel. We could also show that multivariate methods such as factor analysis can identify miRNA signatures in serum able to classify bvFTD, AD and control cases with acceptable diagnostic accuracy. Due to their genomic organization and transcriptional expression pattern we therefore think that it is more likely to identify a biomarker consisting of either members of a miRNA cluster or family compared to single miRNAs. Another interesting observation was that particularly miRNAs associated with our factor model also correlated with CSF amyloid-beta$_{1\text{-}42}$ and phosphorylated neurofilament heavy chain levels either with our control- or bvFTD group. This suggested that de-regulated miRNAs of a family or cluster may possibly be able to monitor the neurodegenerative progression seen in AD or bvFTD. However, further evidence from cell culture experiments is necessary to better describe the functional associations of these miRNAs. Overall, when analysing miRNA clusters and families, it must be noted that it is based on current understanding and annotation and that this relationship will change over time whenever new miRNA species are identified. However, unsupervised clustering is based on what was actually measured, thus reduces this bias in part and is therefore well suited for the detection of networks. However, one shortcoming towards the identification of miRNA signatures using cluster and factor analysis is the limited number of signals in our study. The (factor-) structures identified by us result from a small part of the

miRNome that circulate in serum. However, there are other related (S5 Table) and novel miRNAs that we did not measure but that may further increase complexity. The last miRBase update was 2014. Novel miRNAs have been identified by NGS, but their expression levels have not yet extensively been investigated using qPCR and are not yet available on PCR panels. An updated selection of abundant signals could thus provide novel candidates for miRNA profiling studies. A shortcoming towards our study group was that not all individual were tested for one of the disease causing mutations. However, the probability of carrying one of the tested mutations is generally low and the majority was tested negative so that a few possible mutation carriers should not bias the results. Furthermore, some variation at baseline miRNA expression was observed across the different study sites. We think that (possible) centre effects should affect all miRNAs equally (due to a harmonized and standardised protocol for sampling and extraction) so that variation on a single miRNA basis should not occur. The variation may possibly be the result of a numerically unequal distribution of the patient groups from the different centres. Multi-centre studies should therefore make sure that the proportion of patient groups from the various centres is as equal as possible. Considering the discussed points in follow up studies will greatly contribute to identify other relevant miRNAs to better understand the complex expression pattern overserved in body fluids, and may further improve the classification performance to separate AD and bvFTD cases.

Supporting information

S1 Fig. Baseline expression across all miRNAs between different study sites. A main effect for baseline expression levels (across all miRNAs) between study sites was found in our serum ($F(9, 121) = 5.21$, $p < 0.001$) and CSF ($F(9, 130) = 4.50$, $p < 0.001$) data. Serum expression levels from the study site in Ulm ($M = 29.17$, $SD = 1.06$) were lower compared to München-tu ($M = 27.86$, $SD = 0.61$), Homburg ($M = 27.74$, $SD = 0.29$), Erlangen ($M = 27.9$, $SD = 0.46$) and Hamburg ($M = 28.3$, $SD = 0.93$). In contrast, CSF expression levels from the study site in Göttingen ($M = 32.42$, $SD = 1.23$) were higher compared to Ulm ($M = 33.59$, $SD = 0.65$), München-tu ($M = 33.56$, $SD = 0.49$) and Erlangen ($M = 33.95$, $SD = 0.54$). However, each difference was below the critical threshold of $ddCt < |0.58|$ except for Göttingen vs. Erlangen ($ddCt = 0.62$).
(TIF)

S2 Fig. Pre-analytical variation of circulating miRNA in CSF and serum samples. a) Box plot (whiskers: 2.5–97.5 percentile) of synthetic miRNAs display low technical variation with acceptable intra-assay variation of UniSp2: Cq_{CSF} 17.59 ± 0.37 and Cq_{serum} 17.49 ± 0.49; UniSp4: Cq_{CSF} 24.92 ± 0.52 and Cq_{serum} 24.42 ± 0.59 and with a trend of increasing variation towards the isolation of lower expressed transcripts like UniSp5: Cq_{CSF} 30.65 ± 0.92 and Cq_{serum} 31.23 ± 0.51. UniSp6: Cq_{CSF} 18.63 ± 0.46 and Cq_{serum} 17.59 ± 0.16 and cel-miR-39 Cq_{serum} 24.07 ± 0.25 were used to monitor the cDNA synthesis reactions and indicated constant RT efficiency with no signs of inhibition. b) The hemolysis plot indicates expression ratios of constant miR-23a and red blood cell sensitive miR-451a to monitor serum samples for signs of cellular contamination or hemolysis. With a mean $dCq_{miR\text{-}23a\ -miR\text{-}451} = 4.05 \pm 1.07$, most of the serum samples did not display signs of hemolysis ($dCq \leq 5$, yellow line). Only a few signals showed a $dCq > 5$ but none of the samples appeared at high risk of hemolysis ($dCq \geq 7$, red line). c,d) The cumulative distribution plots display different miRNA normalisation strategies applied on the serum and CSF data. Normalization with reference miRNAs identified by NormFinder and GeNorm resembled normalisation to the global mean and considerably reduced technical variation compared to un-normalized data or data normalized to internal standards.
(TIF)

S3 Fig. miRNA standard curves for SYBR green miRCURY PCR assays. Plotted are mean Cq values from n = 4 replicate standard curves vs the log2 copy numbers. Standard curves were generated for a subset of assays by using a dilution series of a pool of known input amounts of synthetic miRNA oligonucleotides corresponding to the target sequence of the assay. Red error bars depict mean Cq ± CI. R^2 = coefficient of determination, E = PCR efficiency ± CI, CI = confidence interval.
(TIF)

S4 Fig. Day-to-Day reproducibility. RNA from A) CSF and B) serum isolated with miRNeasy serum Kit on different days was measured on each corresponding 96-well panel. Interplate calibrated Cq values were plotted against each other to assess the degree of inter-assay variability by linear regression. R^2 = coefficient of determination.
(TIF)

S5 Fig. Scree plots of serum and CSF data. The scree plot shows the variance explained by each factor in a factor analysis and is used to assess the optimum number of factors to take into account for further analysis.
(TIF)

S1 Table. Raw Cq values of endogenous miRNAs (cut-off Cq < 37) detected in CSF and serum samples. Listed is the FOC (number of positive miRNAs), arithmetic mean of raw Cq values of each subgroup and the standard deviation, the miRBase accession and the miRNA sequence. bvFTD = behavioural variant frontotemporal dementia, Cq = quantification cycle, FOC = frequency of occurrence, HC = cognitively healthy controls, SD = standard deviation.
(XLSX)

S2 Table. MIQE checklist. Provided are necessary information recommended by the MIQE guidelines to increase experimental transparency of quantitative real-time PCR experiments.
(XLSX)

S3 Table. Sequence information of synthetic oligos used for absolute quantification.
(XLSX)

S4 Table. Top 20 list of differentially coexpressed miRNAs from the serum dataset. Shown are significantly pair-wise differential correlations (FDR < 0.05) of miRNA expression levels in serum between cognitively healthy controls and bvFTD cases using the comp.2.cc.fdr function from the DiffCorr package. r = Pearson correlation coefficient, lfdr = local false discovery rate.
(XLSX)

S5 Table. Table of miRNAs used for hierarchal cluster analysis. Depicted are 1) miRNAs, 2) sequence information, 3) clustered miRNAs on genome (http://www.mirbase.org/, <10 kb), 4) paralogous miRNAs, 5) location on chromosome, 6) miRNA family and 7) correlation with Factor 1–3 from factor analysis. miRNAs in red were not included on our serum panel.
(XLSX)

S6 Table. Factor loadings and communalities based on a principal factor analysis for n = 29 miRNAs detected in n = 131 serum samples. Only miRNAs based on factor loadings ≥ |0.5| were considered significant in contributing to the respective factor. F = Factor.
(XLSX)

S7 Table. Differentially expressed miRNAs detected in serum and CSF. Listed are miRNAs that displayed significantly different expression levels in either serum or CSF samples between cognitively healthy control, bvFTD and AD cases. Fold change is calculated as $ddCt = dCt_{CtRef-CtmiRNA} - dCt_{CtRef-CtmiRNA}$ where larger values reflect higher abundance. AD = Alzheimer's disease, bvFTD = behavioural variant frontotemporal dementia, HC = cognitively healthy controls, p = p-value.
(XLSX)

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5 Summary / Zusammenfassung

Currently, there is not a single parameter that meets the high requirements of a reliable biomarker to diagnose severe forms of dementia, such as Alzheimer's disease. MicroRNAs are small, noncoding ribonucleic acids that regulate the activity of genes and, through their beneficial properties, are promising candidates as biomarkers in diagnostics and as potential targets for therapeutic intervention.

In this cumulative dissertation I summarize my contribution to two research articles. They describe the characterization of circulating microRNAs as alternative biomarkers for Alzheimer's disease and frontotemporal dementias.

In the first publication, a broad range of different microRNAs was measured in the cerebrospinal fluid of Alzheimer's disease patients and a heterogeneous control group by means of pre-amplification-based quantitative PCR high-throughput technology (TaqMan OpenArray, ABI). It became clear that only a small part of the microRNAs could be detected, whereby the proportion of strongly expressed and thus frequented signals was lower. Accordingly, a greater portion of the data showed incomplete expression levels, which correlated more strongly with the expression frequency. Relative quantitation identified a variety of differentially regulated microRNAs in the Alzheimer’s group. Using the exploratory "MoR" algorithm, the abundant signals miR-1274a, miR-146a, and miR-100 were found to be reliable and the less abundant signals miR-505-5p, miR-4467, miR-766, miR-375, miR-708, miR-3622b-3p, miR-296, miR-219 and miR-103 were identified as informative and by means of MANCOVA also as statistically significant biomarkers. Interestingly, the degree of information of some microRNA candidate biomarker was identical to that of the traditional Alzheimer protein biomarkers tau, pTau and even higher compared to $A\beta_{42}$. The combination of miR-100, miR-103 and miR-375 finally demonstrated a classification rate of 96%, whereby

controls and Alzheimer's patients were correctly classified with an accuracy of 96.4% and 95.5%, respectively. In addition, a complex correlation pattern of miR-146a with Tau and $A\beta_{42}$ was identified, suggesting miR-146a to be involved in the pathogenesis of Alzheimer's disease. In summary, the study showed that the detection of circulating microRNAs in the cerebrospinal fluid by high-throughput PCR is possible but careful consideration should be given to the microRNAs identified as potential biomarker candidates, since the majority of microRNAs are expressed only in small amounts. Based on the findings and limitations of the first study as well as on further literature research, in the follow-up study I 1) changed detection chemistry (miRCURY, Exiqon) and profiled miRNAs without pre-amplification, 2) increased the number of samples to 44 healthy controls and 48 patients with Alzheimer's disease and frontotemporal dementia, 3) incorporated improved quality controls and 4) measured two comparable panels of 96 microRNAs each in cerebrospinal fluid and also in serum. Overall, the signals were significantly more expressed in the serum and thus better to detect in comparison to those in cerebrospinal fluid. Standard curves and inter-assay experiments showed that the miRCURY assays also measured linearly towards the low range, have consistently high amplification efficiencies, and the day-to-day reproducibility of the signals in serum was ~ 99% and in cerebrospinal fluid ~ 97%, respectively. Further quality measures identified reference microRNAs for normalization and controlled potential contamination by hemolytic serum samples. One goal was to compare the microRNA pool of cerebrospinal fluid and blood serum. In contrast to other protein biomarkers such as neurofilaments, however, no association could be observed globally between microRNA expression levels in cerebrospinal fluid and serum. The result of a cluster analysis suggested that the circular microRNA profile closely resembles that in the cell, which would enable and support the detection of intracellular changes also in body fluids. While factor analysis of serum data ultimately

resulted in a complex 3-factor model classifying healthy controls with superior performance, the cerebrospinal fluid microRNAs displayed rather moderate differences between Alzheimer's disease and frontotemporal dementias. Interestingly, a significant portion of microRNAs from the 3-factor model correlated on the one hand with cerebrospinal fluid protein levels of neurofilaments and on the other hand with those of $A\beta_{42}$ (R^2~20-40%).

Overall, the experiments show that microRNAs in serum and CSF can are promising biomarker candidates for Alzheimer’s disease and frontotemporal dementia. However, further studies, with larger cohorts and stricter quality controls, are needed to improve and ensure the reliability and reproducibility of the data as well as their comparability in the field.

Aktuell gibt es keinen einzigen Parameter, der den hohen Anforderungen eines aussagekräftigen Biomarkers zur Diagnose von schwerwiegenden Formen von Demenzen wie der Alzheimer Krankheit entspricht. MikroRNAs sind kleine, nicht-kodierende Ribonukleinsäuren, die die Aktivität von Genen regulieren und durch ihre positiven Eigenschaften vielversprechende Kandidaten als Biomarker in der Diagnostik und als mögliche Ziele für therapeutische Maßnahmen darstellen.

In der vorliegenden kumulativen Dissertation fasse ich meinen Beitrag zu zwei Forschungsartikeln zusammen. Sie beschreiben die Charakterisierung von zirkulierenden mikroRNAs als alternative Biomarker für die Alzheimer Krankheit und frontotemporale Demenzen.

In der ersten Veröffentlichung wurde zunächst ein breites Spektrum an verschiedenen mikroRNAs in der Gehirn-Rückenmarks-Flüssigkeit von Alzheimer Patienten und einer heterogenen Kontrollgruppe mittels auf quantitativer PCR und Präamplifikation basierender Hochdurchsatz Technologie (TaqMan OpenArray, ABI) gemessen. Es hat sich herausgestellt, dass nur ein kleiner Teil der mikroRNAs nachgewiesen werden konnte, wobei der Anteil stark exprimierter und damit frequentierter Signale geringer war. Dementsprechend zeigte ein größerer Teil der Daten unvollständige Expressionslevel, wobei diese mit der Expressionsfrequenz stärker korrelierten. Relative Quantifizierung konnte eine Vielzahl differentiell regulierter mikroRNAs in der Alzheimer Gruppe nachweisen. Durch den explorativen „MoR“ Algorithmus konnten die abundanten Signale miR-1274a, miR-146a und miR-100 als reliable und die weniger abundanten Signale miR-505-5p, miR-4467, miR-766, miR-375, miR-708, miR-3622b-3p, miR-296, miR-219 und miR-103 als informative und mittels MANCOVA auch als statistisch signifikante Biomarker identifiziert werden. Interessant war, dass der Informationsgehalt einiger mikroRNA Biomarker Kandidaten im Vergleich zu den traditionellen Alzheimer Proteinbiomarkern Tau, pTau identisch und gegenüber Aβ42

sogar höher erschien. Die Kombination aus miR-100, miR-103 und miR-375 demonstrierte schließlich eine Klassifikationsrate von 96%, wobei Kontrollen und Alzheimer Patienten jeweils mit einer Genauigkeit von 96.4% und 95.5% korrekt klassifiziert werden konnten. Darüber hinaus wurde ein komplexes Korrelationsmuster von miR-146a mit Tau und Aβ42 identifiziert, wobei miR-146a eine mögliche Regulation der Alzheimer Pathogenese beigemessen wurde. Zusammenfassend ist der Nachweis von zirkulierenden mikroRNAs in der Gehirn-Rückenmarks-Flüssigkeit mittels Hochdurchsatz PCR möglich, wobei die als potentielle Biomarker Kandidaten identifizierten mikroRNA vorsichtig zu bewerten sind, da die überwiegende Anzahl an mikroRNAs nur in geringen Mengen exprimiert sind.

Basierend auf den Befunden und Limitierungen der ersten Studie als auch auf weiteren Literaturrecherchen wurde in einer Folgestudie 1) die Nachweismethode gewechselt (miRCURY, Exiqon) und auf eine Präamplifikation verzichtet, 2) die Anzahl der Proben auf 44 gesunde Kontrollen und je 48 Patienten mit Alzheimer und frontotemporaler Demenz erhöht, 3) verstärkt Qualitätskontrollen eingebaut und 4) zwei vergleichbare Panel von je 96 mikroRNAs in der Gehirn-Rückenmarks-Flüssigkeit und auch im Blutserum gemessen. Insgesamt waren die Signale im Serum deutlich stärker exprimiert und damit im Vergleich zu denen in der Gehirn-Rückenmarks-Flüssigkeit besser zu detektieren. Standardkurven und inter-assay Experimente zeigten, dass die miRCURY assays auch im niedrigen Bereich linear messen können, konsistent hohe Amplifikationseffizienzen besitzen und die Reproduzierbarkeit (Tag-zu-Tag) der Signale im Serum bei ~ 99% und in der Gehirn-Rückenmarks-Flüssigkeit bei ~97% lag. Weitere Qualitätsmaßnahmen identifizierten Referenz-mikroRNAs zur Normalisierung und kontrollierten mögliche Verunreinigungen durch hämolytische Serumproben. Ein Ziel war es, den mikroRNA Pool von Gehirn-Rückenmarks-Flüssigkeit und Blutserum zu vergleichen. Im Gegensatz zu anderen

Proteinbiomarkern wie den Neurofilamenten, konnte global jedoch keine Assoziation zwischen Gehirn-Rückenmarks-Flüssigkeit und Bluterserum beobachtet werden. Das Ergebnis einer Clusteranalyse legte nahe, dass das zirkuläre mikroRNA Profil stark dem in der Zelle ähnelt, wodurch intrazelluläre Veränderungen auch in Körperflüssigkeiten nachzuweisen wären. Während eine Faktorenanalyse der Serum Daten schließlich in einem komplexen 3-Faktor Modell resultierte, und gesunde Kontrollen mit hoher diagnostischer Güte klassifizierte, zeigten die mikroRNAs in der Gehirn-Rückenmarks-Flüssigkeit eher moderate Unterschiede zwischen der Alzheimer Krankheit und den frontotemporalen Demenzen. Interessant war, dass ein erheblicher Teil der mikroRNAs des 3-Faktor Modells einerseits mit den Proteinkonzentrationen von Neurofilamenten und andererseits mit denen von $A\beta_{42}$ aus der Gehirn-Rückenmarks-Flüssigkeit korrelierte (R^2~20-40%).

Insgesamt zeigen die Versuche, dass mikroRNAs im Serum und in der Gehirn-Rückenmarks-Flüssigkeit als potentielle Biomarker Kandidaten für die Alzheimer Krankheit oder frontotemporale Demenzen fungieren können. Allerdings sind weitere Studien, mit größeren Kohorten und strengeren Qualitätskontrollen notwendig, um die Reliabilität und Reproduzierbarkeit der Daten als auch ihre Vergleichbarkeit im Feld zu verbessern und zu gewährleisten.

6 Declaration of personal contribution to cumulated publications

Die Arbeit wurde in der Klinik für Psychiatrie und Psychotherapie des Universitätsklinikum Hamburg-Eppendorf unter Betreuung von Prof. Dr. med. Klaus Wiedemann durchgeführt. Die Konzeption der Studie erfolgte in Zusammenarbeit mit Dr. med. Holger Jahn (leitender Oberarzt).

Sämtliche Versuche wurden von mir eigenständig und mit Unterstützung von Christine Siegismund, Ph.D, durchgeführt.

Die statistische Auswertung erfolgte eigenständig durch mich und mit Unterstützung von Dr. rer. nat. Alexander Yassouridis.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Hamburg, den 14.09.2018

Unterschrift: .....

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8 Curriculum Vitae

Entfällt aus datenschutzrechtlichen Gründen.

9 Declaration on oath

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

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