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Plasma microRNA profiling distinguishes patients with frontotemporal dementia from healthy subjects

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ABSTRACT

The purpose of this study was to develop an easy and minimally invasive assay to detect a plasma miRNA profile in frontotemporal dementia (FTD) patients, with the final aim of discriminating between FTD patients and healthy controls (HCs). After a global miRNA profiling, significant downregulation of miR-663a, miR-502-3p, and miR-206 ($p = 0.0001$, $p = 0.0002$, and $p = 0.02$ respectively) in FTD patients was confirmed when compared with HCs in a larger case-control sample. Moreover, miR-663a and miR-502-3p showed significant differences in both genders, whereas miR-206, only in male subjects. To obtain a discriminating measure between FTD patients and HCs, we calculated a combined score of the 3 miRNAs by applying a Bayesian approach and obtaining a classifier with an accuracy of 84.4%. Moreover, for men, combined miRNA levels showed an excellent sensitivity (100%) and a good specificity (87.5%) in distinguishing FTD patients from HCs. All these findings open new hypotheses in the pathophysiology and new perspectives in the diagnosis of a complex pathology as FTD.

1. Introduction

Frontotemporal lobar degeneration (FTLD) includes a group of progressive brain diseases, etiologically and neuropathologically heterogeneous but sharing atrophy of the prefrontal and anterior temporal lobes, involving the right and left hemispheres, in some cases asymmetrically. The clinical features involve behavior and personality disturbances, language impairment, and in some cases, concomitant motor neuron disease or parkinsonism. This group of diseases accounts for 5%–15% of all cases of dementia and is the second most common cause of early-onset dementia after Alzheimer's disease (AD) (Cruts and Van Broeckhoven, 2008). Clinically, it is possible to distinguish 2 major clinical subtypes, behavioral-variant frontotemporal dementia (bvFTD), and primary progressive aphasia (PPA). The first subtype is characterized by behavioral symptoms, and the second one is constituted by a semantic variant PPA (svPPA) and nonfluent variant PPA (nfvPPA) in which the main feature is a progressive impairment of speech and language. In a neuropathological context, disease-specific protein aggregates are observed: hyperphosphorylated tau protein in neurons and glia (FTLD-tau) (Lee et al., 2001; Lee and Leugers, 2012), TDP-43 (FTLD-TDP) (Arai et al., 2006; Neumann et al., 2006), FUS-positive inclusions (FTLD-FUS) (Munoz et al., 2009; Neumann et al., 2009a, b), and ubiquitin proteasome system-positive inclusions (FTLD-UPS) (Holm et al., 2007, 2009). Finally, from a genetic point of view, mutations were found in different genes, including those encoding the microtubule-associated protein tau (*MAPT*), progranulin (*GRN*), chromosome 9 open reading frame 72 (*C9ORF72*), valosin-containing protein (*VCP*), charged multivesicular body protein 2B (*CHMP2B*), TAR DNA-binding protein 43 (*TDP-43*), and fused in sarcoma-binding protein (*FUS*) (Fontana et al., 2015; Sieben et al., 2012).

Despite the significant advances made in the last years, clinical diagnosis of neurodegenerative diseases (NDs) remains a challenge.

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Proteins in the cerebrospinal fluid can be measured: beta-amyloid protein 1–42, total tau, and phosphorylated tau protein are biomarkers supporting clinical diagnosis (Blazer, 2013; Fagan et al., 2011), whereas DJ1, alpha-synuclein, and BDNF diagnostic value in Parkinson's disease (PD) patients still needs to be confirmed (van Dijk et al., 2010); however, the invasiveness of the procedure makes this assay challenging for everyday clinical use.

In addition to proteins, microRNAs (miRNAs) have a demonstrated role as noninvasive markers in biofluids for several human pathologies (Keller et al., 2011). miRNAs are a class of small (18–25 nt-long) noncoding RNAs acting as negative regulators of gene expression (Bartel, 2004). miRNAs function via base-pairing with complementary sequences generally located within the 3′UTR of the mRNA target, but sometimes present in the coding sequence (CDS): an imperfect base-pairing results in a block of translation, whereas a perfect pairing causes mRNA degradation (Lewis et al., 2003). Over the last few years, a growing number of publications reported a dysregulation of miRNA expression in several diseases, including neurodegenerative disorders such as AD, PD, amyotrophic lateral sclerosis (ALS), and Huntington's disease (reviewed in Basak et al., 2016; Maciotta et al., 2013). Moreover, several studies support the potential of circulating miRNAs as biomarkers in biofluids, such as cerebrospinal fluid or plasma, derived from patients with different NDs (Grasso et al., 2014).

Also in frontotemporal dementia (FTD), miRNAs are playing an important role (reviewed in Piscopo et al., 2016a). For example, the progranulin gene has been reported to be under the post-transcriptional control of miR-29b, miR-107, and miR-659 (Hébert et al., 2008; Noren Hooten et al., 2010; Piscopo et al., 2016b).

To date, only two articles were published on circulating miRNAs as FTD biomarkers in biofluids (Sheinerman et al., 2017) (Denk et al., 2018). In the first study, the authors chose 37 miRNAs known from the literature to be brain-enriched and inflammation-associated and studied their levels in plasma, finding a dysregulation of 3 miRNA pairs compared with healthy subjects: miR-335/let-7e, miR-99b/let-7e and miR-9*/miR-181a (Sheinerman et al., 2017). In the second study, Denk et al. analyzed 96 miRNAs, with custom qRT-PCR panels, finding miR-320a and miR-26b-5p able to discriminate bvFTD cases with 96% sensitivity and 90% specificity and AD cases with 89% sensitivity and specificity.

The lack of information on wide miRNA profiling prompted us to embark in a nonbiased screening of the circulating miRNome in patients with FTD, to identify miRNAs useful as potential biomarkers in this pathology.

2. Materials and methods

2.1. Subject recruitment and assessment

For the pilot study, we randomly selected 10 of 150 patients diagnosed with FTD according to international criteria for diagnosis (Neary et al., 1998; Rascovsky et al., 2011), who were screened at the "Memory Clinic"—Department of Neurology and Psychiatry—University of Rome, "Sapienza," from September 2008 to May 2012. The healthy control (HC) group ($n = 10$) includes subjects free of dementia or cognitive impairment. For the replication study, 48 FTD cases were further selected from the same population and 46 HCs were spouses of the patients. Moreover, all patients underwent a standard evaluation that included (1) a detailed clinical history recorded from the patients and/or caregivers; (2) an extensive physical examination; (3) neurological examination; (4) an extensive neuropsychological testing including the Mini–Mental State Examination (MMSE); (5) laboratory tests to exclude secondary causes of dementia; (6) brain imaging (magnetic resonance imaging or computerized tomography); and (7) in most cases, 99mTc-HMPAO SPECT or FDG PET scan. HCs underwent clinical and neurological assessment including the administration of MMSE. Written informed consent was obtained from all subjects participating in the study; the protocol of this study was carried out according to the principles of the Declaration of Helsinki and approved by the local ethical committee. All patients were screened for possible mutations in *MAPT*, *GRN*, and *C9ORF72* genes.

2.2. RNA isolation from plasma

Whole blood was recovered in tubes treated with EDTA and centrifuged for 15 minutes at 2500 rpm at 4 °C. The plasma layer was carefully collected without disturbing the buffy coat. Total RNA was extracted from 250 μL of plasma using a miRNeasy mini kit (Qiagen), following the manufacturer's recommendations; 1 μg of MS2 bacteriophage RNA (Roche Diagnostics) was added to each sample to improve endogenous RNA recovery. Qubit RNA HS Assay Kit (Thermo Fisher Scientific) was used with the Qubit fluorometer, to provide an accurate method for the quantification of low-abundance RNA samples. Evaluation of possible hemolysis contamination was carried out by comparing the level of an miRNA highly expressed in red blood cells (hsa-miR-451a) with an miRNA unaffected by hemolysis (hsa-miR-23a-3p) (Blondal et al., 2013). ΔCt (miR-23a-3p − miR-451a) is a measure of hemolysis degree: ΔCt values of more than 5 indicate a possible erythrocyte miRNA contamination, and values of 7–8 or more, a high risk of hemolysis.

2.3. MicroRNA reverse transcription and quantitative real-time PCR (qPCR)

Eighty ng of extracted RNA were retrotranscribed using the Universal cDNA Synthesis kit (Exiqon) according to the manufacturer's protocol. cDNA template was then diluted 50X in nuclease-free water and mixed 1:1 with 2X PCR Master Mix (Exiqon).

miRNome expression was assayed using a V.3M miRCURY LNA Universal RT microRNA PCR Human panel I + II and SYBR Green Universal Master Mix (Exiqon) on a CFX384 real-time PCR detection system (Bio-Rad Laboratories). To normalize the expression of the entire panel of analyzed miRNAs, we selected miRNAs, checking their expression levels and stability in our samples by using the NormFinder and geNorm software (Andersen et al., 2004; Vandesompele et al., 2002). For individual miRNA quantitative PCR assays, samples were analyzed in technical duplicates using miRCURY LNA PCR primers set (Exiqon): hsa-let-7e-5p (205711), hsa-miR-10b-5p (205637), hsa-miR-122-5p (205664), hsa-miR-206 (206073), hsa-miR-375 (204362), hsa-miR-454-5p (204279), hsa-miR-502-3p (204043), hsa-miR-548c-5p (205882), hsa-miR-663a (204284), hsa-miR-877-5p (205626), and endogenous control hsa-miR-93-5p (204715).

Quantitative real-time PCR reactions were performed using ExiLENT SYBR Green master mix (Exiqon) in a CFX384 real-time PCR detection system (Bio-Rad Laboratories). 10 μL of PCR reaction contained 2 μL of the 1:80 diluted cDNA template, 5 μL of SYBR Green master mix and 1 μL of PCR primer mix. The reaction protocol was as follows: 95 °C for 10 minutes, followed by 40 amplification cycles of 95 °C for 10 seconds and 60 °C for 1 minute. Raw Ct values were normalized using the ΔCt method with respect to the endogenous control.

2.4. Statistical analysis

In the discovery study, variables with $Ct < 37$ and expressed in at least 25% in each group were selected. Resultant miRNAs with a higher fold change (FC) and significance were analyzed in the replication study. Moreover, miRNAs already described in literature

were also considered in the replication study, but just if they reached a p close to significance. Study variables were explored by a two-tailed t test according to the Dunn-Bonferroni correction method and by a two-tailed t test, in the discovery and in the replication study, respectively. Furthermore, we presented the receiver operating characteristic curve analysis and calculated the area under the curve (AUC) of each miRNA and for combined score. Finally, an exploratory study was performed to stratify by gender and FTD clinical subtypes (bvFTD and PPA) in which variables were explored through analysis of variance (ANOVA).

2.5. Target gene prediction and pathway analysis

The DIANA-mirPath v3.0 (http://www.microrna.gr/miRPathv3) functional annotation tool was used to study target genes of the differentially expressed miRNAs (Vlachos et al., 2015). By using this tool, we performed a pathway enrichment analysis on validated targets in TarBase 7.0 using conservative statistics or predicted miRNA targets based on the DIANA-microT-CDS algorithm (threshold 0.8), specifically designed to identify miRNA targets both in 3′ untranslated region (3′UTR) and in CDSs. In both analyses, we then created a union set of the KEGG pathways in which these target genes are present and performed an enrichment analysis using Fisher's exact test (hypergeometric test) and p value threshold 0.05.

3. Results

3.1. Samples' characteristics

Demographic and clinical features of patients and controls included in the discovery and replication study are summarized in Table 1. No differences regarding age at baseline and sex were observed between FTD patients and HCs. No *MAPT*, *GRN,* and *C9ORF72* mutation was found in FTD patients.

3.2. Discovery study

To successfully perform the miRNome profiling, we estimated possible cell and hemolysis contamination, by comparing the levels of an miRNA highly expressed in red blood cells (hsa-miR-451a) and one unaffected by hemolysis (hsa-miR-23a-3p), as detailed in Materials and Methods (Blondal et al., 2013). In all our samples, we have found the ΔCt (miR-23a-3p − miR-451a) less than 5, showing the absence of hemolysis. We profiled the expression of 752 miRNAs in the RNA isolated from plasma samples of 10 patients with FTD and 10 HC subjects (Table 1). We identified 421 miRNAs that had Ct < 37 and were detected in at least one subject (Table A.1): 211 miRNAs of these 421 miRNAs were present in at least 25% of subjects in each group, and 116 were detected in all 20 samples (Fig. 1).

Several other studies underlined how selection of reference genes is critical for miRNA expression analyses (Liu et al., 2014; Masè et al.,2017; Peltier and Latham, 2008; Tang et al., 2015). Because the validity of a miRNA as normalizer depends on the tissue and the disease analyzed, it was necessary to identify a reference miRNA measurable in plasma and equally expressed in FTD and HC samples. To this end, of the 116 miRNAs, we selected 5 miRNAs (hsa-miR-93-5p, hsa-miR-103, hsa-miR-191, hsa-miR-423-3p, and hsa-miR-425-5p) and we tested their expression levels, finding miR-93-5p as the most highly expressed miRNA (Fig. A.1). Furthermore, we measured the stability of the 5 miRNAs among FTD patients and HCs, and between the 2 groups by using the NormFinder and geNorm software. Our results showed that miR-93-5p was the most stable among the 5 selected miRNAs. Using miR-93-5p as normalizer, we calculated the FC values for all the detected miRNAs, (miR-x), as $FC = 2^{-\Delta\Delta Ct} = 2\ EXP - [(AvgCt_{miR\text{-}x} - AvgCt_{miR\text{-}93\text{-}5p})_{FTD} - (AvgCt_{miR\text{-}x} - AvgCt_{miR\text{-}93\text{-}5p})_{HC}]$. Levels of significance and change in expression are represented in a volcano plot in Fig. 2. From our top ten differentially expressed miRNAs, 9 showed a good level ($p < 0.05$), and one showed a higher level of significance ($p < 0.005$). Moreover, we found 8 downregulated and 2 upregulated miRNAs in FTD patients with respect to HCs (Table 2).

3.3. Validation study

From the results of differentially expressed miRNAs, we selected 7 miRNAs, for the validation study. Six of these (hsa-miR-663a, hsa-miR-502-3p, hsa-miR-375, hsa-miR-10b-5p, hsa-let-7e-5p, hsa-miR-548c-5p, and hsa-miR-206) showed an expression level approximately 2-fold lower in the FTD group, whereas the miRNA, hsa-miR-877-5p, was more than 2-fold higher in the FTD group. We also selected miR-206, as very close to significance ($p = 0.07$) and with a good FC ($2^{-\Delta\Delta Ct} = 0.411$; $\log_2 2^{-\Delta\Delta Ct} = -1.28$) (Table 2). We chose to include miR-206 in the validation study also because it has already been described in literature as a biomarker for other NDs, such as AD and ALS (Moon et al., 2016; Toivonen et al., 2014; Waller et al., 2017). Moreover, miR-206 plays a key role in the regulation of BDNF (Lee et al., 2012; Tian et al., 2014), an important molecule linked to the pathophysiology of FTLD (Zanardini et al., 2016).

Using individual qPCR kits, and normalizing on miR-93-5p, we tested these 8 miRNAs in a larger case-control sample consisting of 48 FTD patients and 46 HCs. Significant downregulation of miR-663a, miR-502-3p, and miR-206 levels ($p = 0.0001$, $p = 0.0002$, and $p = 0.02$, respectively) in FTD patients was confirmed when compared with HCs (Table 3 and Fig. 3A, D, and G). The other analyzed miRNAs showed the same trend found in the discovery profiling, but the results were not statistically significant (Table 3). As evaluated by the NormFinder and geNorm software, miR-93-5p reference remained stable also in samples of the validation group.

Based on a recent work by our group (Ricci et al., 2015), we used a Bayesian method to identify possible normalized class-discriminating miRNAs. First, employing an analysis of the duplicate variability that relies on fitting the experimental variances with a χ^2 distribution via the minimization of a suitable Kolmogorov-Smirnov statistic, outliers were spotted and removed from further analysis. Thereupon, the class-discriminating miRNA distributions were assessed and characterized by means of a Bayesian approach. As shown in Fig. 4, we found that, as expected, only miR-663a, miR-502-3p, and miR-206 showed a significant difference between the mean of the 2 groups; p value and accuracy were, respectively, 0.000035 and 73.6%, 0.0000046 and 72.9%, and 0.0013 and 74.5%.

As a further step, we tried several different combinations of the 3 miRNAs as a possible discriminating measure between the 2 groups: the sum of the measure concerning miR-663a, miR-502-3p, and miR-206 was the best solution (Fig. 5), providing a Bayesian classifier (p value = 0.000058) with an accuracy of 84.4%. The miRNA sample score was calculated as follows:

$$Score = \Delta Ct663a + \Delta Ct502\text{-}3p + \Delta Ct206.$$

Table 1
Population demographics

Population features	Discovery sample		Replication sample	
	FTD	HC	FTD	HC
N	10	10	48	46
Females (%)	5 (50)	5 (50)	28 (58)	28 (61)
Age at evaluation	66 ± 4	66 ± 5	72 ± 8	73 ± 7
Age at onset	64 ± 6	-	67 ± 8	-

Key: FTD, frontotemporal dementia; HC, healthy control.

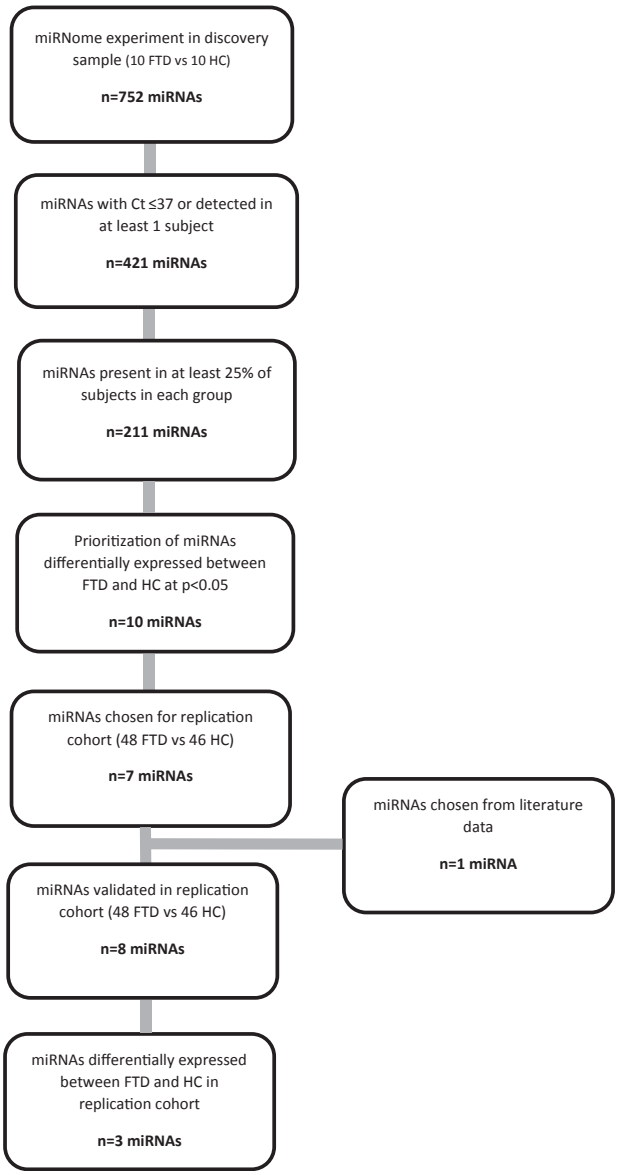

Fig. 1. Flowchart depicting miRNAs selection and sub sequential replication analyses. Abbreviations: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

A receiver operating characteristic curve was then created for each miRNA and for the combination of them to highlight the improvement of the classifier. AUC of the 3 miRNAs in the replication sample were as follows: miR-663a, 0.73 (95% CI, 0.63–0.83) (Fig. 6A); miR-502-3p, 0.73 (95% CI, 0.63–0.84) (Fig. 6D); miR-206, 0.64 (95% CI, 0.53–0.76) (Fig. 6G); miRNA combination, 0.89 (95% CI, 0.7670–1.006) (Fig. 6J). The optimal cutoff point of combined miR-663a, miR-502-3p, and miR-206 was determined as being >26.83 (sensitivity: 87.5%, specificity: 81.3%, positive predictive value (PPV): 82.4%, negative predictive value (NPV): 86.7%).

In a preliminary analysis of 3 validated miRNAs on subgroups by gender, we found that significant differences of miR-206 levels were specific for men (Fig. 3H), so that in women its levels remained similar to the controls (Fig. 3I). On the other hand, miR-663a and miR-502-3p showed significant differences in both genders (Fig. 3B, C, E, and F). Interestingly, although we found a significant difference in hsa-let-7e-5p when comparing HC and FTD females, no difference was found in males and in the overall population (Fig. A.2). By gender, AUCs were the following: miR-663a, male 0.75 (95% CI, 0.59–0.91) (Fig. 6B) and female 0.74 (95% CI, 0.61–0.87) (Fig. 6C); miR-502-3p, male 0.79 (95% CI, 0.65–0.94) (Fig. 6E) and female 0.68 (95% CI, 0.54–0.83) (Fig. 6F); miR-206, male 0.74 (95% CI, 0.58–0.91) (Fig. 6H) and female 0.56 (95% CI, 0.40–0.72) (Fig. 6I); combined miRNAs, male 0.99 (95% CI, 0.96–1.03) (Fig. 6K) and female 0.81 (95% CI, 0.59–1.02) (Fig. 6L). The optimal cutoff point of combined miRNAs in male and female was determined as being >24.64 (sensitivity: 100%, specificity: 87.5%, PPV: 100%, NPV: 88.9%) and >27.59 (sensitivity: 77.8%, specificity: 75.0%, PPV: 77.8%, NPV: 75%), respectively.

No significant correlation was found between miR-663a, miR-502-3p, or miR-206 levels and age at onset and MMSE. Moreover, no difference in the levels of these miRNAs was observed between 2 FTD clinical subtypes: bvFTD (n = 17) and PPA (n = 17) (Fig. 7).

3.4. MicroRNA target prediction and pathway analysis

To reveal which pathways might be regulated by our selected and validated miRNAs, we used DIANA-miRPath v3.0 (Vlachos et al., 2015). This is a functional annotation tool able to perform miRNA pathway analysis by using different algorithms, such as TarBase, a database of experimentally validated miRNA targets, and microT-CDS, the only algorithm available online designed to predict miRNA targets both in 3′ untranslated regions (3′UTR) and in CDSs. We first performed a pathway enrichment analysis on TarBase (Fig. A.3 and Table A.2). Of relevance for FTD, this analysis indicated that 4 genes in the [05020] “prion disease” pathway (PRNP, PRKX, MAPK1, and PRKACB) are targets of miR-502-3p, validated by PAR-CLIP (Skalsky et al., 2012) or HITS-CLIP (Balakrishnan et al., 2014), and that 3 genes in the [04540] “gap junction” pathway (EGFR, GJA1, and ITPR3) are validated targets of miR-206 (Anderson et al., 2006; Hudson et al., 2012; Kedde et al., 2007).

To further explore the role of the selected miRNAs in FTD, we predicted miRNA targets using the DIANA-microT-CDS algorithm. We then created a union set of KEGG pathways in which these target genes are present and performed an enrichment analysis (Fig. 8 and Table A.3).

It is striking to note that the most significantly enriched KEGG pathway (p value 1.298777e-10) is [05031] “amphetamine addiction,” in which 14 genes in total are predicted to be targeted by one or the other of the 3 miRNAs. In fact, two of these genes (CREB5 and DRD1) were predicted to be targeted by both miR-206 and miR-502-3p. One of the predicted targets of miR-206, CALM2, has been validated by HITS-CLIP (Zhang et al., 2014). Even if they were not predicted targets by microT-CDS, other 4 genes in the amphetamine addiction pathway are known to be regulated by the miRNAs under study (Fig. A.4A).

KEGG pathways [04540] “gap junction” (p value 1.248715e-05) and [04360] “axon guidance” (p value 1.354186e-05) targeted by miR-206 and miR-502-3p are also of relevance for NDs in this study.

Two of the 13 predicted targets in the gap junction pathway (DRD1 and SOS1) are common to both miR-206 and miR-502-3p, and 3 have also been experimentally validated in human: TJP1 has been found to be a target of miR-502-3p by HITS-CLIP (Xue et al., 2013), and EGFR and GJA1 have been shown to be targets of miR-206 by microarray (Hudson et al., 2012) and Western blot experiments (Anderson et al., 2006), respectively. Four of the predicted targets, Egfr, Prkacb, Map3k2, and Sos1, have been shown by HITS-CLIP to interact with miR-206 in mice (Zhang et al., 2014). Other 10 genes in the gap junction pathways are known targets of miR-206 (Fig. A.4B).

In the axon guidance pathway, of the 20 targeted genes, one (ROCK1, Rho-associated coiled-coil-containing protein kinase 1) is a

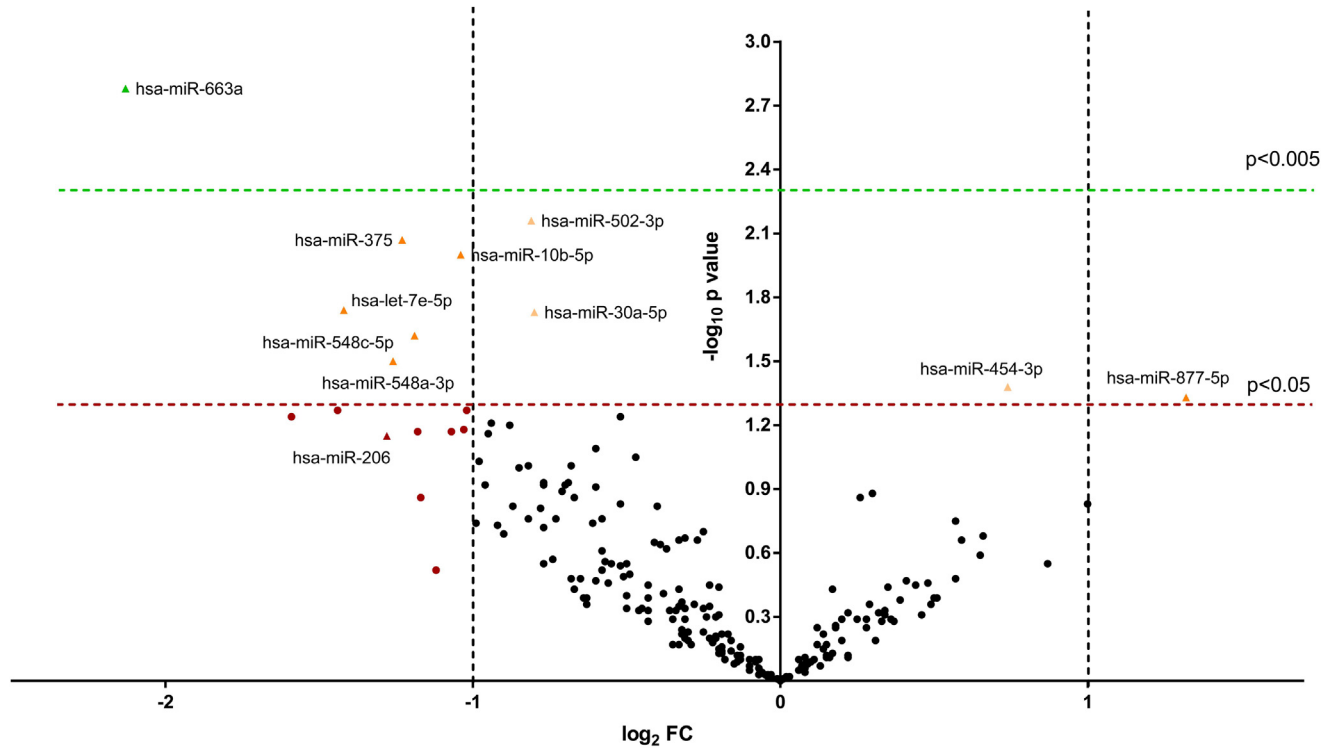

Fig. 2. Volcano plot of differentially expressed miRNAs in FTD patients compared with HCs. Negative $\log_{10}$ *p* values on the y-axis versus mean $\log_2$ fold change (FC) on the x-axis are represented. $FC = 2\ EXP - [(AvgCt_{miR\text{-}x} - AvgCt_{miR\text{-}93\text{-}5p})_{FTD} - (AvgCt_{miR\text{-}x} - AvgCt_{miR\text{-}93\text{-}5p})_{HC}]$. Abbreviations: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

predicted target for both miRNAs and 3 are also experimentally supported in human: SEMA3C was proven to be a target of miR-502-3p by HITS-CLIP (Xue et al., 2013); EFNB2, a target of miR-206 by microarray experiments (Hudson et al., 2012); and MET, a target of miR-206 by Western blot and luciferase assays (Di Leva et al., 2010; Taulli et al., 2009; Yan et al., 2009). In mouse, 5 of the predicted miR-206 targets were validated: Efnb2, Met, Cxcr4, Nrp1, and Nfatc3 by HITS-CLIP (Zhang et al., 2014). Fourteen more genes in the axon guidance pathway are validated targets of miR-206, even if they were not predicted by microT-CDS (Fig. A.4C).

Several other neurologically relevant pathways were found, such as [04724] "glutamatergic synapse" (*p* value 1.460457e-05) and [05032] "morphine addiction" (*p* value 4.161048e-05), targeted by both miR-206 and miR-663a, [05030] "cocaine addiction," [04713] "circadian entrainment," [04,730] "long-term potentiation," [04728] "dopaminergic synapse," [00310] "lysine degradation," [05034] "alcoholism," and [05033] "nicotine addiction." Given the fact that FTD shares with ALS several causative genes, it is also worth mentioning that in this enrichment analysis, miR-502-3p is significantly predicted to regulate 6 genes in the "amyotrophic lateral sclerosis" KEGG pathway (*p* value 0.001867765).

Finally, given that recent preclinical studies demonstrate the involvement of Gram-positive bacteria from the host microbiome in the pathogenesis of several NDs (reviewed in Main and Minter, 2017), it is intriguing to note the enrichment of KEGG pathways [05100] "bacterial invasion of epithelial cells" (*p* value 0.001152536), [05131] "shigellosis" (*p* value 0.002624923), and [05130] "pathogenic *Escherichia coli* infection" (*p*-value 0.02626409).

Table 2
MicroRNAs detectable in plasma obtained from FTD patients and HCs

miRNA	Ct count	FTD count	Ct avg	Ct std	FTD avg	FTD std	FC	*p* value
Downregulated								
hsa-miR-663a	7	9	34.2	0.7	35.3	0.6	0.23	0.0017
hsa-miR-502-3p	8	9	35.1	0.5	35.2	1	0.57	0.0072
hsa-miR-375	8	10	33.9	0.7	34.4	0.9	0.43	0.0084
hsa-miR-10b-5p	10	10	32.2	0.7	32.4	1	0.49	0.0101
hsa-miR-30a-5p	10	10	33.3	0.9	33.3	1.2	0.58	0.0184
hsa-let-7e-5p	9	9	32.7	1.1	33.3	1.9	0.37	0.0182
hsa-miR-548c-5p	8	8	35.9	0.4	36.1	0.7	0.44	0.0240
hsa-miR-548a-3p	7	5	33.9	0.7	34.0	1.1	0.42	0.0314
hsa-miR-206	10	9	34.4	1.3	34.7	1	0.41	0.0713
Upregulated								
hsa-miR-454-3p	10	10	33.4	0.9	31.8	1.1	1.67	0.0412
hsa-miR-877-5p	7	7	35.5	0.9	32.9	0.8	2.50	0.0471

The table shows the number of subjects in each group, the average raw Ct and standard deviation, and fold changes of expression, with the corresponding *p* values (calculated by unpaired two-tailed *t*-test). miRNAs are listed in order of significance from top to bottom and divided into downregulated and upregulated miRNAs.
Key: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

Table 3
Top miRNAs in the discovery sample (10 HCs + 10 FTD patients) analyzed and prioritized based on *t*-test analysis along with validation in the replication sample (48 FTD patients + 46 HCs)

miRNA	*p* value		Fold change	
	Discovery sample	Replication sample	Discovery sample	Replication sample
hsa-miR-663a	0.0017	0.0001	0.228	0.512
hsa-miR-502-3p	0.0072	0.0002	0.569	0.573
hsa-miR-375	0.0084	0.1296	0.425	0.775
hsa-miR-10b-5p	0.0101	0.7377	0.487	0.939
hsa-let-7e-5p	0.0182	0.2642	0.374	0.777
hsa-miR-548c-5p	0.0240	0.5294	0.437	0.818
hsa-miR-877-5p	0.0471	0.7369	2.496	1.068
hsa-miR-206	0.0713	0.0222	0.411	0.593

Key: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

4. Discussion

Currently, the diagnosis of FTD primarily depends on medical history, a full neuropsychological testing evaluation to better assess the pattern of cognitive loss in an individual suspected and the exclusion of other neurodegenerative disorders, besides neuroimaging to determine where and how extensively brain regions have atrophied; however, this clinical diagnostic evaluation is hampered by the considerable overlap of the clinical manifestation within the subtypes and with other types of dementia. At present, there is no single diagnostic test that can confirm or rule out a diagnosis of FTD. Therefore, simple, noninvasive, cost-efficient, and

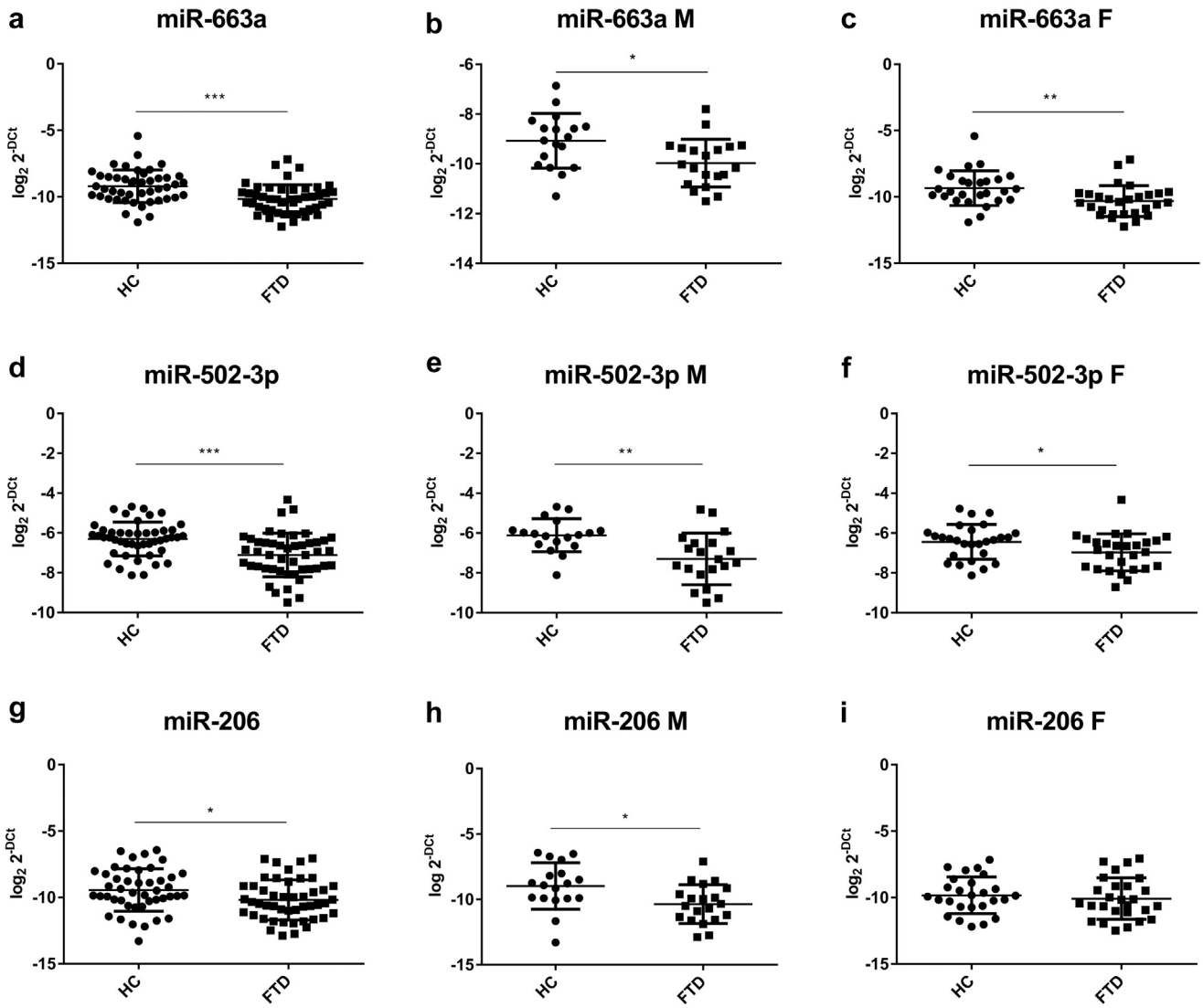

Fig. 3. Validated differentially expressed miRNAs in HCs versus FTD patients. Scatter plots of (A) miR-663a, (B) miR-663a males, (C) miR-663a females, (D) miR-502-3p, (E) miR-502-3p males, (F) miR-502-3p females, (G) miR-206, (H) miR-206 males, and (I) miR-206 females. Abbreviations: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

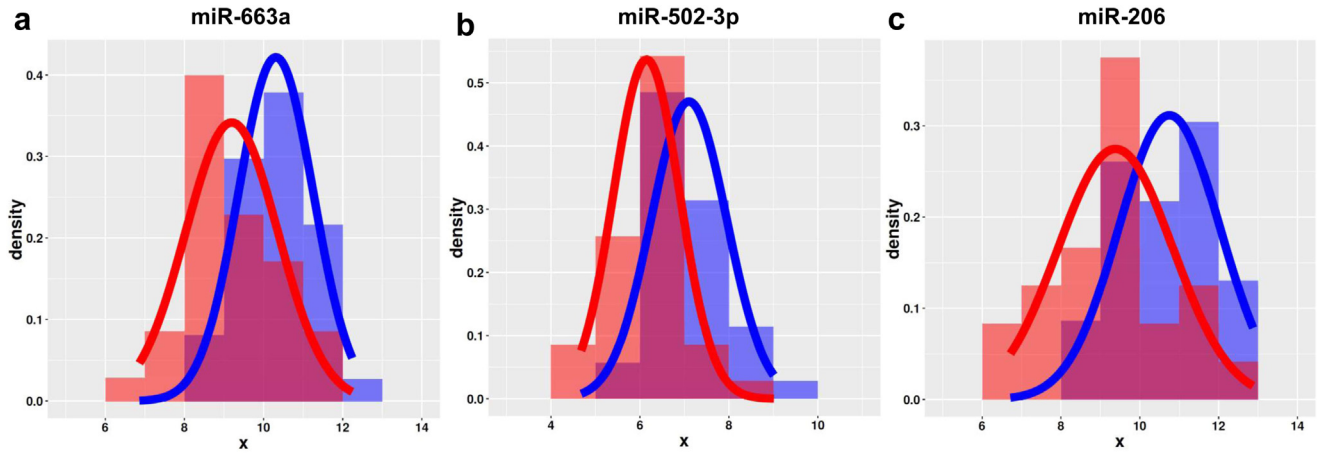

Fig. 4. Histograms of ΔCt for each miRNA candidate. (A) miR-663a, (B) miR-502-3p, and (C) miR-206 for samples belonging to the target class FTD (blue) and to the versus class HCs (red). Overlapping regions are in magenta. The x-axis represents the respective ΔCt. The bin width is equal to 1. Each histogram is normalized to the respective set size and thus corresponds to a sample density. The bold lines represent the gaussian probability densities that fit the data. Abbreviations: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

specific biomarkers that can help in diagnosing early FTD are urgently needed.

Noteworthy, miR-663a is associated with many critical biological processes, including development, inflammatory responses, and carcinogenesis (Jian et al., 2011; Latruffe et al., 2015; Ni et al., 2011; Yi et al., 2012). Moreover, APP intracellular domain (AICD) has been found to act as a transcriptional regulator of miR-663 in human neural stem cells, where it shows a role in the negative modulation of neuronal differentiation (Shu et al., 2015). Microarray data further demonstrate that miR-663 suppresses the expression of genes implicated in neurogenesis, such as FBXL18 and CDK6 (Shu et al., 2015).

With regard to miR-502-3p, it seems to have a role in the suppression of cell proliferation, migration, and invasion in hepatocellular carcinoma (Sun et al., 2016) and in the inhibition of autophagy and tumor growth in colon cancer (Zhai et al., 2013). Interestingly, a variant in the precursor of miR-502-3p, predisposing males to schizophrenia, was identified (Feng et al., 2009). Controversial literature was found regarding miR-502-3p dysregulation in AD. In a study, Satoh et al. reanalyzed a publicly available

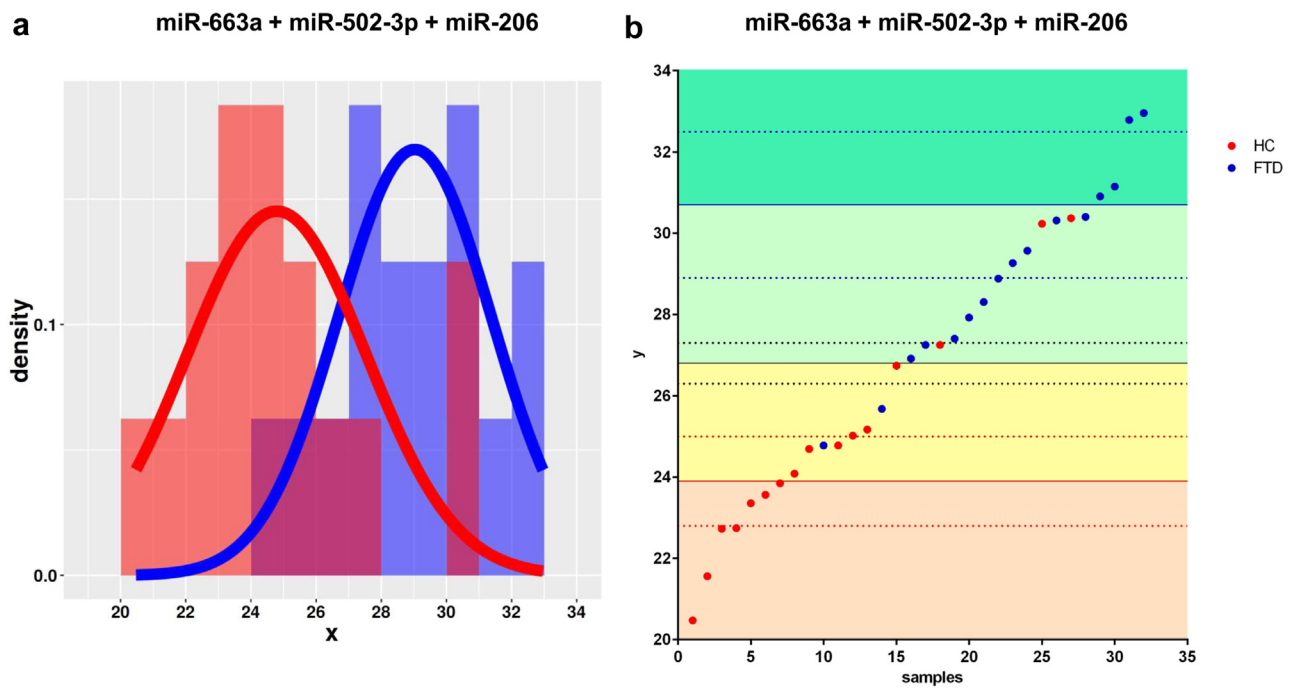

Fig. 5. Combined score (ΔCt663a + ΔCt502-3p + ΔCt206) as discriminating measure between FTD patients and HCs. (A) Histogram of ΔCt for the combined score (ΔCt663a + ΔCt502-3p + ΔCt206). (B) Scatter plot of target (FTD) and versus (HC) samples. Dots corresponding to samples of the target class FTD patients and the class HCs are colored in blue and red, respectively. The plot contains 4 different regions, bounded by 3 thresholds: orange (vs. class with odds larger than 90:10); yellow (vs. class with odds between 50:50 and 90:10); light green (target class with odds between 50:50 and 90:10); and green (target class with odds larger than 90:10). The black bold line represents the main discrimination threshold (26.8); the 2 black, dashed lines correspond to the excursion of this threshold due to its uncertainty (±0.5) as computed by means of standard error propagation (35). Similarly, the 3 red lines and the 3 blue lines represent the threshold of the (10, 90) region with the related uncertainty excursion (23.9 ± 1.1) and the threshold of the (90, 10) region with the related uncertainty excursion (30.7 ± 1.8), respectively. Abbreviations: FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

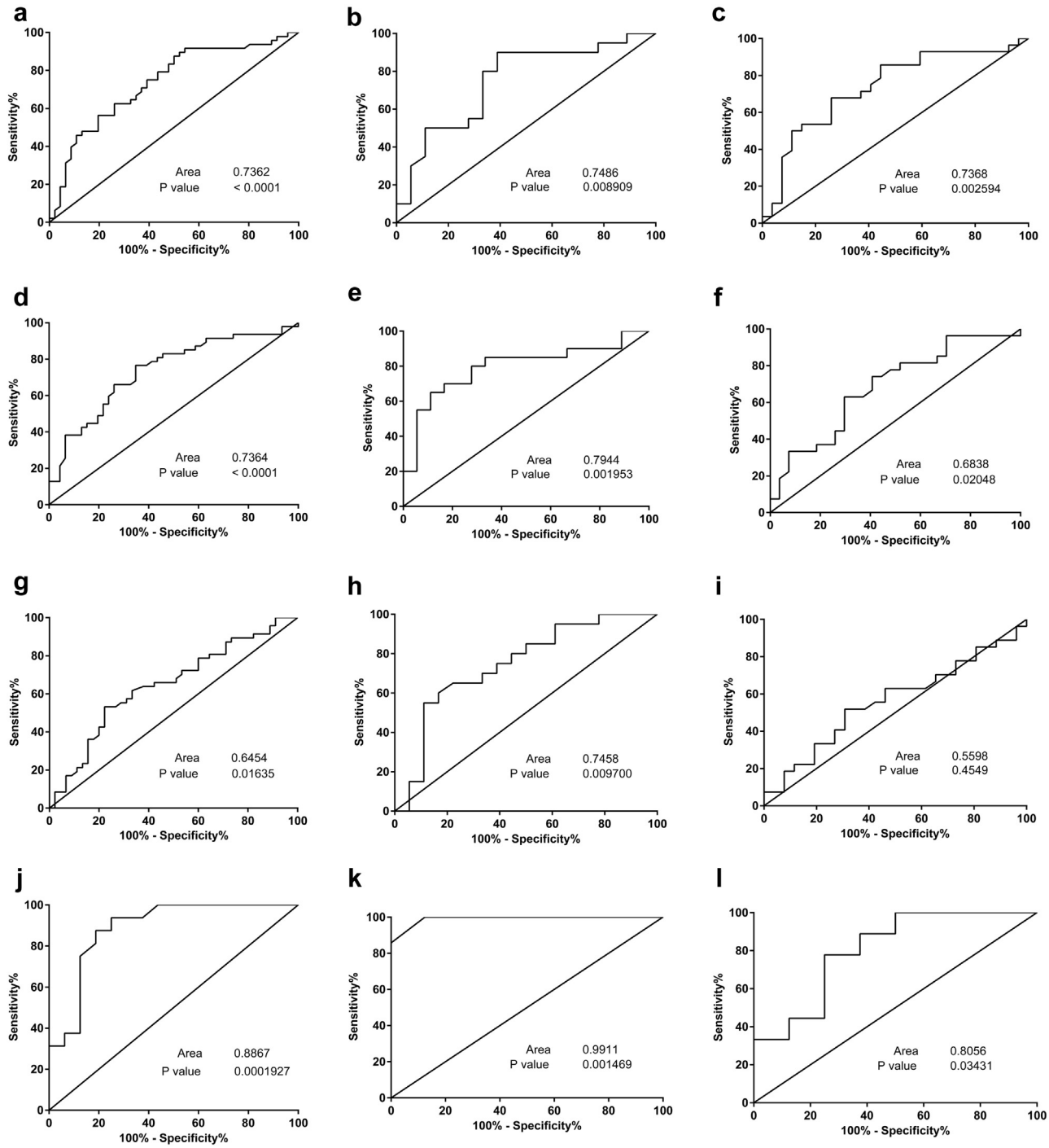

Fig. 6. Receiver operating characteristic (ROC) curve of differentially expressed miRNAs in HCs versus FTD patients. ROC curves of (A) miR-663a, (B) miR-663a males, (C) miR-663a females, (D) miR-502-3p, (E) miR-502-3p males, (F) miR-502-3p females, (G) miR-206, (H) miR-206 males, (I) miR-206 females, (J) combined miRNAs, (K) combined miRNAs males, and (L) combined miRNAs females. Abbreviations: AUC, area under the ROC curve; FTD, frontotemporal dementia; HC, healthy control; miRNA, microRNA.

small RNA-Seq data set identifying 27 miRNAs differentially expressed in AD blood samples including downregulated miR-502-3p (Satoh et al., 2015). On the contrary, in a more recent study, miR-502-3p was found consistently upregulated in plasma of early AD patients. This discrepancy in miR-502-3p expression could be due to the difference in the methodologies used in the 2 studies (Nagaraj et al., 2017).

miR-206 is known as a key modulator of skeletal muscle development and disease (Ma et al., 2015). Moon et al. demonstrated miR-206 overexpression in the olfactory epithelium of patients with early dementia, also describing its sharp increase as dementia progressed (Moon et al., 2016). Xie et al. observed miR-206 increase in serum of subjects with mild cognitive impairment (MCI) (Xie et al., 2015). In 2017, in a follow-up study, they found higher levels of miR-206 in the amnestic MCI AD-converting group than aMCI-stable group, thus suggesting that miR-206 might be a potential predictor of conversion from aMCI to AD (Xie et al., 2017). Several lines of evidence have suggested that this miRNA may regulate BDNF protein

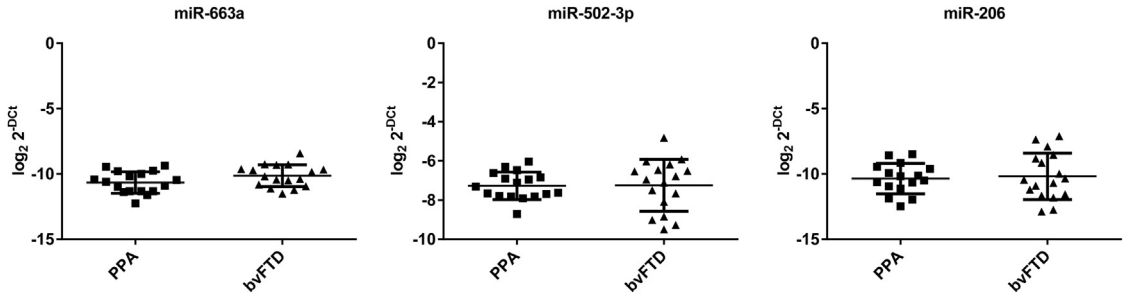

Fig. 7. Scatter plots of miR-663a, miR-502-3p, and miR-206 FTD clinical subtypes: bvFTD and PPA. Abbreviations: bvFTD, behavioral-variant frontotemporal dementia; PPA, primary progressive aphasia.

synthesis and that its inhibitor AM206 enhances BDNF levels, synaptogenesis, and neurogenesis (Lee et al., 2012).

FTD has been related in many ways to RNA dysregulation (Fontana et al., 2015); for example, several genes mutated in familial FTD (and in familial ALS) are RNA-binding proteins related to RNA processing. One of these proteins, TDP-43, has been reported to selectively disrupt miRNA-1/206 incorporation into RISC (King et al., 2014). TDP-43 has also been shown to bind to the

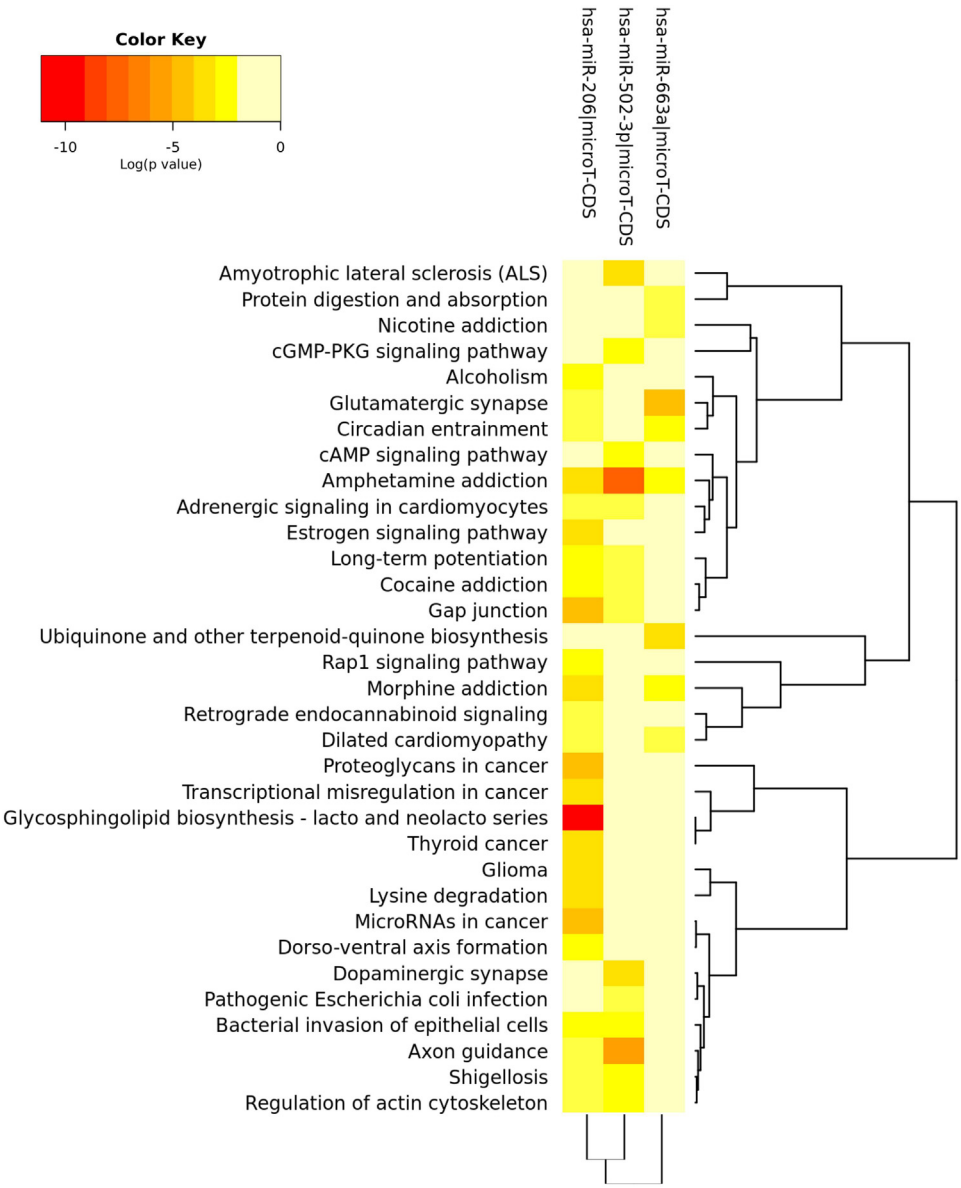

Fig. 8. MicroiRNAs versus pathways heatmap. miRPath v3.0 KEGG enrichment was computed using "pathways union," DIANA-microT-CDS algorithm.

hairpin precursor of miR-663, and the knockdown of TDP-43 resulted in miR-663 upregulation (Buratti et al., 2010). Some evidences in the literature show that both miR-663a and miR-206 can be encapsulated in exosomes and subsequently released outside the cells of origins in the bloodstream (De Gasperi et al., 2017; Koutsoulidou et al., 2017; Muroya et al., 2015; Ramezani et al., 2015). miR-206 was also detected in extracellular vesicles and as a component of protein/lipoprotein complexes (Guescini et al., 2015; Matsuzaka et al., 2016). To date, very little attention, to the best of our knowledge, has been given to the state of miR-502-3p in the blood. By analyzing all subjects on subgroups characterized by gender, we found that miR-663a and miR-502-3p showed significant differences in both genders. On the other hand, specific significant miR-206 differences were found only in male subjects, whereas, interestingly, a significant difference in hsa-let-7e-5p was found by comparing HC and FTD females. Gender differences constitute a phenomenon greatly studied in the last years. Several human diseases display gender differences related to pathogenic mechanisms, age of onset, progression, and response to therapy. Moreover, it is well-known that women are at higher risk of developing AD than men (Gorelick, 2004). Recent studies suggest that the cause may lay in miRNA expression levels and that these differences might be influenced by hormonal and genetic background (Sharma and Eghbali, 2014). Ignoring gender differences can alter the meaning of the results obtained because cluster or families of important genes, which have the potential to cause different disease occurrence, can be lost or not further investigated concerning their function and roles. For most of our validated miRNAs, as far as we know, there are no indications in the literature of their involvement in gender-specific discrimination between patients and controls. The only exception is provided by a few studies that have described a link between miR-206 and estrogens. This miRNA seems to have an inhibitory role in estrogen-dependent ovarian cancer cells and is a promising candidate for the endocrine therapy of ERα-positive ovarian cancer (Li et al., 2014).

With regard to the age at onset or the MMSE, no significant correlation was found with our validated miRNA levels. Concerning MMSE, it tests mainly memory impairment rather than behavioral disturbances, so the lack of correlation with miRNA levels is not unexpected. Moreover, no difference was observed between the 2 different phenotypes bvFTD and PPA. Probably, these 3 miRNAs are not able to distinguish between the two major FTD clinical subtypes; however, a larger cohort of patients is needed to verify this assumption. Finally, by using DIANA-miRPath, we found that the predicted target genes of the differentially expressed miRNAs are involved in several neurological pathways: amphetamine addiction, gap junction, axonal guidance, glutamatergic synapse, long-term potentiation, dopaminergic synapse, etc. As already described in our results, several of these targets are also experimentally validated.

Among the validated target genes for miR-206, ERK2 is well known to be involved in the Ras/MEK/ERK pathway of tau phosphorylation in several dementias (Sun et al., 2017). Moreover, in a previous study, we demonstrated a reciprocal functional interaction of PGRN and MEK pathways in neuroblastoma cell lines (Piscopo et al., 2010). Another validated target of miR-206 is EGFR, a gene playing pivotal roles in cell proliferation, differentiation, and tissue development and linked with CHMP2B and TMEM106B, 2 genes that are involved in FTD (Jun et al., 2015).

5. Conclusion

Our data demonstrate that the combined score of 3 differentially expressed circulating miRNAs *(ΔCt663a + ΔCt502-3p + ΔCt206)* is a robust diagnostic biomarker of FTD, especially for men. In fact, in males, combined miRNA levels showed an excellent sensitivity (100%) and a good specificity (87.5%) for the diagnosis of FTD. In females, however, the same miRNA combined score shows lower sensitivity and specificity (77% and 75%, respectively) leaving a place for further improvement of a female-specific miRNA score. If evaluated in a larger population, including AD and patients with other NDs, these miRNAs could help to diagnose FTD in a gender-specific manner. Moreover, understanding the expression divergence due to gender could help us to disclose mechanisms underlying the occurrence and the development of complex diseases as FTD.

Disclosure

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2019.01.024.

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