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(54) Title: METHODS OF USING miRNA FROM BODILY FLUIDS FOR EARLY DETECTION AND MONITORING OF MILD COGNITIVE IMPAIRMENT (MCI) AND ALZHEIMER'S DISEASE (AD)

(57) Abstract: Described are methods for early diagnosis and progression monitoring of Mild Cognitive Impairment (MCI) and Alzheimer's Disease (AD) by quantifying neurite and/or synapse miRNAs in bodily fluids.

METHODS OF USING miRNA FROM BODILY FLUIDS FOR EARLY DETECTION AND MONITORING OF MILD COGNITIVE IMPAIRMENT (MCI) AND ALZHEIMER'S DISEASE (AD)

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 61/476,591 filed on April 18, 2011, U.S. Provisional Application Serial No. 61/478,766 filed on April 25, 2011, and U.S. Provisional Application Serial No. 61/546,431 filed on October 12, 2011, all of which are incorporated herein by reference in their entirety.

TECHNICAL FIELD OF THE INVENTION

The present invention is directed to methods for early diagnosis and progression monitoring of Mild Cognitive Impairment (MCI) and Alzheimer's Disease (AD) by quantifying neurite and/or synapse miRNAs in bodily fluids.

BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is the most common neurodegenerative disease, which comprise a large group of pathologies caused by metabolic changes in brain cells, loss of synapses and other compartments of neurons, and finally neuronal death (for review see *Neurodegenerative diseases: From Molecular Concepts to Therapeutic Targets*. Editors: R. von Bernhardi, N.C. Inestrosa, Nova Publishers, 2008). Due to increased lifespan, neurodegenerative diseases in general and AD in particular have become very common in developed countries. In the US alone, there are currently more than 5.4 million (and 36 million worldwide) people living with AD, and estimated 70-80 million people, who are over 55 years old, are considered to be at risk of developing the disease. In 2011, the annual cost of healthcare services for AD patients in the US was estimated at $183 billion (Rocca, W.A. et al. Alzheimer's & Dementia. 2011, 7:80-93; http://www.alz.org/downloads/Facts_Figures_2011.pdf).

Drug development and successful treatment of AD and other neurodegenerative diseases are significantly complicated by the absence of effective methods for their early diagnosis and monitoring. Development of effective diagnostic methods is further complicated by the strong brain potential to compensate for the dysfunction and loss of neurons over a long period of time. This results in late clinical manifestation of disease symptoms when treatment cannot be very successful due to serious morphologic changes in the brain including the massive loss of neurons. Thus, diagnostic methods based on detection of early events in the disease development are particularly desirable.

Alzheimer's disease is characterized by neuronal death in several disease-specific areas of the brain, such as hippocampus and cortex. However, the neuronal loss is a relatively late event in the disease progression that typically is preceded by synaptic dysfunction, synaptic loss, neurite retraction, and the appearance of other abnormalities such as axonal transport defects (See, e.g., Crews, Masliah, Human Mol Gen., 2010, 19:R12-R20; Bredesen, Molecular Neurodegeneration 2009, 4:27; Nimmrich and Ebert, Rev Neurosci. 2009, 20:1-12; Yoshiyama et al., Neuron. 2007, 53:337-351; Wishartet al., J Neuropathol Exp Neurol. 2006, 65:733-739; Gylys et al., Neurochem Int. 2004;44:125-131; Conforti et al., Trends Neurosci. 2007, 30:159-166; Revuelta, et al. Am J Alzheimers Dis Other Demen 2008, 23: 97-102). Numerous studies are devoted to description of axon destruction with shedding of membrane-enclosed "axosomes", axon, dendrite and spine pruning, and disassembly of synapses (Goda, Davis, Neuron 2003, 40:243-264; Eaton, Davis, Genes Development, 2003, 17:2075-2082; Koirala, Ko, Neuron, 2004, 44:578-580; Bishop et al., Neuron, 2004, 44:651-661; Low, Cheng, Phil. Trans. R. Soc. B 2006 361, 1531-1544).

Currently there are attempts to develop anti-AD therapeutics capable of restoring dendritic spine density and synapses (Adlard et al., PLoS ONE, 2011, 6:e17669).

The first symptomatic stage of Alzheimer's disease that is manifested by mild clinical symptoms is Mild Cognitive Impairment (MCI), which is usually defined as an intermediate state between normal aging and dementia (DeCarli, Lancet Neurol.,

2003, 2:15-21; Stephan et al., Alzheimer's Res Therapy, 2009, 1:1-9; Apostolova et al., Human Brain Mapping, 2010, 31:786-797). On average, MCI patients convert to dementia at a rate of 10-15% annually (Petersen et al., Arch Neurol. 2001, 58:1985-1992; Apostolova et al., Human Brain Mapping, 2010, 31:786-797). However, currently the MCI outcome is not reliably predictable. First, up to 40% of MCI patients revert to normal status (Larrieu et al., Neurology, 2002, 59:1594-1599; Brooks, Loewenstein, Alzheimer's Res Therapy, 2010, 2:28-36), and autopsy studies demonstrate that a substantial percentage of MCI patients do not have evidence of AD pathology (Jicha et al., Arch Neurol, 2006, 63:674-681; Khan, Alkon, Neurobiol. Aging, 2010, 31:889-900). Second, about 20% of MCI patients who convert to dementia are diagnosed not with AD but other neurodegenerative diseases, such as vascular, Lewy body, Huntington, Parkinson, and other dementias (Jicha et al., Arch Neurol, 2006, 63:674-681; Stephan et al., Alzheimer's Res Therapy, 2009, 1:1-9). Third, disease progression varies for AD patients from slow to intermediate and rapid (Doody et al., Alzheimer's Res Therapy, 2010, 2:2-10). Even clinically MCI is not a homogeneous pathology and can be described as two conditions, with amnestic symptoms (aMCI) and without amnestic symptoms (Dlugaj et al., Dement Geriatr Cogn Disord., 2010, 30:362-373; Brooks, Loewenstein, Alzheimer's Res Therapy, 2010, 2:28-36). Some publications have demonstrated that aMCI converts to dementia much more often and is a better predictor of AD (Mariani et al., J Alzheimer's Dis., 2007, 12:23-35; Luck et al., Psychiatr Prax., 2008, 35:331-336; Koivunen et al., Neurology, 2011, 76:1085-1099). However, other authors have not found significant difference in the conversion rate for two MCI forms (Rountree et al., Dement Geriatr Cogn Disord., 2007, 24:476-482).

Currently, diagnosis of AD and other forms of dementia is based on analysis of the patient's cognitive function. As mentioned above, due to effective compensatory mechanisms in the brain, the decrease of cognitive function is usually registered when a disease is in its later stages and fewer treatments are available. Amyloid plaques between neurons, neurofibrillary tau-tangles, and an overall shrinkage of brain tissue are the hallmarks of AD, and there were many attempts to develop diagnostic tests based on these phenomena. New imaging techniques,

including in vivo detection of β-amyloid deposition (e.g., positron emission tomography (PET), computed tomography (CT), magnetic resonance imaging (MRI), multiphoton imaging, magnetoencephalography (MEG), electroencephalography (EEG) etc.) (Mucke, Nature, 2009, 461:895-897; Mistur et al., J. Clin. Neurol., 2009, 5:153-166; Miller, Science, 2009, 326:386-389; Perrin et al., Nature, 2009, 461: 916-922) are becoming increasingly popular, but cannot be used for screening purposes.

The existing diagnostic molecular tests for AD and other forms of dementia can be divided into two groups. The first group is based on analysis of single nucleotide polymorphisms (SNP), which is helpful for predicting a higher risk of a disease but not for diagnostics (Bettenset al., Hum Mol Genet. 2010, 19(R1):R4-R11). The second group uses analysis of proteins involved in AD pathogenesis or brain-specific proteins, such as neural thread protein (NTP), in bodily fluids (Schipper, Alzheimer's & Dementia. 2007, 3:325-332). However, these tests are not sufficiently sensitive and specific. Recently published data have demonstrated high sensitivity of AD detection by measuring concentrations of three protein biomarkers (beta-amyloid protein 1-42, total tau protein, and phosphorylated tau181P protein) in the cerebrospinal fluid (CSF) (Meyer et al., Arch Neurol. 2010, 67:949-956; Fagan A.M. et al. Arch. Neurol. 2011, 68:1137-1144). The high invasiveness of the CSF collection procedure makes such tests impractical and challenging for everyday clinical use. Several groups have reported diagnostic assays for AD based on analysis of a large number of proteins or antibodies in human blood (Ray S. et al. 2007, Nat. Med. 13, 1359-1362; Reddy M.M. et al. 2011, Cell 144, 132-142; Nagele E. et al. 2011, PLoS One 6, e23112). However, other researchers were not able to confirm the results of these studies (Bjorkqvist M et al. 2012, PLoS One 7, e29868).

On the 19th of April, 2011 The National Institute on Aging/Alzheimer's Association provided new Diagnostic Guidelines for Alzheimer's Disease (Khachaturian ZS, 2011 Alzheimer's and Dementia. 7, 253-256). The new guidelines were published in four papers devoted to: (i) classification of the AD phases, namely the dementia phase, the symptomatic pre-dementia phase (MCI), and the asymptomatic, preclinical phase of AD (pre-MCI) (Jack et al., 2011, Alzheimer's and Dementia. 7, 257-262); (ii) Recommendations from NIA for the diagnosis of

dementia due to AD (McKhann et al., 2011, Alzheimer's and Dementia. 7, 263-269); (iii) Recommendations from NIA for the diagnosis of MCI due to AD (Albert et al., 2011, Alzheimer's and Dementia. 7, 270-279); and (iv) Recommendations from NIA toward defining pre-MCI (Sperling et al., 2011, Alzheimer's and Dementia. 7, 280-292). The new guidelines stress the current lack of and a great need for reliable biomarkers which can be used for detection of pre-MCI and pre-symptomatic AD, as well as MCI and AD.

Thus, there is a huge need in a non-invasive or minimally invasive molecular test(s) capable to detect MCI or even earlier asymptomatic stages of AD (pre-MCI). Further, it would be even better if such a test could be used for prognosis of the disease outcome and disease and treatment monitoring.

Metabolic changes occurring in AD and other neurodegenerative diseases cause the destruction of spines, dendrites, axons, and synapse loss, and the latter likely induces neuronal death (Bredesen, Molecular Neurodegeneration 2009, 4:27; Crews, Masliah, Human Mol Gen., 2010, 19:R12-R20). Similar processes happen during embryonic brain development. Numerous neurons are trying to establish intercellular contacts, those neurons that do it successfully survive, and other neurons die (Butts et al., Cell Death Differ. 2008, 15:1178-1186; Enokido and Hatanaka, Gan To Kagaku Ryoho. 1994, 21:615-620; Gasic and Nicotera, Toxicol Lett. 2003, 139:221-227).

Axon destruction with shedding of membrane-enclosed "axosomes", axon, dendrite and spine pruning, and disassembly of synapses lead to appearance of cell-free vesicles containing cytoplasmic components of neurons, axons, neurites, spines and synapses, including proteins, RNA and their degradation products. There are other processes leading to liberation of these compounds into the extracellular medium, in particular, blebbing (Charras et al., Biophys. J. 2008, 94:1836–1853; Fackler, Grosse, J. Cell Biol. 2008, 181:879-884), exocytosis (Skog et al. Nat Cell Biol., 2008, 10:1470-1476) and other forms of active secretion (Wang et al. Nucleic Acids Res., 2010, 38:7248-7259; Kosaka et al., J Biol Chem., 2010, 285:17442-17452; Pigati et al., PLoS ONE, 2010, e13515).

MicroRNAs (miRNAs) are a class of non-coding RNAs whose final product is an approximately 22 nt functional RNA molecule. They play important roles in the regulation of target genes by binding to complementary regions of messenger transcripts to repress their translation or regulate degradation (Griffiths-Jones Nucleic Acids Research, 2006, 34, Database issue: D140–D144). Frequently, one miRNA can target multiple mRNAs and one mRNA can be regulated by multiple miRNAs targeting different regions of the 3' UTR. Once bound to an mRNA, miRNA can modulate gene expression and protein production by affecting, e.g., mRNA translation and stability (Baek et al., Nature 455(7209):64 (2008); Selbach et al., Nature 455(7209):58 (2008); Ambros, 2004, Nature, 431, 350-355; Bartel, 2004, Cell, 116, 281-297; Cullen, 2004, Virus Research., 102, 3-9; He et al., 2004, Nat. Rev. Genet., 5, 522-531; and Ying et al., 2004, Gene, 342, 25-28). There are other classes of less characterized small RNAs (reviewed in Kim, Mol. Cells, 2005, 19: 1-15).

Many of miRNAs are specific to or over-expressed in certain organs / tissues / cells (see, e.g., Hua *et al.,* BMC Genomics, 2009, 10:214; Liang et al., BMC Genomics. 2007, 8:166; Landgraf et al., Cell. 2007, 129:1401-1414; Lee et al., RNA. 2008, 14:35-42).

Some miRNAs, including those that are cell-specific, are enriched in certain cellular compartments, particularly in axons, dendrites and synapses (see, e.g., Schratt *et al.*, Nature. 439:283-289, 2006; Lugli *et al.*, J Neurochem. 106:650-661, 2008; Bicker and Schratt, J Cell Mol Med., 12:1466-1476, 2008; Smalheiser and Lugli, Neuromolecular Med. 11:133-140, 2009; Rajasethupathy, Neuron. 63:714-716, 2009; Kye, RNA 13:1224-1234, 2007; Yu *et al.*, Exp Cell Res. 314:2618-2633, 2008; Cougot, *et al.*, J Neurosci. 28:13793-13804, 2008; Kawahara, Brain Nerve. 60:1437-1444, 2008; Schratt G. Rev Neurosci. 2009; 10:842-849; Pichardo-Casas et al. Brain Research. 1436:20-33, 2012).

Expression and concentrations of miRNAs are regulated by various physiological and pathological signals. Changes in expression of some miRNAs were found in neurons of Alzheimer's and other neurodegenerative disease patients (Hébert and De Strooper, Trends Neurosci. 32:199-206, 2009; Saba et al., PLoS One. 2008;

3:e3652; Kocerha et al., Neuromolecular Med. 2009; 11:162-172; Sethi and Lukiw, Neurosci Lett. 2009, 459:100-104; Zeng, Mol Pharmacol. 75:259-264, 2009; Cogswell et al., Journal of Alzheimer's Disease. 14: 27-41, 2008; Schaefer et al., J. Exp. Med. 204:1553-1558, 2007; Hébert, Proc Natl Acad Sci USA 2008; 105:6415-6420; Wanget al., J Neurosci. 2008, 28:1213-1223; Nelson et al., Brain Pathol. 2008; 18:130-138; Lukiw, Neuroreport. 2007; 18:297-300).

Due to their small size, miRNAs can cross the blood-brain, placental and kidney barriers. miRNA release can be activated by pathology, e.g. malignancy (Pigati et al., PLoS ONE, 2010, e13515). Analysis of cell/tissue-specific miRNAs in bodily fluids was proposed for detection of *in vivo* cell death (U.S. Patent Pub. No 20090081640; Laterza et al., Clin Chem. 2009, 55:1977-1983).

Cognitive function testing and brain imaging, which are currently used as main methods for diagnosis of neurodegenerative diseases such as AD, allow only detection of later stages of disease and are not sufficiently specific. There is still a great need in the art to develop methods for early diagnosis of MCI and AD prior to occurrence of major morphological changes and massive neuronal cell death.

SUMMARY OF THE INVENTION

As specified in the Background Section above, there is a great need in a noninvasive or minimally invasive test for early detection and monitoring of Alzheimer's Disease (AD), Mild Cognitive Impairment (MCI), and preceding asymptomatic stages, as well as other neurodegenerative diseases. The present invention addresses this need by providing novel, highly sensitive and noninvasive or minimally invasive diagnostic and monitoring methods based on quantification of synapse and/or neurite miRNAs in bodily fluids. The methods of the present invention allow diagnosis and monitoring of pre-MCI, MCI, AD, and other neurodegenerative diseases prior to occurrence of major morphological changes and massive neuronal cell death and thus have numerous clinical implications. For example, the use of the methods of the present invention can lead to enhanced effectiveness of currently available treatments for neurodegenerative diseases as such

treatments could be administered at significantly earlier stages of the diseases. The use of the methods of the present invention can also allow development of new effective therapeutic and/or preventive treatments and can decrease costs and increase efficiency of clinical trials associated with such development (e.g., by simplifying and enhancing certainty in patient selection and stratification, and/or by simplifying and increasing the efficiency of the methods for evaluating drug effect).

In one aspect, the present invention provides a method for detection of pre-MCI or MCI in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with an age-matched control level of said miRNA, and

c. (i) identifying the subject as being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the age-matched control or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is not increased as compared to the age-matched control.

In a related aspect, the invention provides a method for detection of pre-MCI or MCI in a subject, which method comprises:

a. measuring the level of a synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. (i) identifying the subject as being afflicted with pre-MCI or MCI when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when ratio of the levels of the miRNAs calculated in step (c) is not higher than the corresponding age-matched control ratio.

In another aspect, the invention provides a method for predicting likelihood of progression from pre-MCI to MCI in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been obtained at spaced apart time points;

b. comparing the level of said miRNA in each of the bodily fluid samples collected from the subject with an age-matched control level of the said miRNA, and

c. predicting that the disease in the subject will progress from pre-MCI to MCI if the level of said miRNA is increased compared to the age-matched control in two or more consequently obtained bodily fluid samples collected from the subject.

In one embodiment, the bodily fluid samples can be obtained several months apart, e.g., 1, 3, 6, 12, or 24 months apart, preferably 3-6 months apart.

In a related aspect, the invention provides a method for predicting likelihood of progression from pre-MCI to MCI in a subject, which method comprises:

a. measuring the level a synapse or neurite miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been obtained at spaced apart time points;

b. measuring the level of a normalizer miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the miRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. predicting that the disease in the subject will progress from pre-MCI to MCI if the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio in two or more consequently obtained bodily fluid samples collected from the subject.

The age-matched control level or age-matched control ratio of the miRNA used in the above methods can be, for example, a predetermined standard (e.g., an art-accepted level or ratio determined using age-matched population with normal cognitive functions).

In a separate aspect, the invention provides a method for detection of brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with (i) a control level of said miRNA obtained from the same subject in the past or with (ii) a predetermined young age standard, and

c. identifying the subject as being subject to brain aging when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the control (i) or as compared to the predetermined young age standard (ii).

In a related aspect, the invention provides a method for detection of brain aging in a subject, which method comprises:

a. measuring the level of a synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with (i) a corresponding control ratio obtained from the same subject in the past or with (ii) a predetermined young age standard ratio, and

e. identifying the subject as being subject to brain aging when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio (i) or as compared to the predetermined young age standard ratio (ii).

The predetermined young age standard used in the above two methods can be, for example, an art-accepted level or ratio determined using a relevant young population (e.g., 10-20 y.o., 20-30 y.o, 30-40 y.o., 20-50 y.o.) with normal cognitive functions.

In a further aspect, the invention provides a method for determining the effectiveness of pre-MCI or MCI treatment in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) determining that the treatment is effective if the level of the miRNA has decreased in the course of or following the treatment; (ii) determining that the treatment is not effective if the level of miRNA has not decreased in the course of or following the treatment.

In a related aspect, the invention provides a method for determining the effectiveness of pre-MCI or MCI treatment in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to initiation of the treatment;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained in the course of or following the treatment;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained in the course of or following the treatment;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) determining that the treatment is effective if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) determining that the treatment is not effective if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In a separate aspect, the invention provides a method for determining the effectiveness of a treatment to delay brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) determining that the treatment is effective if the level of the miRNA has decreased in the course of or following the treatment; (ii) determining that the treatment is not effective if the level of miRNA has not decreased in the course of or following the treatment.

In a related aspect, the invention provides a method for determining the effectiveness of a treatment to delay brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to initiation of the treatment;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained in the course of or following the treatment;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained in the course of or following the treatment;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) determining that the treatment is effective if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) determining that the treatment is not effective if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In one embodiment of the above methods for determining treatment effectiveness, the samples can be obtained, e.g., every 1 week, 2 weeks, 1 month, 3 months, 6 months, 12 months, or 24 months during or following the treatment.

In an additional aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating pre-MCI or MCI, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject having pre-MCI or MCI, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) identifying that the test compound is useful for slowing down the progression or treating pre-MCI or MCI if the level of the miRNA has decreased after the compound administration; (ii) identifying that the test compound is not useful for slowing down the progression or treating pre-MCI or MCI if the level of miRNA has not decreased after the compound administration.

In a related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating pre-MCI or MCI, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject having pre-MCI or MCI, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to test compound administration;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to test compound administration;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained following administration of the test compound;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained following administration of the test compound;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) identifying that the test compound is useful for slowing down the progression or treating pre-MCI or MCI if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating pre-MCI or MCI if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In a separate aspect, the invention provides a method for identifying a compound useful for delaying brain aging, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) identifying that the test compound is useful for delaying brain aging if the level of the miRNA has decreased after the compound administration; (ii) identifying that the test compound is not useful for delaying brain aging if the level of miRNA has not decreased after the compound administration.

In a related aspect, the invention provides a method for identifying a compound useful for delaying brain aging, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to test compound administration;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to test compound administration;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained following administration of the test compound;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained following administration of the test compound;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) identifying that the test compound is useful for delaying brain aging if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) identifying that the test compound is not useful for delaying brain aging if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

The above test compound screening methods can also comprise a step of administering the test compound to the subject.

In a separate aspect, the invention provides a method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miR-451 in a bodily fluid sample collected from the subject;

b. measuring the level of at least one synapse or neurite miRNA in the same bodily fluid sample;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. determining that the disease in the subject will progress from MCI to dementia stage of AD if the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio.

In a separate aspect, the invention provides a method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of at least one of miR-7, miR-125b, and miR-16 in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. determining that the disease in the subject will progress from MCI to dementia stage of AD if at least one ratio calculated in step (c) is higher than the corresponding age-matched control ratio.

In a related aspect, the invention provides a method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises combination of two biomarker/normalizer miRNA ratios, namely the ratio of miR-451 to synapse or neurite miRNA and the ratio of miR-7, 125b, or miR-16 to a normalizer miRNA, in one test. The disease in the subject will be expected to progress from MCI to dementia stage of AD if both ratios are higher than respective age-matched control ratios.

In a separate aspect, the invention provides a method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miRNA-451 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. comparing the level of miRNA-451 in each of the bodily fluid samples from the subject with a corresponding age-matched control level, and

c. determining that the disease in the subject progresses from MCI to AD if the level of miRNA-451 in each of the bodily fluid samples from the subject is higher than the corresponding age-matched control level.

In a related aspect, the invention provides a method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miR-451 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. measuring the level of at least one synapse or neurite miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the mRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. determining that the disease in the subject progresses from MCI to dementia stage of AD if the ratio of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio for each of the bodily fluid samples collected from the subject.

In a separate aspect, the invention provides a method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of at least one of miR-7, 125b, and miR-16 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. measuring the level of a normalizer miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the mRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. determining that the disease in the subject progresses from MCI to dementia stage of AD if at least one ratio calculated in step (c) is higher than the corresponding age-matched control ratio for each of the bodily fluid samples collected from the subject.

In a related aspect, the invention provides a method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises combining two biomarker/normalizer miRNA ratios, namely (i) the ratio of miR-451 to synapse or neurite miRNA and (ii) the ratio of at least one of miR-7, miR-125b, and miR-16 to a normalizer miRNA (e.g., miR-491-5p or the average of two or more normalizers selected from the group consisting of miR-9, miR-127, miR-181a, miR-370, and miR-491-5p), in one test. The disease in the subject will be expected to progress from MCI to AD dementia if both ratios (i) and (ii) are higher than the corresponding age-matched control ratio for each of the bodily fluid samples collected from the subject.

In one embodiment of the above methods, the bodily fluid samples can be collected several months apart, e.g., 1, 3, 6, 12, or 24 months apart, preferably 3-6 months apart.

Non-limiting examples of synapse or neurite miRNAs useful in the methods of the present invention include, e.g., miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939.

Preferred examples of synapse or neurite miRNAs useful in the pre-MCI and MCI diagnostic, prognostic and screening methods of the present invention include miR-128, miR-132, miR-874, miR-134, miR-323-3p, miR-382, miR-7, and miR-125b.

Preferred examples of synapse or neurite miRNAs useful in the diagnostic, prognostic and screening methods related to brain aging include miR-128, miR-132, miR-874, miR-134, miR-323-3p, and miR-382.

To increase accuracy in the methods of the invention, it is preferable to use levels of two or more synapse or neurite miRNAs. It is further preferable to verify changes in synapse or neurite miRNA levels in two or more consecutively collected bodily fluid samples.

The normalizer miRNAs useful in the methods of the invention include brain-enriched normalizer miRNAs as well as miRNAs which are expressed in numerous tissues but are not significantly expressed in brain (e.g., miR-10b or miR-141). The methods of the invention encompass the use of single normalizers (e.g., miR-491-5p, miR-370, etc.) as well as the average of two or more normalizers (e.g., two or more normalizers selected from the group consisting of miR-9, miR-127, miR-181a, miR-370, and miR-491-5p).

Brain-enriched normalizer miRNAs useful in the methods of the invention include, for example, neuronal body miRNAs; miRNAs, which are mainly expressed in brain areas not involved in a pathology being assessed; miRNAs, which are mainly expressed in glial cells; and brain-enriched miRNAs, which expression, secretion or both are downregulated in a pathology being assessed.

Non-limiting examples of brain-enriched normalizer miRNAs useful in the methods of the invention include, e.g., miR-9, miR-181a, miR-127, miR-370, and miR-491-5p, which can be used alone or in combination.

In one specific embodiment (applicable to each of the methods of the present invention), the synapse or neurite miRNA is selected from the group consisting of miR-128, miR-132, and miR-874 (collectively "miR-132 family"), and the normalizer miRNA is selected from the group consisting of miR-491-5p, miR-9, miR-181a, and miR-141.

In another specific embodiment (applicable to each of the methods of the present invention), the synapse or neurite miRNA is selected from the group consisting of miR-134, miR-323-3p, and miR-382 (collectively "miR-134 family"), and the normalizer miRNA is miR-370 or miR-127.

In another specific embodiment (applicable to all methods of the invention, except for methods related to brain aging), the synapse or neurite miRNA is miR-7,

and the normalizer miRNA is miR-9, miR-27, miR-181a, miR-370, miR-491-5p, or the average of plasma concentrations of all these normalizers.

In another specific embodiment (applicable to all methods of the invention, except for methods related to brain aging), the synapse or neurite miRNA is miR-125b, and the normalizer miRNA is miR-9, miR-181a, miR-370, miR-491-5p, or the average of plasma concentrations of all these normalizers.

Subjects used in the methods of the present invention include, e.g., humans, veterinary animals and experimental animal models of neurodegenerative diseases or other neuronal pathologies. For diagnostic, prognostic and treatment monitoring methods of the invention, the subject is preferably a human. For screening methods, the subject is preferably an experimental animal.

Non-limiting examples of bodily fluids which can be used in the methods of the invention include, e.g., blood plasma or serum, urine, and saliva. In some embodiments, miRNA is purified from the bodily fluid sample.

In some embodiments, the methods of the invention comprise (e.g., as an initial step) the step of collecting a bodily fluid sample from the subject.

In the methods of the invention, the level of miRNA can be determined using any suitable technique, for example, hybridization, RT-PCR, or sequencing.

In some embodiments, the methods of the invention can further comprise the step of reducing or eliminating degradation of the miRNA.

In some embodiments, the diagnostic methods of the invention can further comprise the step of administering a therapeutic or preventive treatment to the subject that has been diagnosed as having the condition or as being at risk of progression to a more severe condition.

In some embodiments, the diagnostic methods of the invention can further comprise the step of recruiting the subject in a clinical trial.

In conjunction with the above diagnostic and screening methods, the present invention also provides various kits comprising one or more primer and/or probe sets specific for the detection of target miRNA. Such kits can further include primer

and/or probe sets specific for the detection of normalizer miRNA. Non-limiting examples of primer or probe combinations in kits are as follows:

1. Primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-125b, and miR-16 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-491-5p, miR-9, miR-127, miR-181a, and miR-370).

2. Primers or probes specific for miR-451 (optionally, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939).

3. Primers or probes specific for at least two miRNAs selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p).

4. Primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, miR-874, miR-134, miR-323-3p, miR-382, miR-7, and miR-125b (optionally, further comprising primers or probes specific for at

least one normalizer miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p).

5. Primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, and miR-874 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-491-5p, miR-9, miR-181a, and miR-141).

6. Primers or probes specific for at least one miRNA selected from the group consisting of miR-134, miR-323-3p, and miR-382 (optionally, further comprising primers or probes specific for at least one normalizer of miR-370 or miR-127).

7. Primers or probes specific for miR-7 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-9, miR-27, miR-181a, miR-370, and miR-491-5p).

8. Primers or probes specific for miR-125b (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-9, miR-181a, miR-370, and miR-491-5p).

Such kits can be useful for direct miRNA detection in bodily fluid samples isolated from patients or can be used on purified RNA samples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-E are graphs showing comparison of concentrations of miR-7 (**A**), miR-874 (**B**), miR-9 (**C**), miR-181a (**D**) and miR-491-5p (**E**) in plasma of MCI patients (MCI) and age-matched controls (AMC). All concentrations were normalized per spiked ath-miR-159a. Here and in other box and whisker plots, the box indicates the distribution of 50% of the results and the bar above and below the box indicates 80% of the results. The points indicate assay values located outside of 80% data. Median value of the assays is indicated by the line inside the box. Normalized miRNA concentrations are presented on ordinate axis in relative units (log scale).

Figures 2A-C are graphs showing comparison of concentrations of miR-874 (**A**), miR-134 (**B**) and miR-539 (**C**) in plasma of MCI patients (MCI) and age-matched controls (AMC). All concentrations were normalized per miR-141.

Figures 3A-C are graphs showing comparison of miRNA concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). Concentrations of miR-7 (**A**), miR-128 (**B**) and miR-134 (**C**) were normalized per miR-181a.

Figure 4 is a graph showing comparison of miR-125b concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). Concentrations of miR-125b were normalized per miR-9.

Figures 5A-C are graphs showing comparison of miRNA concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). Concentrations of miR-128 (**A**), miR-134 (**B**) and miR-874 (**C**) were normalized per miR-491-5p.

Figures 6 is a graph showing comparison of miRNA concentrations in plasma of MCI (MCI) and age-matched controls (AMC). Concentration of miR-134 was normalized per miR-127

Figures 7A-B are graphs showing comparison of miRNA concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). Concentrations of miR-132 (**A**) and miR-323-3p (**B**) were normalized per miR-16.

Figures 8A-C are graphs showing comparison of miRNA concentrations in plasma of MCI (MCI) and age-matched controls (AMC). Concentrations of miR-134 (**A**), miR-874 (**B**), miR-539 (**C**) were normalized per miR-10b.

Figures 9A-C are graphs showing comparison of miRNA concentrations in plasma of MCI (MCI) and AD patients (AD) and age-matched controls (AMC). Concentrations of miR-7 (**A**), miR-132 (**B**), miR-874 (**C**) were normalized per miR-141.

Figures 10A-E are graphs showing comparison of miRNA concentrations in plasma of MCI (MCI) and AD patients (AD) and age-matched controls (AMC). Concentrations of miR-7 (**A**), miR-128 (**B**), miR-132 (**C**), miR382 (**D**), miR-874 (**E**) were normalized per miR-9.

Figures 11A-E are graphs showing comparison of miRNA concentrations in plasma of MCI and AD patients and age-matched controls. Concentrations of miR-132 (**A**), miR-134 (**B**), miR-323-3p (**C**), miR-382 (**D**) and miR-874 (**E**) were normalized per miR-127-3p.

Figures 12A-G are graphs showing comparison of miRNA concentrations in plasma of MCI and AD patients and age-matched controls. Concentrations of miR-7 (**A**), miR-128 (**B**), miR-132 (**C**), miR-134 (**D**), miR323-3p (**E**), miR-382 (**F**), and miR-874 (**G**) were normalized per miR-181a.

Figures 13A-H are graphs showing comparison of miRNA concentrations in plasma of MCI and AD patients and age-matched controls. Concentrations of miR-7 (**A**), miR-125 (**B**), miR-128 (**C**), miR-132 (**D**), miR-134 (**E**), miR323-3p (**F**), miR-382 (**G**), and miR-874 (**H**) were normalized per miR-370.

Figures 14A-H are graphs showing comparison of miRNA concentrations in plasma of MCI and AD patients and age-matched controls. Concentrations of miR-7 (**A**), miR-125 (**B**), miR-128 (**C**), miR-132 (**D**), miR-134 (**E**), miR323-3p (**F**), miR-382 (**G**), and miR-874 (**H**) were normalized per miR-491-5p.

Figures 15A-C present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between MCI patients (MCI) and age-matched controls

(AMC) obtained with miR-128 (**A**), miR-132 (**B**) and miR-874 (**C**) normalized per miR-491-5p. The areas under the ROC curve (AUC) are reported. Sensitivity, specificity and accuracy for each biomarker/normalizer pair are calculated for the "cutoff" point (indicated as a dot on each plot); the cutoff point is the biomarker / normalizer ratio, at which a sample is equally likely to belong to the AMC or the MCI groups.

Figures 16A-C present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between MCI patients (MCI) and age-matched controls (AMC) obtained with miR-134 (**A**), miR-323-3p (**B**) and miR-382 (**C**) normalized per miR-370. The areas under the ROC curve (AUC) are reported. Sensitivity, specificity and accuracy for each biomarker/normalizer pair are calculated for the "cutoff" point (indicated as a dot on each plot); the cutoff point is the biomarker / normalizer ratio, at which a sample is equally likely to belong to the AMC or the MCI groups.

Figures 17A-F present analysis of associations betweenmiR128 and miR-132 (**A**), miR-128 and miR-874 (**B**), miR-132 and miR-874 (**C**), miR-134 and miR-323-3p (**D**), miR-134 and miR-382 (**E**), and miR-382 and miR-323-3p (**F**). The Ct values of various biomarker pairs were compared and Spearman's rank correlation coefficients *r* along with 95% confidence intervals (MIN & MAX) were calculated.

Figures 18A-J are graphs showing comparison of miRNA concentrations in plasma of 21-50 (CY) and 76-86 (CO) years old controls. Concentrations of biomarker miRNAs were normalized per various miRNA normalizers and presented in relative units (ordinate axis). **A**: miR-128/miR-181a; **B**: miR-132/miR-181a; **C**: miR-874/miR-181a; **D**: miR-134/miR-370; **E**: miR-323-3p/miR-370; **F**: miR-382/miR-370; **G**: miR-132/miR-9; **H**: miR-382/miR-127-3p; **I**: miR-132/miR-491-5p; **J**: miR-874/miR-491-5p.

Figure 19 presents analysis of concentrations of biomarkers in the plasma of elderly subjects with initially normal cognitive function over the course of 2-5 years. Levels of miR-128, miR-132 and miR-874 (biomarkers) were measured and normalized per miR-491-5p. Patients were considered pathology-positive if concentrations of at least two of the three biomarkers were higher than control values

predetermined as cutoff points from ROC curves (Fig. 15). Grey and black colors indicate control and pathology, respectively. Small boxes provide results of the plasma miRNA test, and outer colors represent clinical diagnosis; thus, two colors are seen only for cases, in which clinical diagnosis differs from the predictions of the current method.

 - MCI free patients, as diagnosed by cognitive function testing, who were also determined negative by the plasma miRNA test;  - MCI free patients, who were determined positive by the plasma miRNA test;  - patients with clinical symptoms of MCI, who were determined negative by the plasma miRNA test;  - patients with clinical symptoms of MCI, who were determined positive by the plasma miRNA test. miRNA isolated from the sample collected from Patient 1 at time point 0 did not pass quality control (QC) because of strong inhibition of RT-PCR; no analysis was performed for this sample.

Figures 20A-G are graphs showing comparison of miR-451 concentrations in plasma of MCI (MCI) and AD patients (AD) and age-matched controls (AMC). Concentrations of miR-451 were normalized per miR-141 (**A**), miR-9 (**B**), miR-181a (**C**), miR-370 (**D**), miR-491-5p (**E**), the average of normalizers miR-9, miR-127-3p, miR-181a, miR-370, and miR-491-5p (**F**), and the average (AVER) of all 15 miRNA (see Example 3) analyzed in the study (**G**) and presented in relative units (ordinate axis).

Figures 21A-B are graphs showing the ratio of miR-451 and neurite/synapse miR-132 (**A**) or miR-874 (**B**) concentrations in plasma of MCI and AD patients.

Figures 22A-F are graphs showing comparison of two miRNA concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). All concentrations are normalized per miR-451-5p and presented in relative units (log scale). **A**: miR-7 and miR-451; **B**: miR-16 and miR-451; **C**: miR-125b and miR-451; **D**: miR-7 and miR-16; **E**: miR-7 and miR-125b; **F**: miR-16 and miR-125b.

Figures 23A-F are graphs showing comparison of two miRNA concentrations in plasma of MCI patients (MCI) and age-matched controls (AMC). All concentrations are normalized per the average of 5 normalizers (miR-9, miR-127,

miR-181a, miR-370, and miR-491-5p) and presented in relative units (log scale). **A**: miR-7 and miR-451; **B**: miR-16 and miR-451; **C**: miR-125b and miR-451; **D**: miR-7 and miR-16; **E**: miR-7 and miR-125b; **F**: miR-16 and miR-125b.

Figure 24 compares data presented in Fig. 21, Fig. 14 and Fig. 20. In columns "MCI compared to AD" the grey cells indicate clinically diagnosed MCI patients, whose plasma miR-451/miR-132 and miR-451/miR-874 ratios are in the range characteristic of AD dementia patients. In columns "MCI compared to AMC" the grey cells indicate patients, whose plasma concentrations of miR-7, miR-16, and miR-451 normalized per miR-491-5p differentiate them from aged-matched controls and other MCI patients. Both approaches reveal the same MCI patient, which validates their capability to predict MCI-dementia progression.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the inventors' realization that since neurite (axon and/or dendrite and/or spine) destruction and synapse loss as well as some metabolic events precede neuronal death in the course of development of AD (MCI and a preceding period) and other neurodegenerative diseases, methods based on detection of those phenomena could be used for earlier disease diagnosis than the ones based on detecting cell death. Moreover, since such a test(s) will reflect important events in the pathology development, it could be used for disease and treatment monitoring.

The instant invention is further based on the inventors' discovery that levels of synapse and/or neurite miRNAs increase in bodily fluids of patients with Mild Cognitive Impairment (MCI) compared to respective age-matched controls reflecting excessive destruction of neurites and/or loss of synapses.

Within the meaning of the present invention, the term "synapse and/or neurite miRNA" refers to miRNA which (i) is "brain-enriched", i.e., is present in increased amounts (e.g., at least 5-times higher concentrations) in the brain, as compared to other organs that can be a source of significant amounts of miRNA in a bodily fluid being tested and (ii) is present in a synapse and/or neurite (i.e., axon and/or dendrite

and/or spine). Brain enrichment of potential miRNA biomarker is important for screening and primary diagnosis purposes, because many miRNA that are expressed in neurons are also expressed in other tissue and cell types. As a result, changes in such miRNA plasma concentrations may reflect pathology of other organs. On the other hand, when a disease, e.g. MCI, has already been detected miRNA, which are expressed in neurons but are not brain-enriched, can be used for differential diagnosis, prognosis of the disease outcome and monitoring, especially in combination with brain-enriched miRNA biomarker and normalizer. For example, since some synapse and/or neurite miRNAs are much more effectively secreted from abnormal cells during neuronal pathology development, such synapse and/or neurite miRNAs can be also tested as potential biomarkers in the methods of the present invention even if they are not brain-enriched. To be useful in the methods of the present invention, such synapse and/or neurite miRNAs should be detectable in bodily fluids as a result of their release from neurons (e.g., due to secretion, neurite/synapse destruction or neuronal death).

The present invention provides novel highly sensitive and noninvasive or minimally invasive methods for diagnosing Mild Cognitive Impairment (MCI) and various neurodegenerative pathologies it can progress to (e.g., Alzheimer's Disease (AD)) in a subject, said methods comprising determining the level in a bodily fluid sample from the subject (e.g., blood plasma or serum, urine, saliva, or other bodily fluids) of one or more synapse and/or neurite miRNA.

The diagnostic methods of the invention make possible early diagnosis of MCI and preceding stages of AD, other neurodegenerative diseases and other neurodegenerative disorders, e.g., prior to occurrence of major morphological changes and/or massive neuronal cell death associated with such diseases and disorders.

Furthermore, analysis of synapse and/or neurite miRNAs significantly enhances the sensitivity of the miRNA detection as compared to detecting neuronal body miRNAs which are not present or depleted in synapses and neurites, because the amount of synapses and neurites in the brain is 10^3 times higher than the amount of neurons. This approach also provides detailed and comprehensive information for

monitoring disease development and treatment effectiveness, since various specific events in neurons (e.g., changes in miRNA profile, their secretion, neurite degradation, synapse loss, and finally neuronal death) can be detected and quantitated.

Although on a smaller scale, similar processes are characteristic of normal aging and can be detected and monitored using the same approach. Experimental data described in the Examples, below, demonstrate that with appropriate normalization plasma concentration of miR-128, miR-132, miR-874, miR-134, miR-323-3p, and miR-382 is 40%-60% higher in elder control group (76-86 years old) than in "young age" group control (20-50 years old). Respective numbers for MCI patients are significantly higher (200%-500% increase of biomarker miRNA concentration in plasma when compared to the "young age" controls).

Differences in levels of synapse and/or neurite miRNAs in bodily fluids of subjects having MCI, pre-MCI, or other neurodegenerative disorders as compared to age-matched healthy individuals detectable by the methods of the present invention may be due to (i) disease-associated destruction of neurites and/or synapses, (ii) disease-associated changes in expression or metabolism of these miRNAs, (iii) disease-associated changes in transport and intracellular distribution of these miRNAs, (iv) disease-associated changes in secretion of these miRNAs (Rabinowits et al. Clin Lung Cancer, 2009, 10:42-46; e.g., miR-451, miR-1246 – see Pigati et al., PLoS ONE, 2010, e13515), (v) disease-associated changes in the blood/brain barrier permeability, as well as other causes.

Since miRNA concentration levels in bodily fluids depend on many factors, data normalization becomes a very important issue. Several approaches can be used for the data normalization: (i) normalization per spiked non-human miRNA (e.g., ath-miR-159a) provides information on miRNA yield during extraction and potential RT-PCR inhibition; (ii) normalization per ubiquitous miRNA (e.g., miR-16), for which a limitation can be disease-related changes in its expression, secretion and so on; (iii) normalization per miRNA, which is expressed in numerous tissues but is under-expressed in brain (e.g. miR-10b, miR-141); (iv) normalization per brain-enriched miRNA, which should compensate such factors as changes in blood supply,

blood/brain barrier permeability, and others. The latter approach could be especially productive when: (1) miRNA biomarker is enriched in neurites and/or synapses and miRNA normalizer is present in glial cells mainly, e.g. miR-127 (Wu et al. 2009; Mol. Therapy, 17: 2058-2066); (2) miRNA biomarker is located in neurites or synapses and miRNA normalizer is specific for neuronal body; in this case in early stages of AD miRNA biomarker will be preferably released due to axon, neurite, spine and synapse destruction, and, for example, brain-enriched miR-9 which is located mainly in the perinuclear area of neurons could be used as a normalizer (Truettner et al. 2011. J. Cerebral Blood Flow & Metabolism, epub. April 20), (3) miRNA biomarker is located in hippocampus, which is afflicted first in AD, and miRNA normalizer is located in other brain areas; (4) expression or secretion of miRNA "normalizer" is downregulated due to AD development; thus, measurement of the biomarker/"normalizer" ratio can be useful for early MCI and AD detection. Another important advantage of using various brain-enriched miRNA as normalizers is their absence or very low expression in cells of the peripheral blood which prevents data distortion caused by hemolysis; (v) normalization per the average of several normalizers or, if many, e.g. >15, miRNA are analyzed, normalization per the average of all brain-enriched miRNA tested.

As discussed in detail in the Examples, below, for selection of best biomarker and normalizer miRNAs concentrations of many brain-enriched miRNA, including neurite/synapse ones, in plasma of MCI and AD patients and age-matched control group were analyzed by RT-PCR. Then all miRNA analyzed were tested as potential biomarkers and normalizers and combinations, which provided statistically significant differentiation between MCI patients and age-matched controls, were selected as most promising. The data have demonstrated that the best potential biomarkers are neurite/synapse miRNA and best normalizers are other brain-enriched miRNA. Two families of biomarkers and several normalizers have demonstrated the highest sensitivity (84%-92%) and specificity (84%-90%) in MCI detection, miR-132 family and miR-134 family. High correlation between members of miR-134 family can be easily explained by the fact that all members of this family, namely miR-134, miR-323-3p and miR-382, belong to the same cluster and are expressed in the same cell

types. Close relationships between members of miR-132 family, namely miR-128, miR-132 and miR-874, have not been described before. It is also interesting that miR-132 and miR-134 biomarker families give better results with different normalizers. miR-132 family works better than miR-134 family with normalizers miR-491-5p, miR-181a, miR-9, and miR-141. On the other hand, miR-134 family demonstrates better results than miR-132 family with normalizers miR-370 and miR-127.

As disclosed herein, retrospective longitudinal study of MCI development in eldery patients with normal cognitive function at enrollment demonstrated that the increase in plasma miRNA biomarker is detectable in asymptomatic disease stage, preceding MCI clinical manifestation by 1 to 5 years.

Since MCI/AD progression and normal aging share certain common processes, e.g. neurite and synapse destruction and ultimately neuronal death, the present inventors analyzed whether normal aging could also be detected using the same combinations of miRNA biomarkers and normalizers. miRNA in plasma samples from two groups of cognitively normal subjects, Group 1 (21-50 years old) and Group 2 (76-86 years old) were compared. The analysis showed that median concentrations of neurite/synapse miR-132 and miR-134 families were 40-80% higher in the plasma of Group 2 subjects compared to Group 1 ($p<0.05$ to $p<0.001$).

Other promising biomarkers, such as miR-7 and miR-125b, detect smaller subpopulations of MCI patients (about 60% sensitivity) but with high specificity (86%-93%). These miRNA do not detect age-related brain changes, which means that the increase in their plasma concentrations during MCI and AD development is due to less common processes, maybe, those characteristic of AD.

During progression from MCI to the dementia stage of AD the biomarker/normalizer ratio in bodily fluids is changing due to various factors. First, since numerous synapse and neurites are destroyed during early asymptomatic stage of MCI progression, during the later stages of AD there are fewer synapse and neurites and the total amount of excreted synapse/neurite miRNA decreases. Second, due to increased neuronal cell death during the later stages of AD, concentration of

neuronal body miRNA in bodily fluids increases. Third, as disease progresses, new brain areas and glial cells become involved in the pathology with disease progression, which lead to further increase in concentration of respective miRNA normalizers in bodily fluids. The phenomena described above can be used to monitor MCI-dementia transition during AD development.

An increase in the level of miR-451, whose secretion from pathologic cells is significantly higher, is statistically significant in the plasma of AD patients when compared to the plasma of MCI patients. The ratios of miR-451 to miRNA of miR-132 and miR-134 families as well as to other brain-enriched miRNA ensure the best differentiation of AD from MCI. However, in about 40-50% of MCI cases these parameters overlap with numbers obtained for AD patients. It is likely that these patients will progress from MCI to the AD dementia. When compared with the age-matched controls after normalization per various miRNA, miR-7, 125b and miR-16 detect the same MCI cases that were characterized as AD by miR-451 analysis, which indicates that both approaches can be used for predicting MCI progression to dementia.

Since different brain areas are involved in various neurodegenerative diseases leading to development of dementia (Geldmacher & Whitehouse, Neurology. 1997, 48:S2-9; Levy & Chelune, J Geriatr Psychiatry Neurol. 2007 20:227-238; Gong & Lippa, Am J Alzheimer's Dis Other Demen, 2010, 25:547-555) and due to different miRNA expression profile in various brain areas (Landgraf et al., Cell. 2007, 129:1401-1414;The miR-Ontology Data Base: http://ferrolab.dmi.unict.it/miro/), analysis of neurite and/or synapse miRNA profile in bodily fluids can be used for differentiation of pre-MCI and MCI that will result in AD dementia or dementia caused by other neurodegenerative diseases.

The methods of the present invention can be used to diagnose pre-MCI and MCI and predict and/or monitor a progression from pre-MCI and MCI to various more severe neurodegenerative diseases such as, e.g., Alzheimer's disease (AD), Parkinson's disease (PD), Lewy Body dementia, Huntington's disease (HD),

frontotemporal dementia (FTD), vascular dementia, HIV Associated Neurocognitive Disorders (HAND), mixed dementia, etc.

Non-limiting examples of brain-enriched miRNAs useful in the methods of the present invention include, e.g., 7, 9, 96, 98, 99a, 103, 107, 124a, 125a, 125b, 127, 128a, 132, 134, 137, 138, 149, 153, 154, 181a, 181b, 181c, 182, 183, 204, 212, 213, 218, 219, 221, 222, 299-3p, 299-5p, 323-3p, 324-5p, 328, 329, 330, 331, 335, 337, 338, 342, 346, 369-3p, 369-5p, 370, 379, 381, 382, 383, 409-3p, 411, 425, 432, 433-5p, 485-3p, 485-5p, 487b, 488, 491-5p, 494, 495, 496, 504, 539, 541, 543, 584, 656, 668, 758, 874, 889, 935, 939, 1193, 1197, 9*.

Neurite and/or synapse miRNAs useful in the methods of the present invention include, without limitation, miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939 (see Schratt et al., Nature 439:283-289, 2006; Lugli et al., J Neurochem. 106:650-661, 2008; Bicker and Schratt, J Cell Mol Med. 12:1466-1476, 2008; Smalheiser and Lugli, Neuromolecular Med. 11:133-140, 2009; Rajasethupathy, Neuron, 63:714-716, 2009; Kye, RNA, 13:1224-1234, 2007; Yu, et al., Exp Cell Res. 314:2618-2633, 2008; Cougot et al., J Neurosci. 28:13793-13804, 2008; Kawahara, Brain Nerve, 60:1437-1444, 2008; http://ferrolab.dmi.unict.it/miro/). Additional miRNAs useful in the methods of the invention can be identified, for example, based on their enrichment in neurons (and in certain regions of the brain depending on a disease) and intracellular localization in axons and/or dendrites and/or spines and/or synapses. If urine samples are selected for conducting diagnostic methods of the invention, preferred miRNAs for detection would be those miRNAs which are not significantly expressed in cells of the urinary system. Similarly, if blood samples (e.g., serum or plasma) are used for conducting diagnostic methods of

the invention, preferred miRNAs for detection would be those miRNAs which are not expressed or are present at very low levels in blood cells.

The methods of the instant invention are based on measurement of levels of certain miRNAs in bodily fluids. The use of bodily fluids that can be collected by non-invasive or minimally invasive techniques (e.g., as opposed to detection in the brain or CSF) allows for a cost effective and minimally invasive or noninvasive diagnostic procedure. Preferred bodily fluids for use in the methods of the invention are blood plasma, serum, urine, and saliva. However, any other bodily fluid can also be used.

Examples of useful methods for measuring miRNA level in bodily fluids include hybridization with selective probes (e.g., using Northern blotting, bead-based flow-cytometry, oligonucleotide microchip [microarray], or solution hybridization assays such as Ambion mirVana miRNA Detection Kit), polymerase chain reaction (PCR)-based detection (e.g., stem-loop reverse transcription-polymerase chain reaction [RT-PCR], quantitative RT-PCR based array method [qPCR-array]), or direct sequencing by one of the next generation sequencing technologies (e.g., Helicos small RNA sequencing, miRNA BeadArray (Illumina), Roche 454 (FLX-Titanium), and ABI SOLiD). For review of additional applicable techniques see, e.g., Chen et al., BMC Genomics, 2009, 10:407; Kong et al., J Cell Physiol. 2009; 218:22-25.

In some embodiments, miRNAs are purified prior to quantification. miRNAs can be isolated and purified from bodily fluids by various methods, including the use of commercial kits (e.g., miRNeasy kit [Qiagen], MirVana RNA isolation kit [Ambion/ABI], miRACLE [Agilent], High Pure miRNA isolation kit [Roche], and miRNA Purification kit [Norgen Biotek Corp.]), Trizol extraction (see Example 1, below), concentration and purification on anion-exchangers, magnetic beads covered by RNA-binding substances, or adsorption of certain miRNA on complementary oligonucleotides.

In some embodiments, miRNA degradation in bodily fluid samples and/or during miRNA purification is reduced or eliminated. Useful methods for reducing or eliminating miRNA degradation include, without limitation, adding RNase inhibitors

(e.g., RNasin Plus [Promega], SUPERase-In [ABI], etc.), use of guanidine chloride, guanidine isothiocyanate, N-lauroylsarcosine, sodium dodecylsulphate (SDS), or a combination thereof. Reducing miRNA degradation in bodily fluid samples is particularly important when sample storage and transportation is required prior to miRNA quantification.

To account for possible losses of a given miRNA during purification, potential RT-PCR inhibition, miRNA contaminants derived from dying or damaged blood or urine cells during sample isolation and treatment, variations in kidney filtration, etc., various additional methods of experimental data normalization can be employed. For example, the following normalization methods can be used in the present invention:

a) Concentration of a target miRNA can be normalized to one of the ubiquitous miRNAs (e.g., miR-16), small nucleolar RNAs (snoRNAs), U6 small nuclear RNA (U6 RNA), and others).

b) Synthetic small RNA (e.g., non-human miRNA) oligonucleotides can be synthesized and used as controls for losses during purification and RT-PCR inhibition (by adding them to bodily fluid samples before RNA purification).

c) To account for variations in kidney filtration (when working with urine samples), miRNA concentration in urine can be normalized on creatinine and/or albumin level.

The following approach for selecting miRNA biomarkers for early detection of MCI and AD was developed in the current invention:

1. In addition to known neurite/synapse-enriched miRNAs other brain-enriched miRNAs were included in the preliminary study and analyzed in plasma from AD and MCI patients and compared to age-matched controls.

2. Data for each miRNA were normalized per all other individual miRNAs and miRNA biomarkers and normalizers most promising for MCI detection were selected.

3. These miRNAs were used for a larger study which included plasma samples from younger and age-matched donors, MCI and AD patients.

4. Finally, the retrospective longitudinal study was performed using plasma collected from individuals originally enrolled when they had no symptoms of MCI or AD and then followed for several years. Later some donors developed MCI, some developed AD, and some remained AD and MCI free.

5. In addition to miRNAs enriched in brain, miR-451, which is secreted much more effectively from pathologic cells, was also included in study.

In conjunction with the above diagnostic and screening methods, the present invention provides various kits comprising one or more primer and/or probe sets specific for the detection of target miRNA. Such kits can further include primer and/or probe sets specific for the detection of normalizer miRNA. Non-limiting examples of primer or probe combinations in kits are as follows:

1. Primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-125b, and miR-16 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-491-5p, miR-9, miR-127, miR-181a, and miR-370).

2. Primers or probes specific for miR-451 (optionally, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939).

3. Primers or probes specific for at least two miRNAs selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351,

miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p).

4. Primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, miR-874, miR-134, miR-323-3p, miR-382, miR-7, and miR-125b (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p).

5. Primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, and miR-874 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-491-5p, miR-9, miR-181a, and miR-141).

6. Primers or probes specific for at least one miRNA selected from the group consisting of miR-134, miR-323-3p, and miR-382 (optionally, further comprising primers or probes specific for at least one normalizer of miR-370 or miR-127).

7. Primers or probes specific for miR-7 (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-9, miR-27, miR-181a, miR-370, and miR-491-5p).

8. Primers or probes specific for miR-125b (optionally, further comprising primers or probes specific for at least one normalizer miRNA selected from the group consisting of miR-9, miR-181a, miR-370, and miR-491-5p).

Such kits can be useful for direct miRNA detection in bodily fluid samples isolated from patients or can be used on purified RNA samples.

A kit of the invention can also provide reagents for primer extension and amplification reactions. For example, in some embodiments, the kit may further

include one or more of the following components: a reverse transcriptase enzyme, a DNA polymerase enzyme (such as, e.g., a thermostable DNA polymerase), a polymerase chain reaction buffer, a reverse transcription buffer, and deoxynucleoside triphosphates (dNTPs). Alternatively (or in addition), a kit can include reagents for performing a hybridization assay. The detecting agents can include nucleotide analogs and/or a labeling moiety, e.g., directly detectable moiety such as a fluorophore (fluorochrome) or a radioactive isotope, or indirectly detectable moiety, such as a member of a binding pair, such as biotin, or an enzyme capable of catalyzing a non-soluble colorimetric or luminometric reaction. In addition, the kit may further include at least one container containing reagents for detection of electrophoresed nucleic acids. Such reagents include those which directly detect nucleic acids, such as fluorescent intercalating agent or silver staining reagents, or those reagents directed at detecting labeled nucleic acids, such as, but not limited to, ECL reagents. A kit can further include miRNA isolation or purification means as well as positive and negative controls. A kit can also include a notice associated therewith in a form prescribed by a governmental agency regulating the manufacture, use or sale of diagnostic kits. Detailed instructions for use, storage and trouble shooting may also be provided with the kit. A kit can also be optionally provided in a suitable housing that is preferably useful for robotic handling in a high throughput setting.

The components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container. The container will generally include at least one vial, test tube, flask, bottle, syringe, and/or other container means, into which the solvent is placed, optionally aliquoted. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other solvent.

Where there is more than one component in the kit, the kit also will generally contain a second, third, or other additional container into which the additional

components may be separately placed. However, various combinations of components may be comprised in a container.

Such kits may also include components that preserve or maintain DNA or RNA, such as reagents that protect against nucleic acid degradation. Such components may be nuclease or RNase-free or protect against RNases, for example. Any of the compositions or reagents described herein may be components in a kit.

Definitions

The term "Alzheimer's Disease" or "AD" as used herein refers to post-MCI AD phases characterized by dementia.

The term "pre-Mild Cognitive Impairment" or "pre-MCI" refers to asymptomatic, preclinical phase of AD and other neurodegenerative diseases leading to dementia (Jack et al., Alzheimer's and Dementia. 2011, Epub April 19).

The term "neuronal cell body" refers to the portion of a nerve cell that contains the nucleus surrounded by the cytoplasm and the plasma membrane but does not incorporate the dendrites or axons.

The term "neurite" as used herein refers to any projection from the cell body of a neuron. This projection can be an axon, a dendrite, or a spine.

The term "axon" refers to a long, slender projection of a neuron that conducts electrical impulses away from the neuron's cell body or soma. Axons are distinguished from dendrites by several features, including shape (dendrites often taper while axons usually maintain a constant radius), length (dendrites are restricted to a small region around the cell body while axons can be much longer), and function (dendrites usually receive signals while axons usually transmit them). Axons and dendrites make contact with other cells (usually other neurons but sometimes muscle or gland cells) at junctions called synapses.

The term "dendrite" refers to a branched projection of a neuron that acts to conduct the electrochemical stimulation received from other neural cells to the cell body of the neuron from which the dendrites project.

The terms "spine" or "dendritic spine" refer to a small membranous protrusion from a neuron's dendrite that typically receives input from a single synapse of an axon. Dendritic spines serve as a storage site for synaptic strength and help transmit electrical signals to the neuronal cell body. Most spines have a bulbous head (the spine head), and a thin neck that connects the head of the spine to the shaft of the dendrite. The dendrites of a single neuron can contain hundreds to thousands of spines. In addition to spines providing an anatomical substrate for memory storage and synaptic transmission, they may also serve to increase the number of possible contacts between neurons.

The term "synapse" refers to specialized junctions, through which neurons signal to each other and to non-neuronal cells such as those in muscles or glands. A typical neuron gives rise to several thousand synapses. Most synapses connect axons to dendrites, but there are also other types of connections, including axon-to-cell-body, axon-to-axon, and dendrite-to-dendrite. In the brain, each neuron forms synapses with many others, and, likewise, each receives synaptic inputs from many others. As a result, the output of a neuron may depend on the input of many others, each of which may have a different degree of influence, depending on the strength of its synapse with that neuron. There are two major types of synapses, chemical synapses and electrical synapses. In electrical synapses, cells approach within about 3.5 nm of each other, rather than the 20 to 40 nm distance that separates cells at chemical synapses. In chemical synapses, the postsynaptic potential is caused by the opening of ion channels by chemical transmitters, while in electrical synapses it is caused by direct electrical coupling between both neurons. Electrical synapses are therefore faster than chemical synapses.

Within the meaning of the present invention, the term "synapse and/or neurite miRNA" refers to miRNA which (i) is "brain-enriched", i.e., is present in increased amounts (e.g., at least 5-times higher concentrations) in the brain, as compared to other organs that can be a source of significant amounts of miRNA in a bodily fluid being tested and (ii) is present in a synapse and/or neurite (i.e., axon and/or dendrite and/or spine).

Since some synapse and/or neurite miRNAs are much more effectively secreted from abnormal cells during neuronal pathology development, such synapse and/or neurite miRNAs can be also tested as potential biomarkers in the methods of the present invention even if they are not brain-enriched. To be useful in the methods of the present invention, such synapse and/or neurite miRNAs should be detectable in bodily fluids as a result of their release from neurons (e.g., due to secretion, neurite/synapse destruction or neuronal death).

The term "normalizer miRNA" as used herein refers to miRNA which is used for normalization of neurite/synapse miRNA concentration to account for various factors that affect appearance and stability of neurite/synapse miRNA in plasma.

The term "neuronal body miRNA" as used herein refers to miRNA which (i) is "brain-enriched", i.e., is present in increased amounts (e.g., at least 5-times higher concentrations) in the brain, as compared to other organs that can be a source of significant amounts of miRNA in a bodily fluid being tested and (ii) is absent from or present insignificantly lower concentrations in neurites or synapses than in neuronal cell bodies.

The terms "neuronal pathology" and "pathological changes in neurons" are used herein to refer to metabolic and/or structural changes in neurons associated with neurite and/or synapse dysfunction and/or neurite destruction and/or synapse loss.

The term "associated with" is used to encompass any correlation, co-occurrence and any cause-and-effect relationship.

The term "development of a neuronal pathology" is used herein to refer to any negative change in the extent/severity of a metabolic and/or structural change in individual neurons and/or any increase in the number of neurons affected. The phrase "improvement of a neuronal pathology" and similar terms refer to any positive change in the extent/severity of a metabolic and/or structural change in individual neurons and/or any decrease in the number of neurons affected.

As used herein, the term "small RNA" refers generally to a heterogeneous group of non-coding RNAs with a variety of regulatory functions including chromatin architecture/epigenetic memory, transcription, RNA splicing, RNA editing, mRNA

translation, and RNA turnover. The diagnostic methods of the present invention rely on detecting neurite and/or synapse small RNAs, which can be detected in bodily fluids, such as, for example, microRNAs (miRNAs), Brain Cytoplasmic RNAs BC1/BC200, etc. There are other classes of less characterized small RNAs which can be also useful in the methods of the present invention (reviewed in Kim, Mol. Cells, 2005, 19: 1-15).

The terms "microRNA" or "miRNA" as used herein refer to a class of small approximately 22 nt long non-coding RNA molecules. They play important roles in the regulation of target genes by binding to complementary regions of messenger transcripts (mRNA) to repress their translation or regulate degradation (Griffiths-Jones Nucleic Acids Research, 2006, 34, Database issue: D140-D144). Frequently, one miRNA can target multiple mRNAs and one mRNA can be regulated by multiple miRNAs targeting different regions of the 3' UTR. Once bound to an mRNA, miRNA can modulate gene expression and protein production by affecting, e.g., mRNA translation and stability (Baek et al., Nature 455(7209):64 (2008); Selbach et al., Nature 455(7209):58 (2008); Ambros, 2004, Nature, 431, 350-355; Bartel, 2004, Cell, 116, 281-297; Cullen, 2004, Virus Research., 102, 3-9; He et al., 2004, Nat. Rev. Genet., 5, 522-531; and Ying et al., 2004, Gene, 342, 25-28). Examples of neurite and/or synapse miRNAs useful in the methods of the present invention include, without limitation, miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939. Information on most currently known miRNAs can be found in the miRNA database miRBase (available at the world wide web at mirbase.org). See also Burside et al., BMC Genomics 9:185 (2008); Williams et al., BMC Genomics 8:172 (2007); Landgraf et al., Cell 129:1401 (2007).

The term "miRNA array" refers to a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of multiple (e.g., thousands) microscopic spots of oligonucleotides, each containing a specific sequence (probe) complementary to a particular target miRNA. After probe-target hybridization under high-stringency conditions the resulting hybrids are usually detected and quantified by quantifying fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of miRNA. In the methods of the present invention, both custom-made and commercially available miRNA arrays can be used. Examples of useful commercially available miRNA arrays (based on various methods of target labeling, hybrid detection and analysis) include arrays produced by Agilent, Illumina, Invitrogen, Febit, and LC Sciences.

The term "next generation sequencing technologies" broadly refers to sequencing methods which generate multiple sequencing reactions in parallel. This allows vastly increased throughput and yield of data. Non-limiting examples of commonly used next generation sequencing platforms include Helicos small RNA sequencing, miRNA BeadArray (Illumina), Roche 454 (FLX-Titanium), and ABI SOLiD.

An "individual" or "subject" or "animal", as used herein, refers to humans, veterinary animals (e.g., cats, dogs, cows, horses, sheep, pigs, etc.) and experimental animal models of neurodegenerative diseases or other neuronal pathologies. In a preferred embodiment, the subject is a human.

The term "urinary tract" refers to the organs and ducts, which participate in the secretion and elimination of urine from the body.

The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, i.e., contaminants, including native materials from which the material is obtained. For example, RNA purification includes elimination of proteins, lipids, salts and other unrelated compounds present in bodily fluids. Besides, for some methods of analysis a purified miRNA is preferably substantially free of other RNA oligonucleotides contained in bodily fluid samples (e.g., rRNA and mRNA fragments, ubiquitous

miRNAs, which are expressed at high levels in almost all tissues [e.g., miR-16], etc.). As used herein, the term "substantially free" is used operationally, in the context of analytical testing of the material. Preferably, purified material substantially free of contaminants is at least 50% pure; more preferably, at least 90% pure, and still more preferably at least 99% pure. Purity can be evaluated by chromatography, gel electrophoresis, composition analysis, biological assay, and other methods known in the art.

As used herein, the term "similarly processed" refers to samples (e.g., bodily fluid samples or purified RNAs) which have been obtained using the same protocol.

The term "about" or "approximately" means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, still more preferably within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the term "about" or "approximately" depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989 (herein "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization [B.D. Hames & S.J. Higgins eds. (1985)]; Transcription And Translation [B.D. Hames & S.J. Higgins, eds. (1984)]; Animal Cell Culture [R.I. Freshney, ed. (1986)]; Immobilized Cells And Enzymes [IRL Press, (1986)]; B. Perbal, A Practical Guide To Molecular Cloning (1984); Ausubel, F.M. et al. (eds.). Current Protocols in Molecular Biology. John Wiley & Sons, Inc., 1994. These techniques include site directed mutagenesis as described in Kunkel, Proc. Natl. Acad. Sci. USA 82: 488- 492 (1985), U. S. Patent No. 5,071, 743, Fukuoka et al., Biochem. Biophys. Res. Commun. 263: 357-360 (1999); Kim and Maas, BioTech. 28: 196-198

(2000); Parikh and Guengerich, BioTech. 24: 4 28-431 (1998); Ray and Nickoloff, BioTech. 13: 342-346 (1992); Wang et al., BioTech. 19: 556-559 (1995); Wang and Malcolm, BioTech. 26: 680-682 (1999); Xu and Gong, BioTech. 26: 639-641 (1999), U.S. Patents Nos. 5,789, 166 and 5,932, 419, Hogrefe, Strategies l4. 3: 74-75 (2001), U. S. Patents Nos. 5,702,931, 5,780,270, and 6,242,222, Angag and Schutz, Biotech. 30: 486-488 (2001), Wang and Wilkinson, Biotech. 29: 976-978 (2000), Kang et al., Biotech. 20: 44-46 (1996), Ogel and McPherson, Protein Engineer. 5: 467-468 (1992), Kirsch and Joly, Nucl. Acids. Res. 26: 1848-1850 (1998), Rhem and Hancock, J. Bacteriol. 178: 3346-3349 (1996), Boles and Miogsa, Curr. Genet. 28: 197-198 (1995), Barrenttino et al., Nuc. Acids. Res. 22: 541-542 (1993), Tessier and Thomas, Meths. Molec. Biol. 57: 229-237, and Pons et al., Meth. Molec. Biol. 67: 209-218.

EXAMPLES

The present invention is also described and demonstrated by way of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described here. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and such variations can be made without departing from the invention in spirit or in scope. The invention is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which those claims are entitled.

Example 1: Comparison of different methods used for miRNA purification from serum or plasma.

There are many commercial kits for miRNA isolation, including the miRNeasy kit (Qiagen), the MirVana RNA isolation kit (Ambion/ABI), miRACLE (Agilent), High Pure miRNA isolation kit (Roche), and miRNA Purification kit (Norgen Biotek Corp.). Besides, the in-house techniques based on the use of Trizol

(Invitrogen) can be used. In this technique (Invitrogen's protocol), after Trizol LS deproteinization, RNA is precipitated with isopropyl alcohol or additionally purified on silica columns. In some experiments, purified RNA is treated with RNAse-free DNAse (Qiagen, ABI, Invitrogen or other).

miRNA preparations obtained by different methods were compared using RT-PCR. Using Trizol LS (Invitrogen's protocol) and the MirVana RNA isolation kit (Ambion/ABI protocol)miRNA was purified from plasma and serum samples obtained from the same 5 healthy donors. 10^7 copies of Arabidopsis thaliana miR-159a (ath-miR-159a) were spiked per 1 ml plasma or serum after addition of guanidine-containing solution for evaluation of miRNA yield. Two techniques, one based on MirVana Paris kit (Ambion/ABI), and another based on Trizol (Invitrogen) deproteinization, and subsequent purification on silica columns, were compared. After RNA purification concentrations of spiked miRNA and human endogenous miR-9, miR-16, and miR-134 in final preps were measured by RT-PCR. Both MirVana Paris kit and the Trizol/silica filtration-based technique were effective in miRNA isolation and were used in future experiments. Although all analyzed miRNA were detectable in serum and plasma and both sample types are suitable for miRNA testing, the final PCR Ct values were about 2 cycles lower for plasma, and the latter was used in subsequent experiments. Based on the quantitative measurement of spiked ath-miR-159a, average yield of miRNA from plasma was about 70%.

A similar analysis was performed using plasma samples and the miRNeasy kit (Qiagen). A synthetic non-human miRNA was spiked after guanidine addition for calculating miRNA yield.

Example 2: Selection of miRNA for testing.

Tested miRNAs were initially selected based on literature data on their enrichment in brain compartments and presence in neurites (i.e., axons and/or dendrites and/or spines) and/or synapses (Hua *et al., BMC Genomics* 2009, 10:214; Liang et al., BMC Genomics. 2007, 8:166; Landgraf et al., Cell. 2007, 129:1401-1414; Lee et al., RNA. 2008, 14:35-42; Schratt *et al.*, Nature. 439:283-289,

2006;Lugli *et al.*, J Neurochem. 106:650-661, 2008; Bicker and Schratt, J Cell Mol Med., 12:1466-1476, 2008; Smalheiser and Lugli, Neuromolecular Med. 11:133-140, 2009; Rajasethupathy, Neuron. 63:714-716, 2009; Kye, RNA 13:1224-1234, 2007; Yu *et al.*, Exp Cell Res. 314:2618-2633, 2008; Cougot, *et al.*, J Neurosci. 28:13793-13804, 2008; Kawahara, Brain Nerve. 60:1437-1444, 2008; Schratt G. Rev Neurosci. 2009;10:842-849; Pichardo-Casas et al. Brain Research. 1436:20-33, 2012) as well as on their suggested involvement in neurite- and synapse-associated processes (The miR-Ontology Data Base: http://ferrolab.dmi.unict.it/miro/). For normalization, in addition to spiked miRNA, ubiquitous miRNA, such as miR-16, as well as miRNA expressed in numerous tissues but not in the brain, such as miR-10b and miR-141, were used.

Example 3: Experimental pre-selection of miRNA biomarkers and normalizers.

Plasma samples were obtained from patients diagnosed with MCI with amnestic symptoms (aMCI) (Dlugaj et al., Dement Geriatr Cogn Disord., 2010, 30:362-373; Brooks, Loewenstein, Alzheimer's Res Therapy, 2010, 2:28-36). Profiles of brain-enriched miRNAs from plasma of these patients were analyzed using RT-PCR with primers and probes for each individual miRNA (ABI). The amount of RNA equivalent to 30 µL plasma were taken in each RT reaction, and 1/15 of RT product was taken into final PCR. Thus, the amount of miRNA equivalent to 2 µL plasma was detected. The results obtained for each miRNA were normalized per each potential normalizer miRNA, converted into Relative Concentration (RC) of miRNA according to the ABI protocol (2^{-Ct}), and compared with miRNA profiles from age-matched controls (AMC). Practically, all miRNA analyzed were tested as potential biomarkers and normalizers and combinations, which provided statistically significant differentiation between MCI patients and age-matched controls were selected for further studies. Two conclusions are obvious from data presented below. First, the best potential biomarkers are neurite/synapse miRNA and, second, best normalizers are other brain-enriched miRNA.

When normalization per spiked non-human miRNA (ath-miR-159a) was performed, which gives relative miRNA concentration per 1 ml plasma, some plasma samples from MCI patients contained more neurite and/or synapse miRNAs (Fig. 1, miR-7 (A) and miR-874 (B)).

At the same time concentrations of other brain-enriched miRNAs were not changed in the plasma of MCI patients (Fig. 1, miR-9 (C), miR-181a (D) and miR-491-5p (E)).

Similar results were obtained, when miRNA concentrations in plasma were normalized per miR-141 or miR-10b, which are expressed in many organs but not in the brain (Fig. 2A-C and 8A-C). Ubiquitous miR-16 was not a good potential normalizer for differentiating MCI from AMC (Fig. 7).

At the same time, normalization of neurite/synapse miRNA concentrations in plasma per other brain-enriched miRNAs revealed several promising normalizers. Some of these normalizer miRNAs are neuronal body miRNAs, others are mainly expressed in brain areas not involved in the pathology, or in glial cells. It is also possible that some of them are downregulated in the pathology.

Figures 3A-C, 4, 5A-C and 6 present examples of the results obtained with various neurite/synapse miRNA concentrations in plasma of MCI and AD patients versus controls after normalization per brain-enriched miR-181a, miR-9, miR-491-5p, and miR-127, respectively. Based on data obtained 8 neurite/synapse miRNAs (miR-7, miR-125b, miR-128, miR-132, miR-134, miR-323-3p, miR-382, and miR-874) were selected as the most promising biomarkers and 7 miRNAs (miR-9, miR-127, miR-141, miR-181a, miR-370, and miR-491-5p) were selected as potential normalizers.

Example 4: MCI detection by analysis of miRNAs in plasma.

Plasma from amnestic MCI patients, AD patients and age-matched controls (AMC), 20 in each group, were used in the study. RNA was isolated from two 200 µl aliquots of plasma samples by the Trizol-silica method according to an

Asuragen procedure. Single target TaqMan® miRNA qRT-PCR assays (Applied Biosystems) were run using 2 µl plasma equivalents in triplicate in a reaction volume of 10 µl for final PCR for measuring concentration of a neurite/synapse miRNA biomarker as well as levels of normalizer miRNA selected as described in Example 3. miR-451 was also included in the study due to its presence in neurons, significantly higher secretion from abnormal cells (Pigati et al., PLoSOne, 2010, 5:e13515) and up-regulation in brain of fetuses with anencephaly (Zhang et al., Int. J.Biochem. Cell Biol. 2010; 42:367-374).

Data presented in Figures 9-14 demonstrate the 2-5 times increase in median concentrations of neurite/synapse miRNAs (miR-7, miR-125b, miR-128, miR-132, miR-134, miR-323-3p, miR-382, miR-874) in plasma of MCI and AD patients when compared to age-matched controls. The effect is more prominent when normalization is performed per brain-enriched miRNA, such as miR-9, miR-127, miR-181a, miR-370, and miR-491-5p.

Two families of biomarkers, miR-132 family and miR-134 family, and several normalizers have demonstrated the highest sensitivity and specificity,. Biomarkers miR-128, miR-132 and mir-874 ("miR-132 family") demonstrated 84%-92% sensitivity and 84%-90% specificity when normalized per miR-491-5p (Fig. 15A-C). Receiver-Operating Characteristic (ROC) curves for these combinations of biomarkers and the normalizer are presented in Fig. 15A-C. The area under the ROC curve (AUC) for miR-128, miR-132 and miR-874 is 0.95, 0.93 and 0.95, respectively. The second promising set of biomarkers consists of miR-134, miR-323-3p and miR-382 ("miR-134 family") and demonstrates 78%-91% sensitivity and 85-87% specificity when normalized per miR-370 (Fig. 16A-C). AUC for miR-134, miR-323-3p and miR-382 are 091, 0.94 and 0.92, respectively.

Correlation analysis shown in Fig. 17A-F demonstrates that miR-128, miR-132 and miR-874 form one family of biomarkers (Spearman test **r** values in the pair comparison are in the 0.93-0.95 range) and miR-134, miR-323-p and miR-382 form another family of biomarkers (Spearman test **r** values in the pair comparison are in the 0.87-0.93 range). High correlation between members of miR-134 family can be easily

explained by the fact that all members of this family, namely miR-134, miR-323-3p and miR-382, belong to the same cluster and are expressed in the same cell types (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Cluster.cgi). Close relationships between members of miR-132 family, namely miR-128, miR-132 and miR-874, have not been described before. It is also interesting that biomarker families miR-132 and miR-134 give better results with different normalizers. miR-132 family works better than miR-134 family with normalizers miR-491-5p, miR-181a, miR-9, and miR-141. On the other hand, miR-134 family demonstrates better results than miR-132 family with normalizers miR-370 and miR-127. Correlation between miR-132 and miR-134 biomarker families is relatively low (*r* values in the pair comparison Spearman test are in the 0.56-0.79 range) indicating that they either reflect distinct pathological processes or are located in different brain areas.

Concentrations of two other neurite/synapse miRNA, miR-7 and miR-125b, when analyzed with any normalizer, were increased in plasma of about 40-50% of MCI patients.

Example 5: Detection of age-related changes in plasma concentrations of neurite/synapse miRNA.

There are a number of common processes, e.g. neurite and synapse destruction and finally neuronal death, although on a smaller scale and caused by different factors, characteristic of normal aging and MCI/AD development. Since MCI is detectable by the approach proposed in current invention, it was of interest to investigate whether normal aging could be analyzed and monitored using the same miRNA biomarkers and normalizers.

Plasma samples from Group 1 (21-50 years old) and Group 2 (76-86 years old) subjects with normal cognitive functions, 20 samples in each group, were used in the study. RNA was isolated from two 200 µl aliquots of plasma samples by the Trizol-silica method according to an Asuragen procedure. Single target TaqMan® miRNA qRT-PCR assays (Applied Biosystems) were run using 2 µl plasma equivalents in triplicate in a reaction volume of 10 µl for final PCR for measuring

concentration of a neurite/synapse miRNA biomarker as well as levels of a normalizer miRNA selected as described in Example 3.

Data presented in Fig. 18A-J demonstrate that median concentrations of neurite/synapse miRNA biomarkers described in Example 4, such as miR-128, miR-132, miR-874, miR-134, miR-323-3p, or miR-382, after normalization per various brain-enriched miRNA normalizers, such as miR-9, miR-181a, miR-370, or miR-491-5p, are 40-60% higher in the plasma of Group 2 subjects when compared with those from Group 1. Thus, one can expect that prospective longitudinal analysis of neurite/synapse miRNAs and miRNA normalizers in subject bodily fluids will provide important information on brain processes associated with his/her normal aging. This also means that the two biomarker families detect neuronal processes that are common for normal aging and MCI development, such as neurite and synapse destruction.

Neurite/synapse miR-7 and miR-125b (Fig. 18) as well as miR-451, independent of miRNA normalizer used, are not increased during aging and, thus, do not differentiate between two groups.

Example 6: Retrospective longitudinal study of MCI development in elderly patients with normal cognitive functions at enrollment.

≥70 years old subjects with normal cognitive functions were enrolled in the study. The dynamics of their cognitive function impairment had been investigated and plasma samples had been collected periodically for 4-5 years. Some subjects during this period remained MCI free and others progressed to MCI. miRNAs were extracted and analyzed as described in Example 4. Concentrations of neurite and/or synapse miR-128, miR-132 and miR-874 were measured and normalized per miR-491-5p. Patients were considered MCI-positive if concentrations of at least two of three biomarkers were higher than predetermined control values. Data presented in Fig. 19 demonstrate that in 70% cases the increase in plasma biomarker miRNA is detectable in pre-symptomatic disease stage starting from patient enrollment, which preceded MCI diagnosis by 1 to 5 years.

Example 7: Detection of MCI transition to dementia stage of AD.

miR-451, which although is not brain-enriched is present in neurite and synapses and is secreted significantly more effectively from pathologic cells, was also included in the study.

Fig. 20 shows that the median concentration of miR-451 is slightly higher in plasma of MCI and is significantly (2-4 times) increased in plasma of AD patients when compared to age-matched controls. Ratios of miR-451 to neurite/synapse miRNA biomarkers concentrations differentiate MCI and AD populations even better (Fig. 21). At the same time in about 40-50% MCI patients this parameter is not distinguishable from that in AD patients, which indicates that these MCI patients will progress to the AD dementia. Thus, consecutive measurements of miR-451 concentration, particularly in combination with neurite/synapse miRNA, in plasma can be used as a marker of MCI-AD progression.

Since miR-7 and miR-125b also detect as pathologic about 40-50% of MCI patients (Example 4) and as miR-451 do not distinguish between young and old subjects (Example 5), these biomarkers were compared with miR-451. miR-16, which surprisingly behaved very similar to miR-7 in all experiments, was also included in the study. 2D graphs in Fig. 22 compare concentrations of these miRNA in plasma of MCI patients and age-matched controls after normalization per miR-491-5p. In all cases there is a group of MCI patients with higher plasma concentrations of both compared miRNA. Similar data were obtained when biomarker miRNA were normalized per the average of 5 brain-enriched normalizer miRNA (Fig. 23). Fig. 24 combines data presented in Figures 21-23 and demonstrates that practically the same patients are detected as pathologic (MCI, which will progress to the AD dementia) by described approaches.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

CLAIMS:

1. A method for detection of pre-MCI or MCI in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with an age-matched control level of said miRNA, and

c. (i) identifying the subject as being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the age-matched control or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is not increased as compared to the age-matched control.

2. A method for detection of pre-MCI or MCI in a subject, which method comprises:

a. measuring the level of a synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. (i) identifying the subject as being afflicted with pre-MCI or MCI when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when ratio of the levels of the miRNAs calculated in step (c) is not higher than the corresponding age-matched control ratio.

3. A method for predicting likelihood of progression from pre-MCI to MCI in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been obtained at spaced apart time points;

b. comparing the level of said miRNA in each of the bodily fluid samples collected from the subject with an age-matched control level of the said miRNA, and

c. predicting that the disease in the subject will progress from pre-MCI to MCI if the level of said miRNA is increased compared to the age-matched control in two or more consequently obtained bodily fluid samples collected from the subject.

4. A method for predicting likelihood of progression from pre-MCI to MCI in a subject, which method comprises:

a. measuring the level a synapse or neurite miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been obtained at spaced apart time points;

b. measuring the level of a normalizer miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the miRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. predicting that the disease in the subject will progress from pre-MCI to MCI if the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio in two or more consequently obtained bodily fluid samples collected from the subject.

5. A method for detection of brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with (i) a control level of said miRNA obtained from the same subject in the past or with (ii) a predetermined young age standard, and

c. identifying the subject as being subject to brain aging when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the control (i) or as compared to the predetermined young age standard (ii).

6. A method for detection of brain aging in a subject, which method comprises:

a. measuring the level of a synapse or neurite miRNA in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with (i) a corresponding control ratio obtained from the same subject in the past or with (ii) a predetermined young age standard ratio, and

e. identifying the subject as being subject to brain aging when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio (i) or as compared to the predetermined young age standard ratio (ii).

7. A method for determining the effectiveness of pre-MCI or MCI treatment in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) determining that the treatment is effective if the level of the miRNA has decreased in the course of or following the treatment; (ii) determining that the treatment is not effective if the level of miRNA has not decreased in the course of or following the treatment.

8. A method for determining the effectiveness of pre-MCI or MCI treatment in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to initiation of the treatment;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained in the course of or following the treatment;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained in the course of or following the treatment;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) determining that the treatment is effective if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) determining that the treatment is not effective if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

9. A method for determining the effectiveness of a treatment to delay brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) determining that the treatment is effective if the level of the miRNA has decreased in the course of or following the treatment; (ii) determining that the treatment is not effective if the level of miRNA has not decreased in the course of or following the treatment.

10. A method for determining the effectiveness of a treatment to delay brain aging in a subject, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to initiation of the treatment;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to initiation of the treatment;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained in the course of or following the treatment;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained in the course of or following the treatment;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) determining that the treatment is effective if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) determining that the treatment is not effective if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

11. A method for identifying a compound useful for slowing down the progression or treating pre-MCI or MCI, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject having pre-MCI or MCI, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) identifying that the test compound is useful for slowing down the progression or treating pre-MCI or MCI if the level of the miRNA has decreased after the compound administration; (ii) identifying that the test compound is not useful for slowing down the progression or treating pre-MCI or MCI if the level of miRNA has not decreased after the compound administration.

12. A method for identifying a compound useful for slowing down the progression or treating pre-MCI or MCI, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject having pre-MCI or MCI, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to test compound administration;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to test compound administration;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained following administration of the test compound;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained following administration of the test compound;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) identifying that the test compound is useful for slowing down the progression or treating pre-MCI or MCI if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating pre-MCI or MCI if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

13. A method for identifying a compound useful for delaying brain aging, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

c. comparing the levels of the miRNA measured in steps (a) and (b), and

d. (i) identifying that the test compound is useful for delaying brain aging if the level of the miRNA has decreased after the compound administration; (ii) identifying that the test compound is not useful for delaying brain aging if the level of miRNA has not decreased after the compound administration.

14. A method for identifying a compound useful for delaying brain aging, which method comprises:

a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject, wherein said bodily fluid sample(s) is obtained prior to test compound administration;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained prior to test compound administration;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject obtained prior to test compound administration;

d. measuring the level of the same synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;

e. measuring the level of a normalizer miRNA in the same bodily fluid sample(s) from the subject obtained following administration of the test compound;

f. calculating the ratio of the levels of the miRNAs measured in steps (d) and (e) for each of the bodily fluid samples collected from the subject obtained following administration of the test compound;

g. comparing the ratio of the levels of the miRNAs calculated in steps (c) and (f), and

h. (i) identifying that the test compound is useful for delaying brain aging if the ratio of the levels of the miRNAs calculated in step (f) is lower than the ratio of the levels of the miRNAs calculated in step (c); (ii) identifying that the test compound is not useful for delaying brain aging if the ratio of the levels of the miRNAs calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

15. A method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miR-451 in a bodily fluid sample collected from the subject;

b. measuring the level of at least one synapse or neurite miRNA in the same bodily fluid sample;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. determining that the disease in the subject will progress from MCI to dementia stage of AD if the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio.

16. A method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of at least one of miR-7, miR-125b, and miR-16 in a bodily fluid sample collected from the subject;

b. measuring the level of a normalizer miRNA in the same bodily fluid sample;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);

d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and

e. determining that the disease in the subject will progress from MCI to dementia stage of AD if at least one ratio calculated in step (c) is higher than the corresponding age-matched control ratio.

17. A method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miRNA-451 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. comparing the level of miRNA-451 in each of the bodily fluid samples from the subject with a corresponding age-matched control level, and

c. determining that the disease in the subject progresses from MCI to AD if the level of miRNA-451 in each of the bodily fluid samples from the subject is higher than the corresponding age-matched control level.

18. A method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of miR-451 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. measuring the level of at least one synapse or neurite miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the mRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. determining that the disease in the subject progresses from MCI to dementia stage of AD if the ratio of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio for each of the bodily fluid samples collected from the subject.

19. A method for monitoring progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, which method comprises:

a. measuring the level of at least one of miR-7, 125b, and miR-16 in bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points;

b. measuring the level of a normalizer miRNA in each of the same bodily fluid samples collected from the subject;

c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b) for each of the bodily fluid samples collected from the subject;

d. comparing the ratio of the levels of the mRNAs calculated in step (c) for each of the bodily fluid samples collected from the subject with a corresponding age-matched control ratio, and

e. determining that the disease in the subject progresses from MCI to dementia stage of AD if at least one ratio calculated in step (c) is higher than the corresponding age-matched control ratio for each of the bodily fluid samples collected from the subject.

20. The method of any one of claims 1-16 and 18, wherein the synapse or neurite miRNA is selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939.

21. The method of any one of claims 1-16 and 18, wherein the synapse or neurite miRNA is selected from the group consisting of miR-128, miR-132, miR-874, miR-134, miR-323-3p, and miR-382.

22. The method of any one of claims 1-16 and 18, comprising measuring the level of two or more synapse or neurite miRNA.

23. The method of any one of claims 1, 2, 5, and 6, comprising measuring the level of miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points.

24. The method of any one of claims 3, 4, 7-15, and 17-19, wherein the bodily fluid samples are obtained several months apart.

25. The method of claim 24, wherein the bodily fluid samples are obtained 3-6 months apart.

26. The method of any one of claims 2, 4, 6, 8, 10, 12, 14, 16, and 19, wherein the normalizer miRNA is a brain-enriched normalizer miRNA.

27. The method of claim 26, wherein the brain-enriched normalizer miRNA is selected from the group consisting of neuronal body miRNAs; miRNAs, which are mainly expressed in brain areas not involved in a pathology being assessed; miRNAs, which are mainly expressed in glial cells, and brain-enriched miRNAs downregulated in a pathology being assessed.

28. The method of any one of claims 2, 4, 6, 8, 10, 12, 14, 16, and 19, wherein the normalizer miRNA is selected from the group consisting of miR-9, miR-181a, miR-127, miR-370, and miR-491-5p.

29. The method of any one of claims 2, 4, 6, 8, 10, 12, 14, 16, and 19, wherein the normalizer miRNA is miRNA which is expressed in numerous tissues but is not significantly expressed in brain.

30. The method of any one of claims 2, 4, 6, 8, 10, 12, 14, 16, and 19, wherein the normalizer miRNA is miR-10b or miR-141.

31. The method of claim 16 or 19, wherein the normalizer miRNA is miR-491-5p or the average of two or more normalizers selected from the group consisting of miR-9, miR-127, miR-181a, miR-370, and miR-491-5p.

32. The method of any one of claims 2, 4, 6, 8, 10, 12, and 14, wherein the synapse or neurite miRNA is selected from the group consisting of miR-128, miR-132, and miR-874, and the normalizer miRNA is selected from the group consisting of miR-491-5p, miR-9, miR-181a, and miR-141.

33. The method of any one of claims 2, 4, 6, 8, 10, 12, and 14, wherein the synapse or neurite miRNA is selected from the group consisting of miR-134, miR-323-3p, and miR-382, and the normalizer miRNA is miR-370 or miR-127.

34. The method of claim 16 or 19, wherein the synapse or neurite miRNA is miR-7, and the normalizer miRNA is selected from the group consisting of miR-9, miR-181a, miR-370, and miR-491-5p.

35. The method of claim 16 or 19, wherein the synapse or neurite miRNA is miR-125b, and the normalizer miRNA is selected from the group consisting of miR-9, miR-181a, miR-370, and miR-491-5p.

36. The method of claim 1 or 3, wherein the age-matched control level of the miRNA is a predetermined standard.

37. The method of claim 2 or 4, wherein the age-matched control ratio of the miRNA is a predetermined standard.

38. The method of any one of claims 1-19, wherein the subject is human.

39. The method of any one of claims 11-14, wherein the subject is an experimental animal.

40. The method of any one of claims 1-19, wherein the bodily fluid sample is blood plasma or serum.

41. The method of any one of claims 1-19, which method comprises the step of collecting the bodily fluid sample(s) from the subject.

42. The method of any one of claims 1-19, wherein the level of the miRNA is determined using a method selected from the group consisting of hybridization, RT-PCR, and sequencing.

43. The method of any one of claims 1-19, wherein, prior to measuring miRNA level, the miRNA is purified from the bodily fluid sample.

44. The method of any one of claims 1-19, further comprising the step of reducing or eliminating degradation of the miRNA.

45. The method of any one of claims 7-14, wherein said method comprises the step of administering the test compound to the subject.

46. The method of any one of claims 1, 3, 5, 7, 9, 11, 13, 15, 17, and 18, further comprising normalizing the level of the synapse or neurite miRNA or miRNA-451 to a normalizer miRNA.

47. The method of claim 46, wherein the normalizer miRNA is a brain-enriched normalizer miRNA.

48. The method of claim 47, wherein the brain-enriched normalizer miRNA is selected from the group consisting of neuronal body miRNA; miRNA, which is mainly expressed in brain areas not involved in a pathology being assessed; miRNA, which is mainly expressed in glial cells, and brain-enriched miRNA downregulated in a pathology being assessed.

49. The method of claim 46, wherein the normalizer miRNA is selected from the group consisting of miR-9, miR-181a, miR-127, miR-370, and miR-491-5p.

50. The method of claim 46, wherein the normalizer miRNA is miRNA which is expressed in numerous tissues but is not significantly expressed in brain.

51. The method of claim 46, wherein the normalizer miRNA is miR-10b or miR-141.

52. The method of any one of claims 1-6 and 15-19, further comprising administering a therapeutic or preventive treatment to the subject that has been diagnosed as having the condition or as being at risk of progression to a more severe condition.

53. The method of any one of claims 1-6 and 15-19, further comprising recruiting the subject in a clinical trial.

54. A kit comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-125b, and miR-16.

55. The kit of claim 54, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-491-5p, miR-9, miR-127, miR-181a, and miR-370.

56. A kit comprising primers or probes specific for miR-451.

57. The kit of claim 56, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939.

58. A kit comprising primers or probes specific for at least two miRNAs selected from the group consisting of miR-7, miR-25, miR-26a, miR-26b, miR-98, miR-124, miR-125a, miR-125b, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200b, miR-200c, miR-218, miR-292-5p, miR-297, miR-322, miR-323-3p, miR-329, miR-325, miR-337, miR-339, miR-345, miR-350, miR-351, miR-369-3, miR-369-5p, miR-381, miR-382, miR-409-3p, miR-425, miR-429, miR-433-5p, miR-446, miR-467, miR-483-3p, miR-485-5p, miR-487b, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, and miR-939.

59. The kit of claim 58, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p.

60. A kit comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, miR-874, miR-134, miR-323-3p, miR-382, miR-7, and miR-125b.

61. The kit of claim 60, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-10b, miR-141, miR-9, miR-127, miR-181a, miR-370, and miR-491-5p.

62. A kit comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-128, miR-132, and miR-874.

63. The kit of claim 62, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-491-5p, miR-9, miR-181a, and miR-141.

64. A kit comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-134, miR-323-3p, and miR-382.

65. The kit of claim 64, further comprising primers or probes specific for at least one of miR-370 or miR-127.

66. A kit comprising primers or probes specific for miR-7.

67. The kit of claim 66, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-9, miR-27, miR-181a, miR-370, and miR-491-5p.

68. A kit comprising primers or probes specific for miR-125b.

69. The kit of claim 68, further comprising primers or probes specific for at least one miRNA selected from the group consisting of miR-9, miR-181a, miR-370, and miR-491-5p.

70. The kit of any one of claims 54-69, further comprising miRNA isolation or purification means.

71. The kit of any one of claims 54-69, further comprising instructions for use.

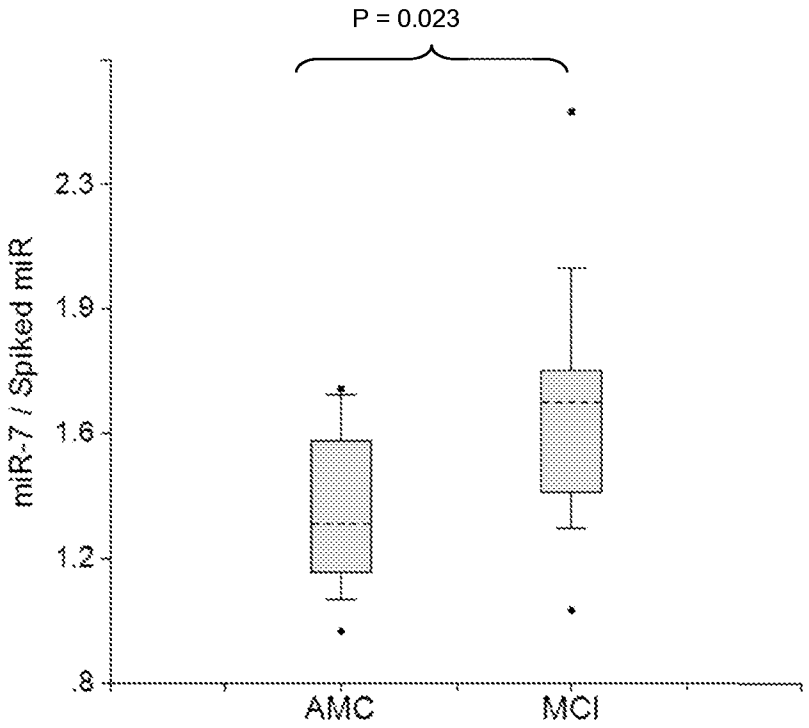


Figure 1A

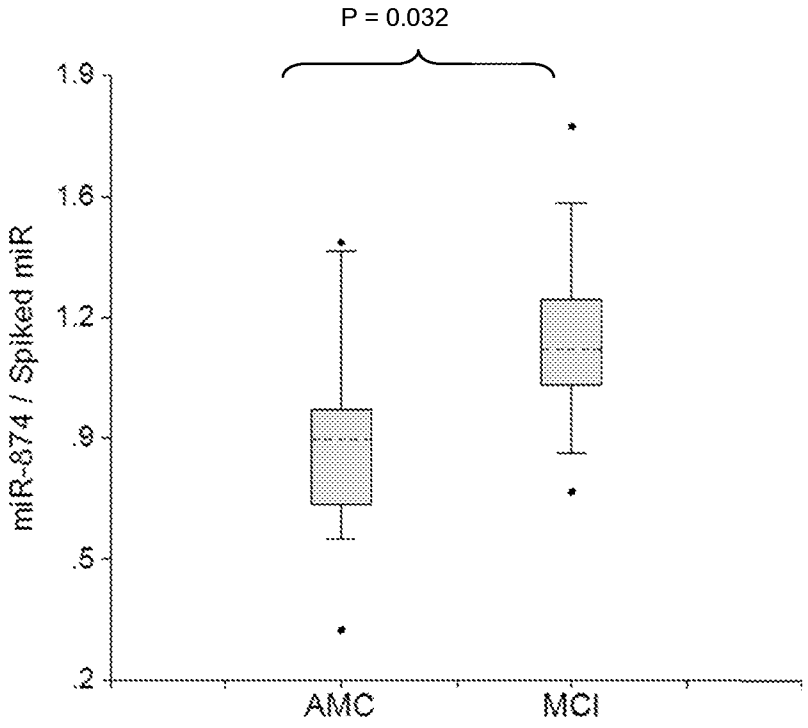


Figure 1B

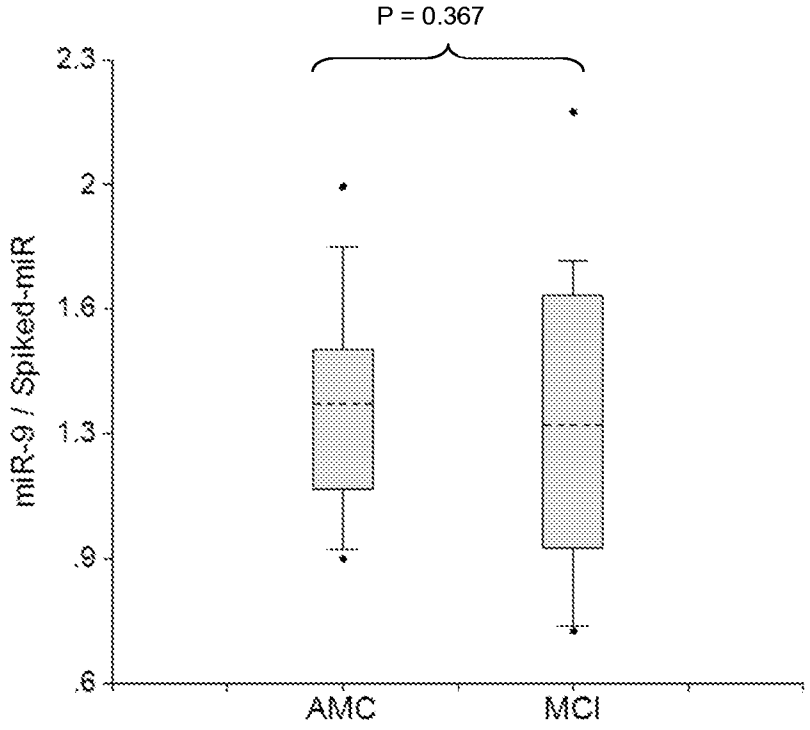


Figure 1C

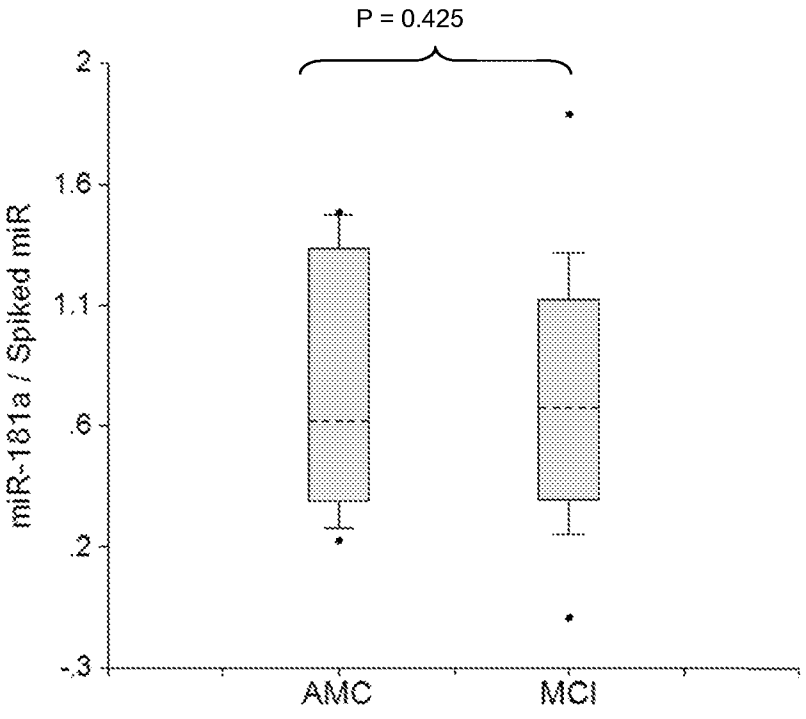


Figure 1D

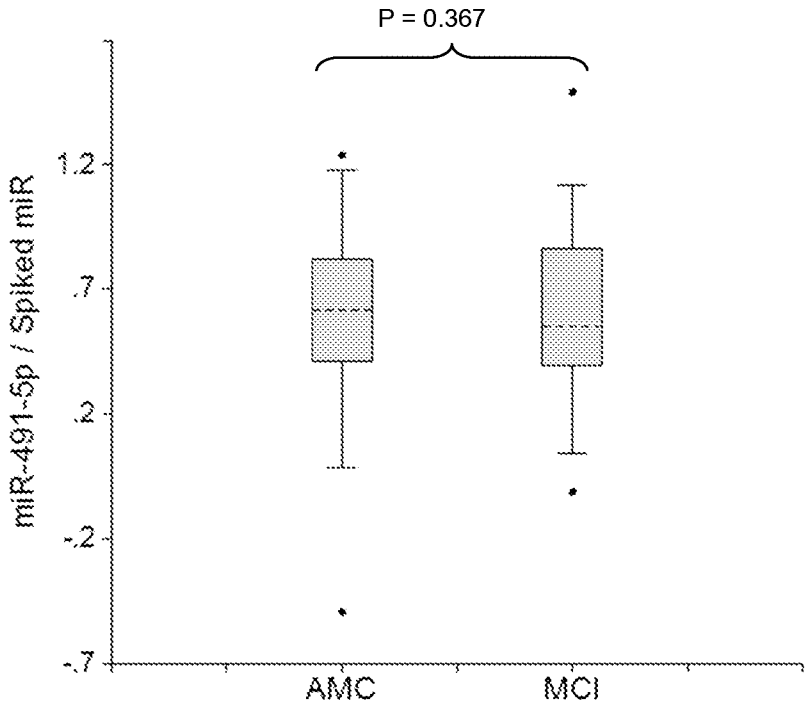


Figure 1E

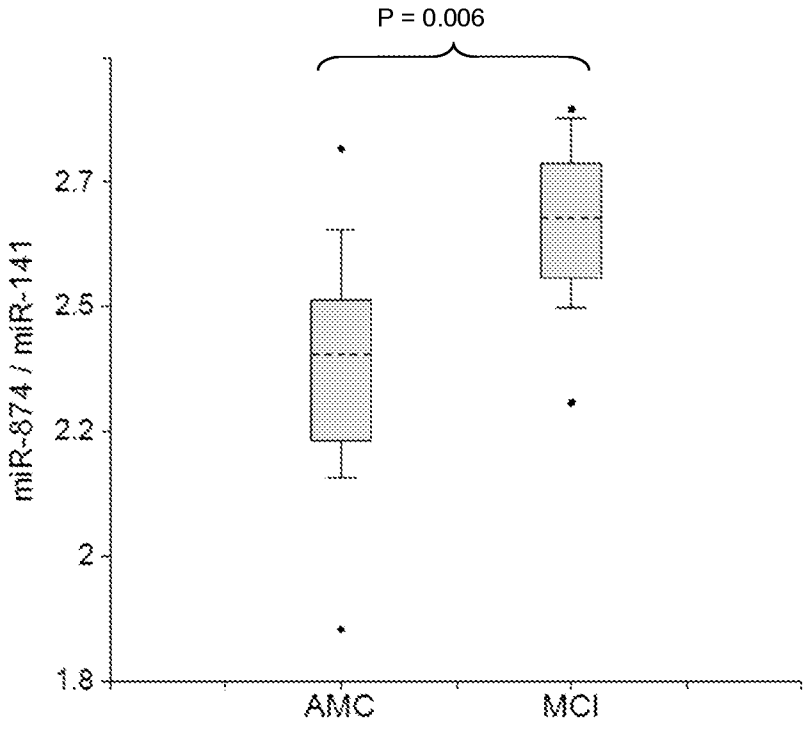


Figure 2A

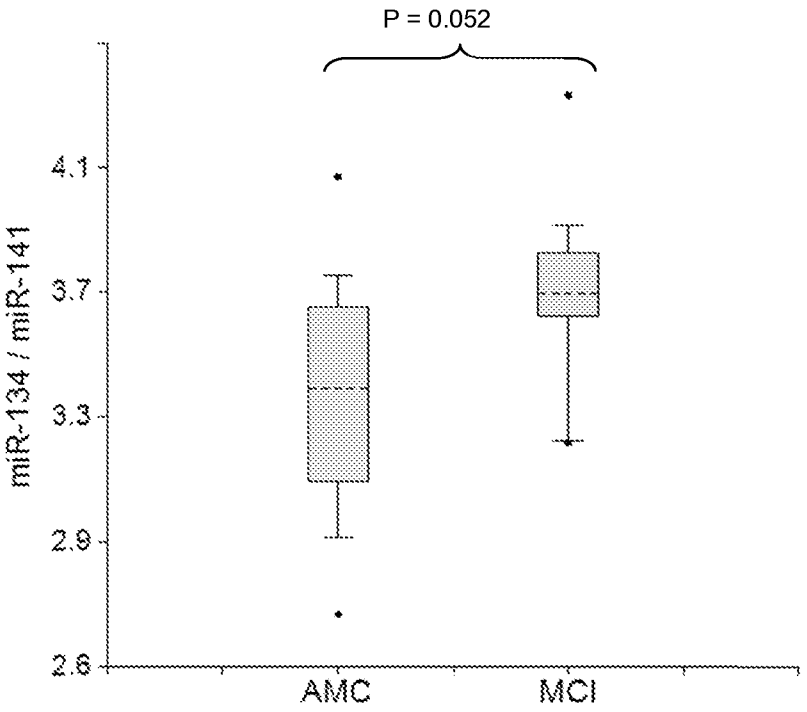


Figure 2B

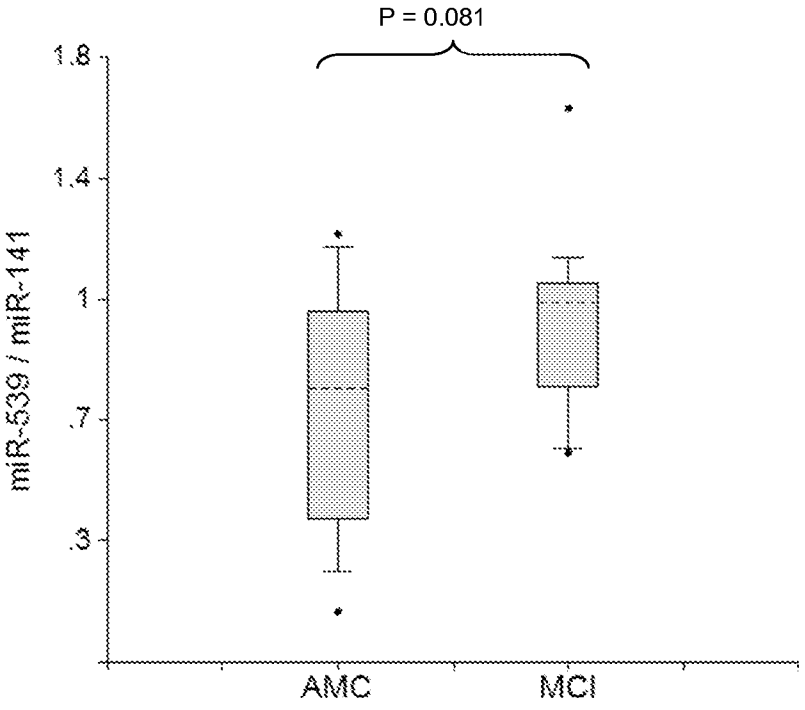


Figure 2C

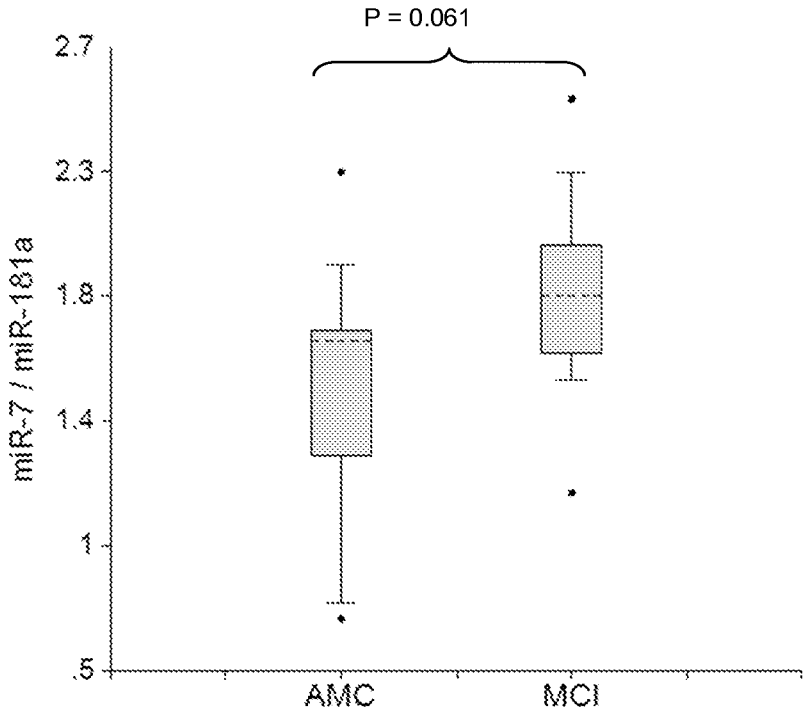


Figure 3A

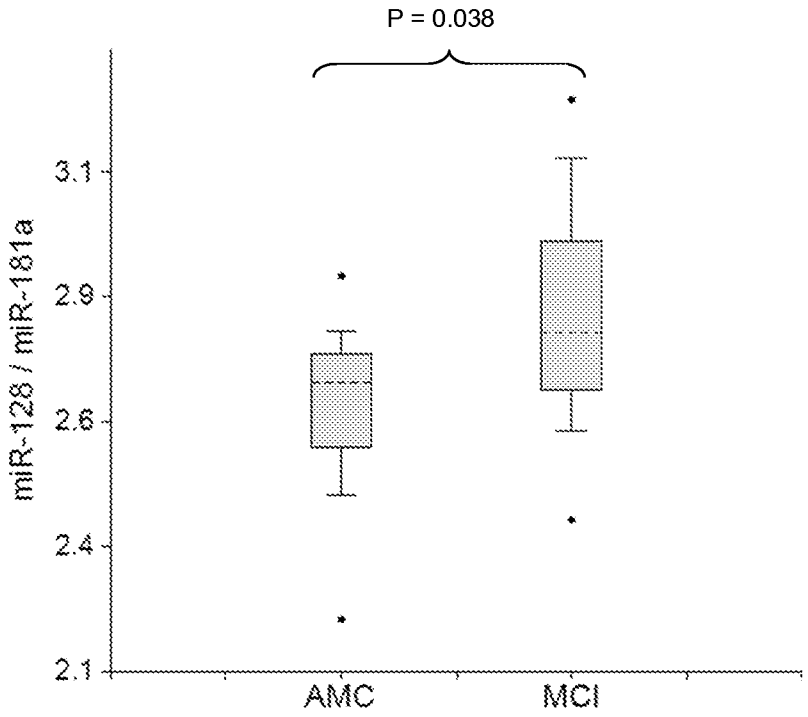


Figure 3B

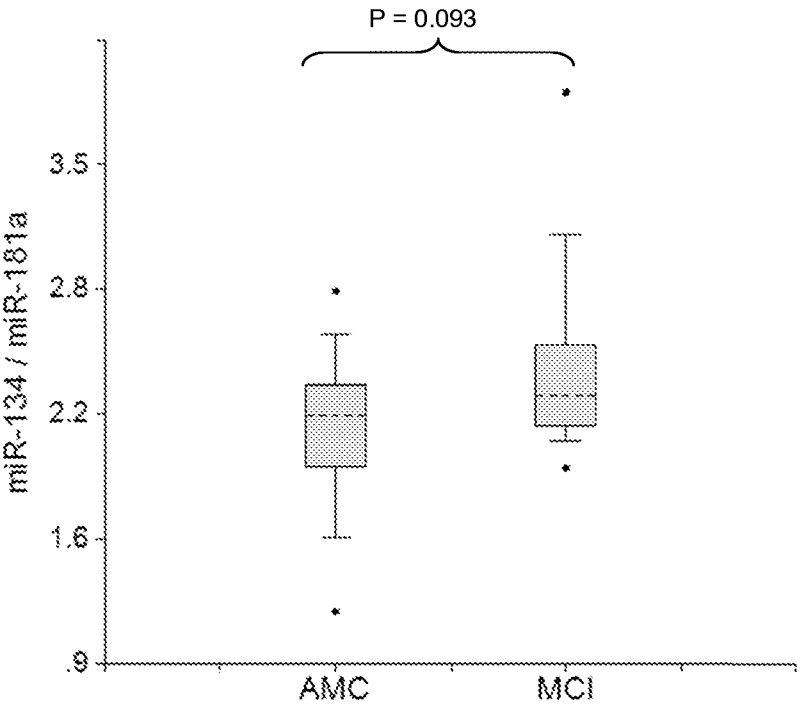


Figure 3C

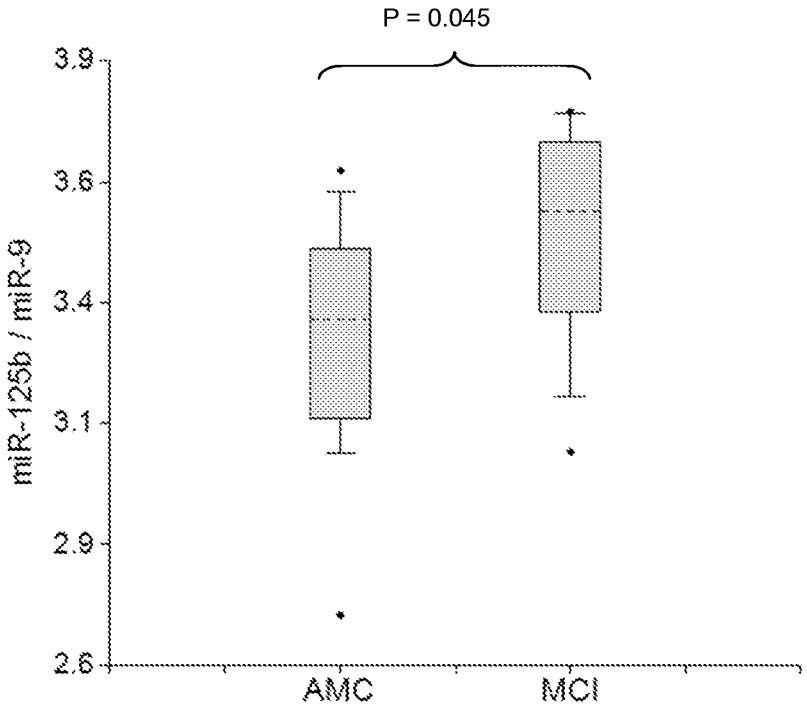


Figure 4

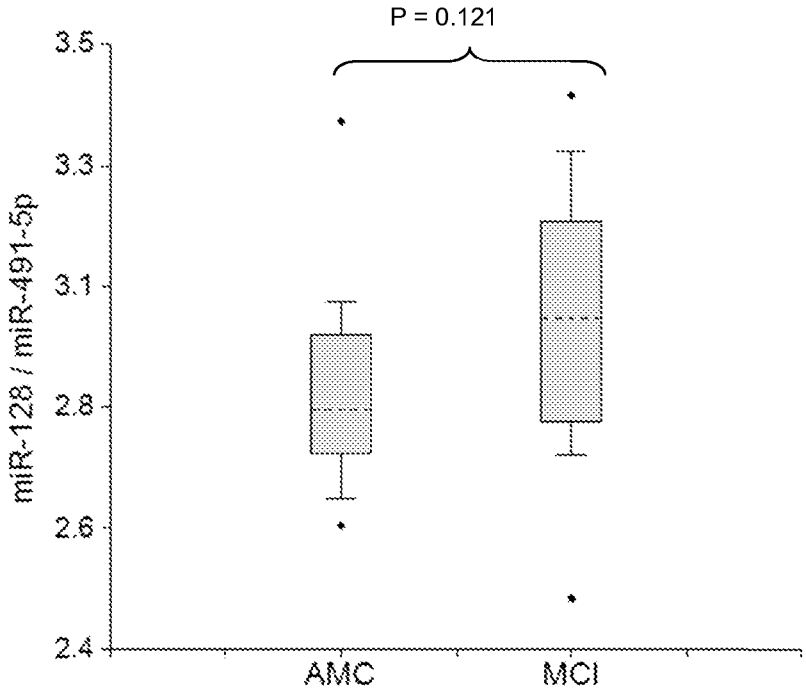


Figure 5A

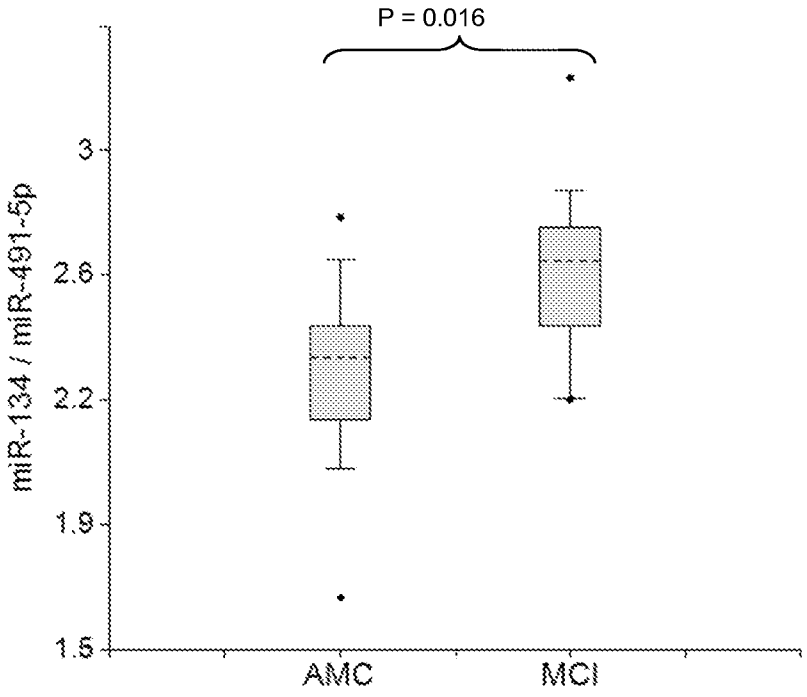


Figure 5B

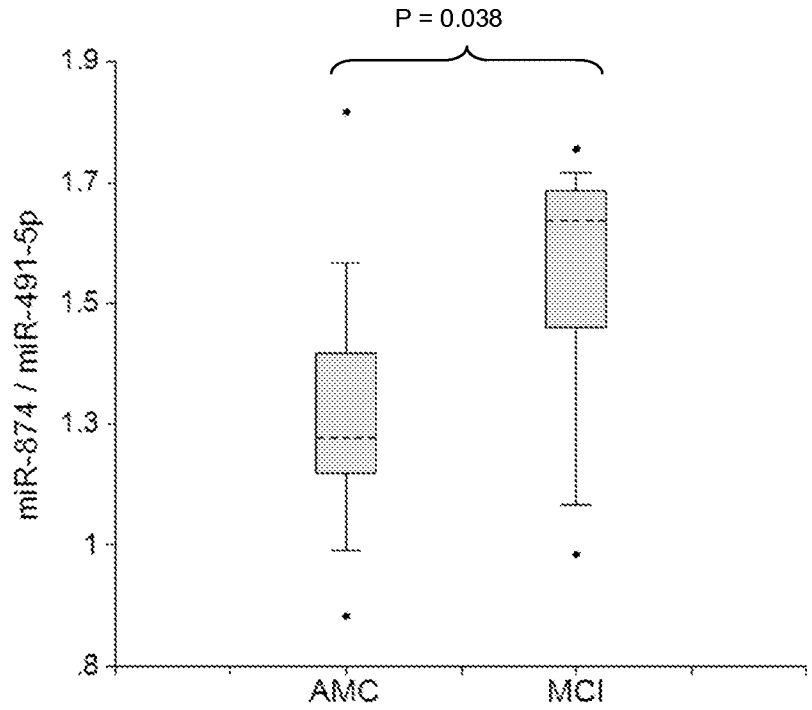


Figure 5C

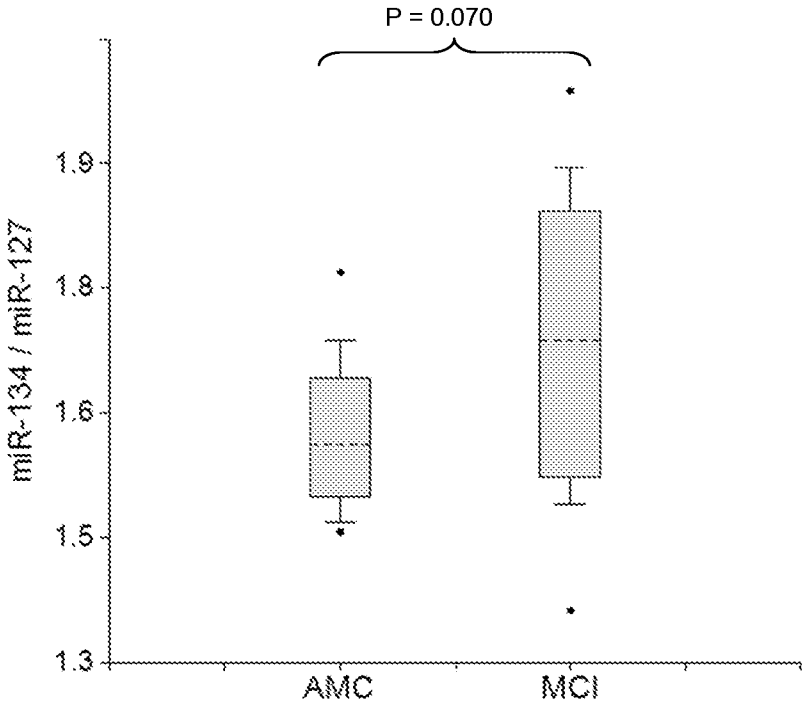


Figure 6

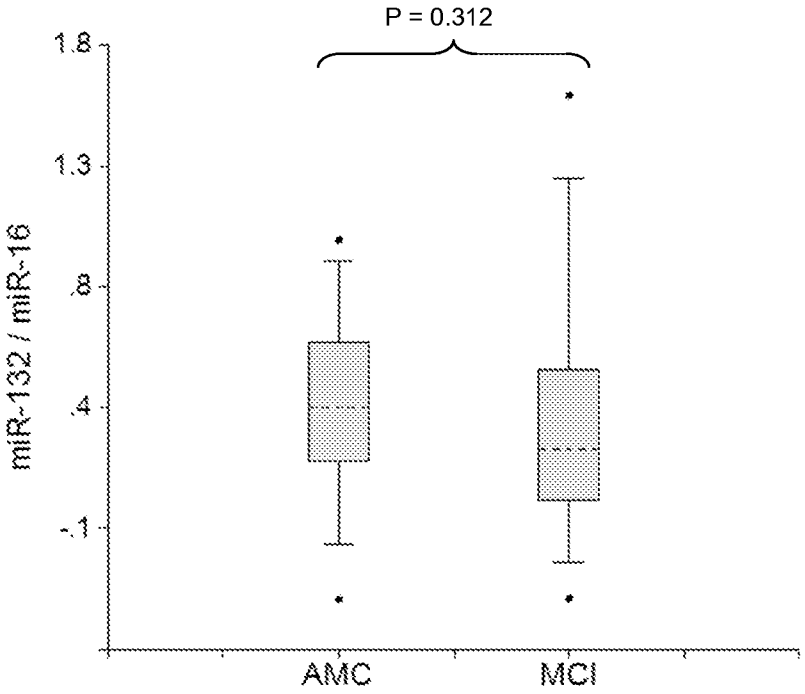


Figure 7A

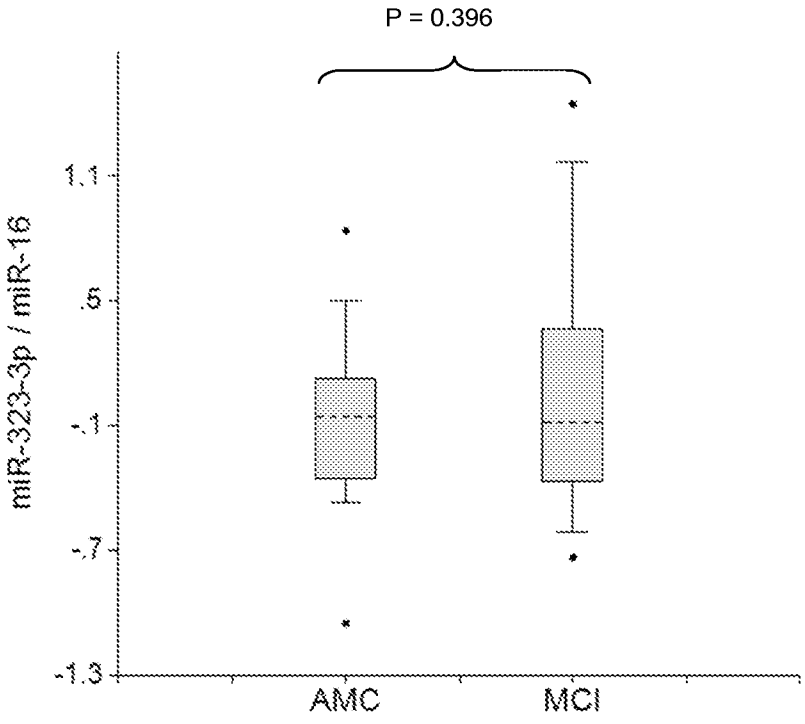


Figure 7B

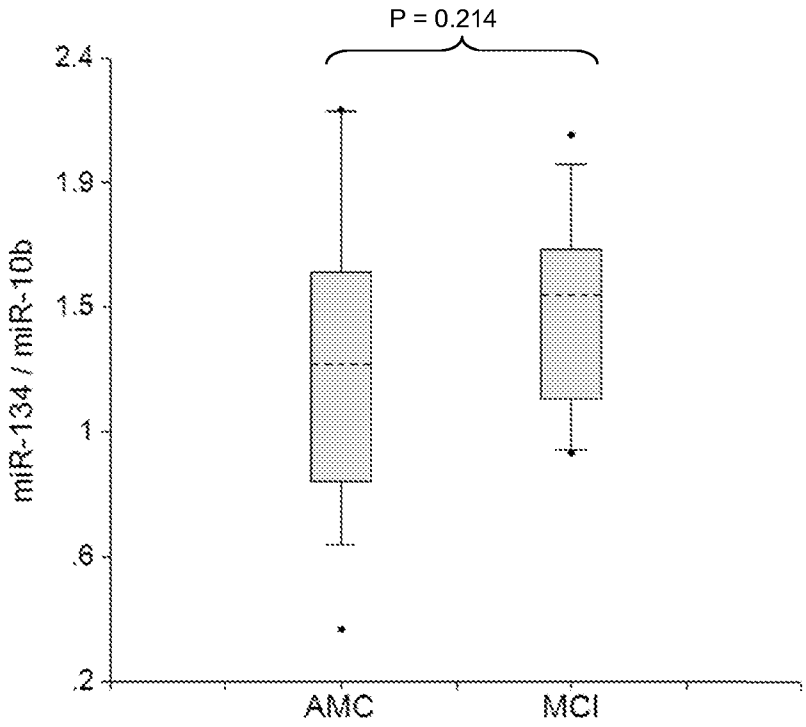


Figure 8A

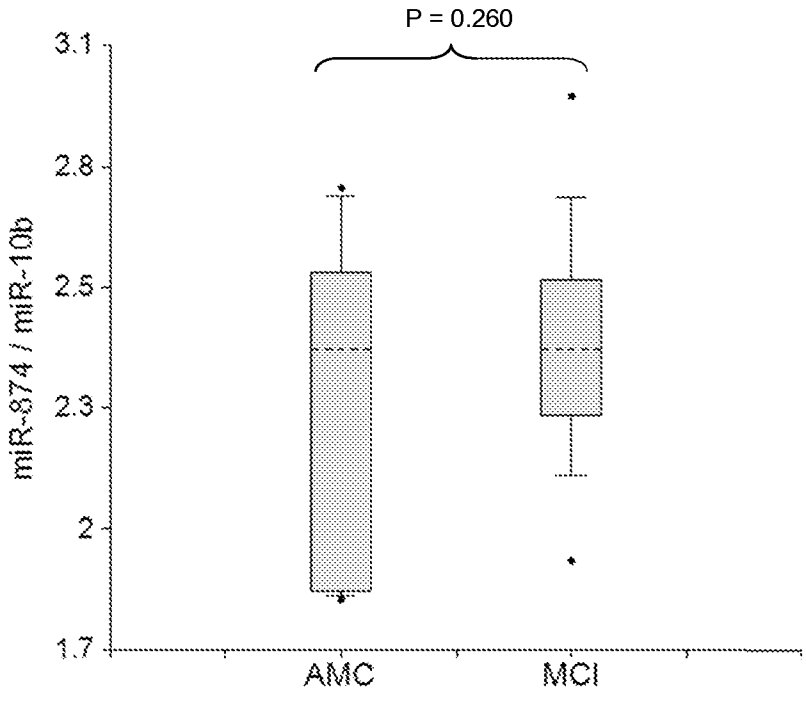


Figure 8B

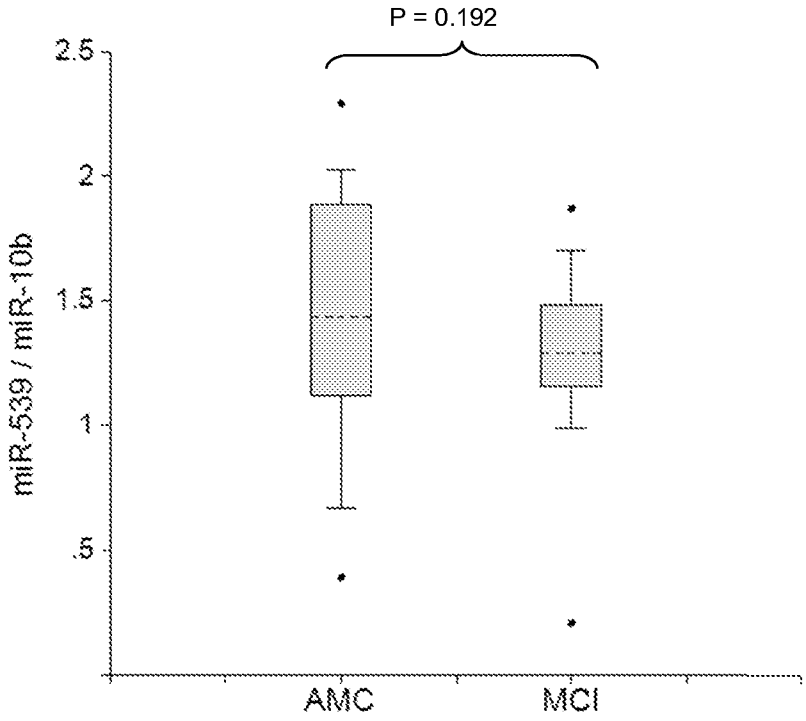


Figure 8C

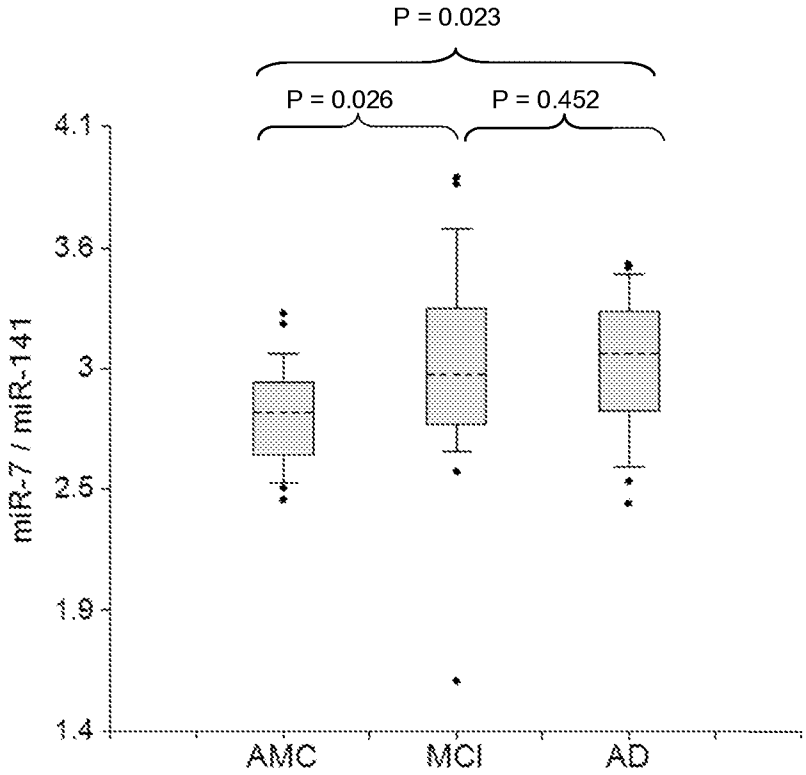


Figure 9A

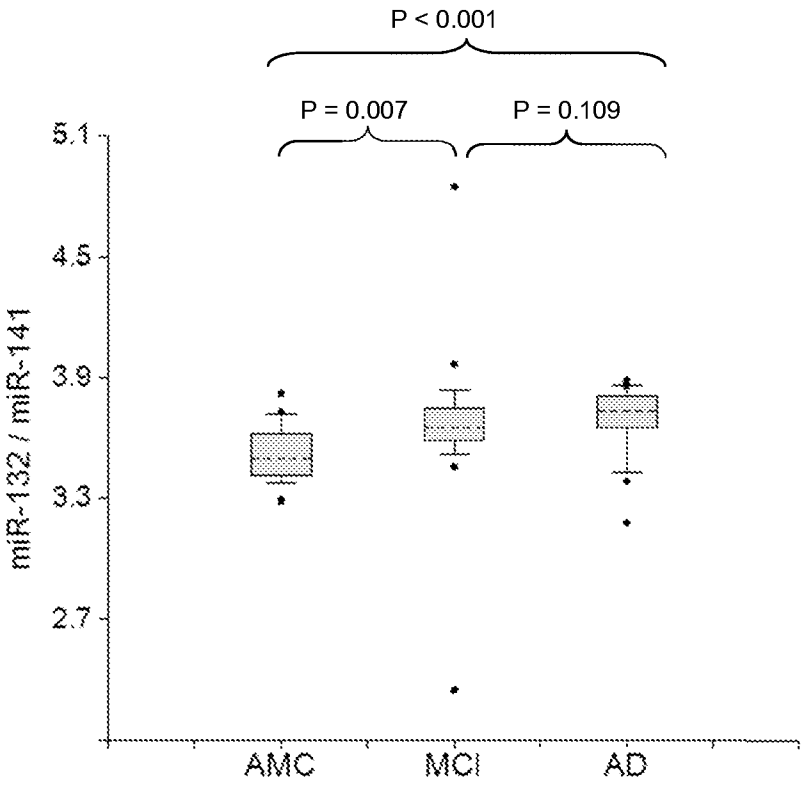


Figure 9B

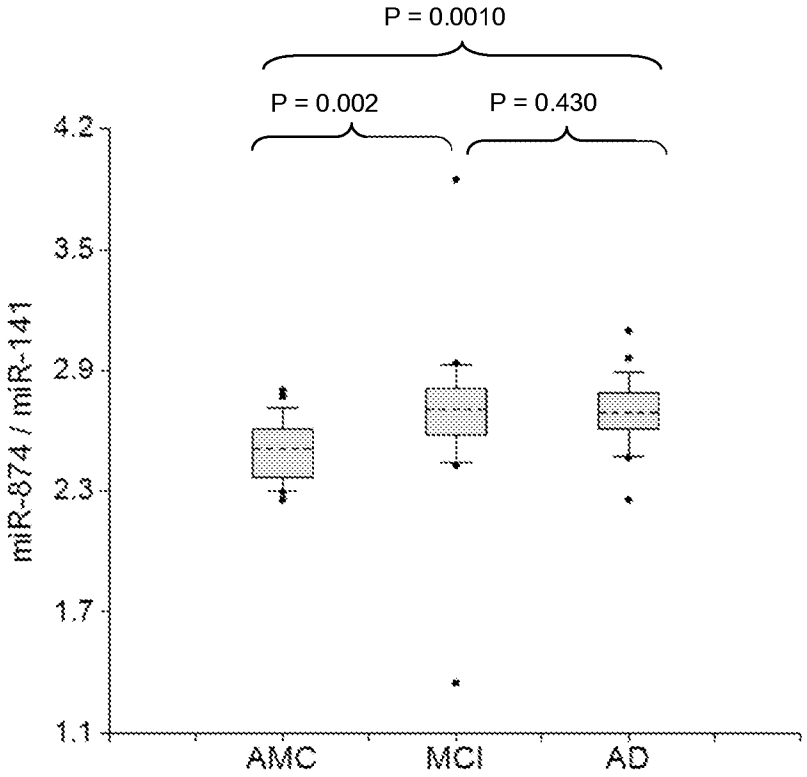


Figure 9C

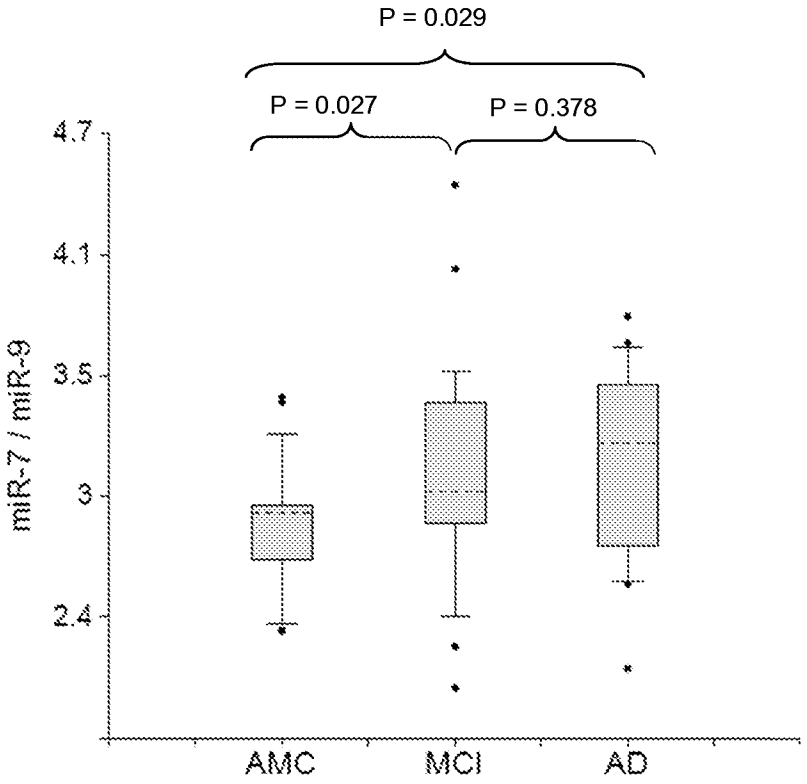


Figure 10A

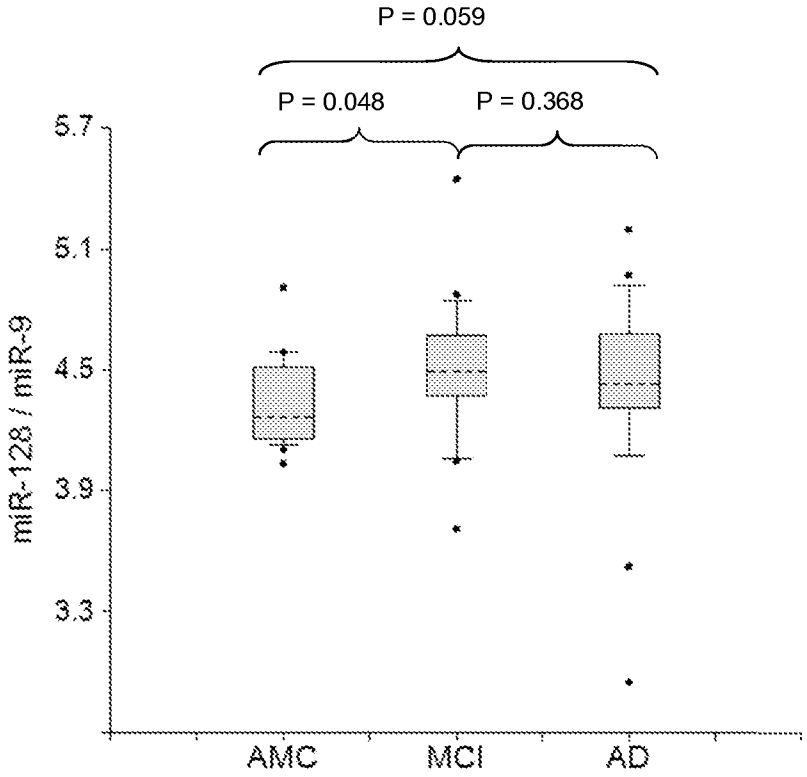


Figure 10B

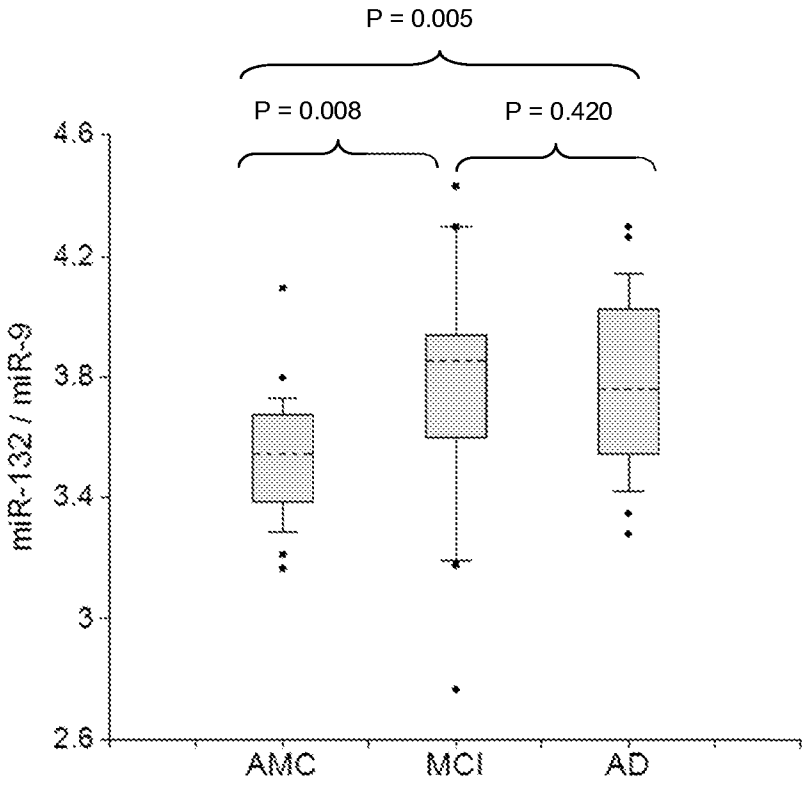


Figure 10C

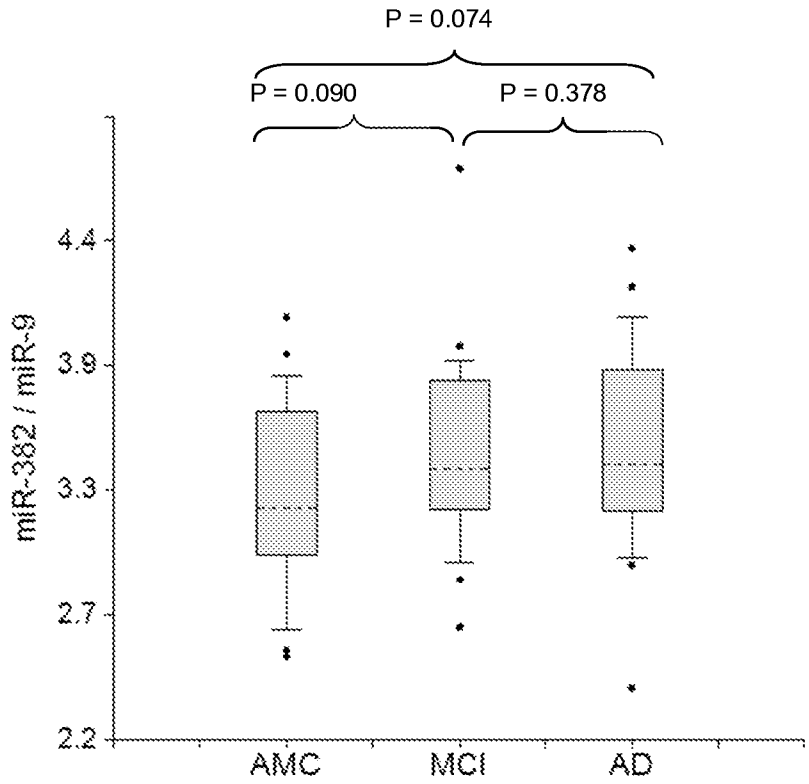


Figure 10D

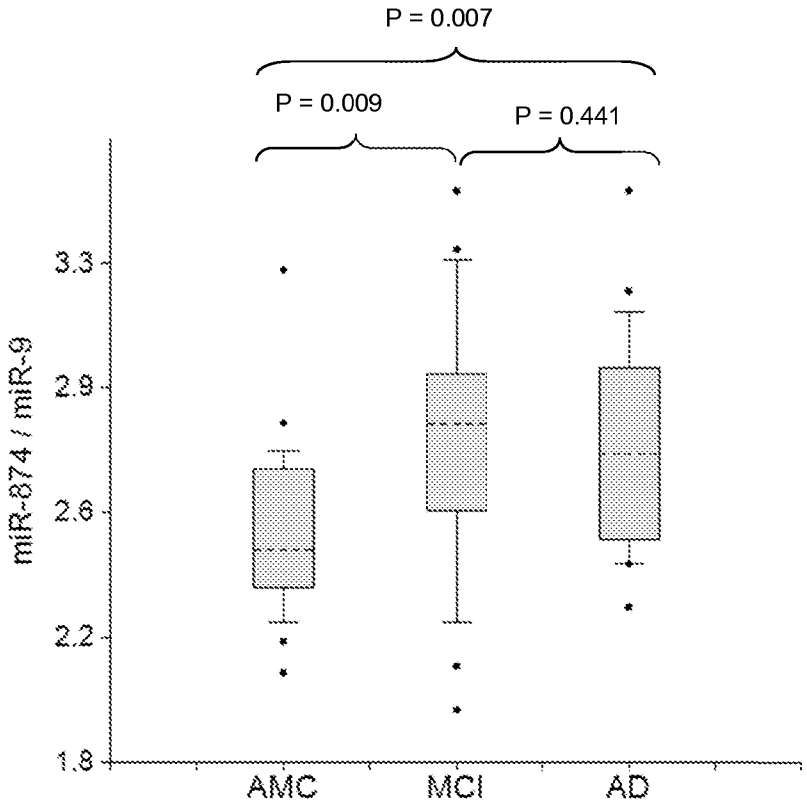


Figure 10E

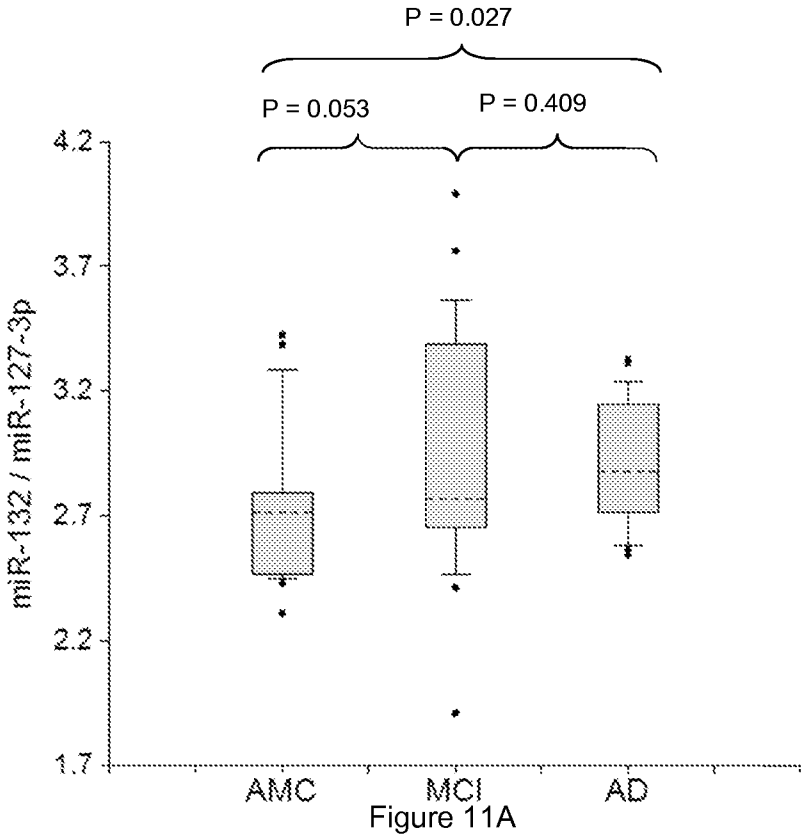


Figure 11A

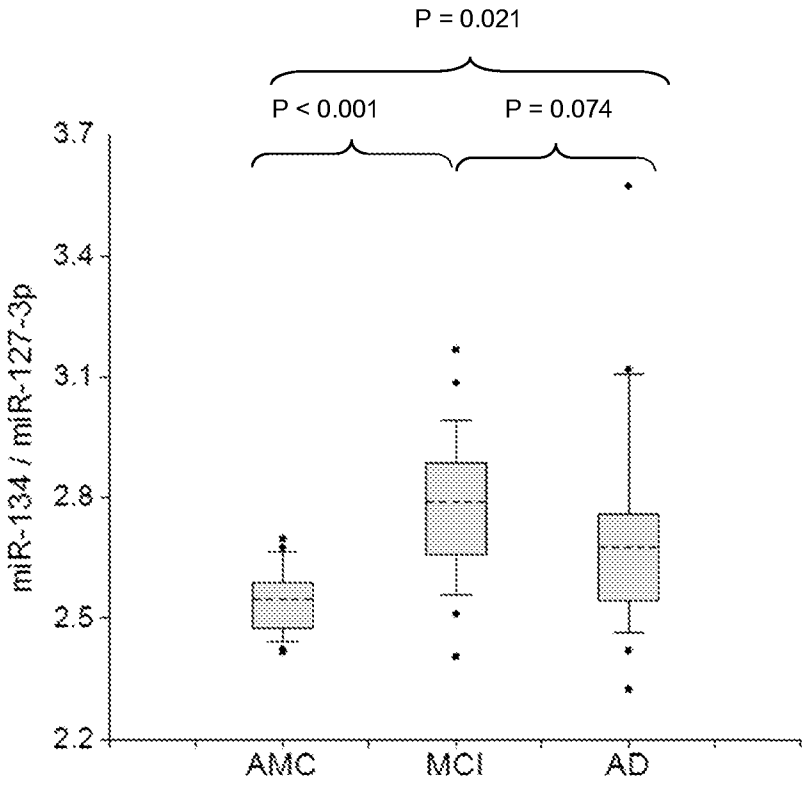


Figure 11B

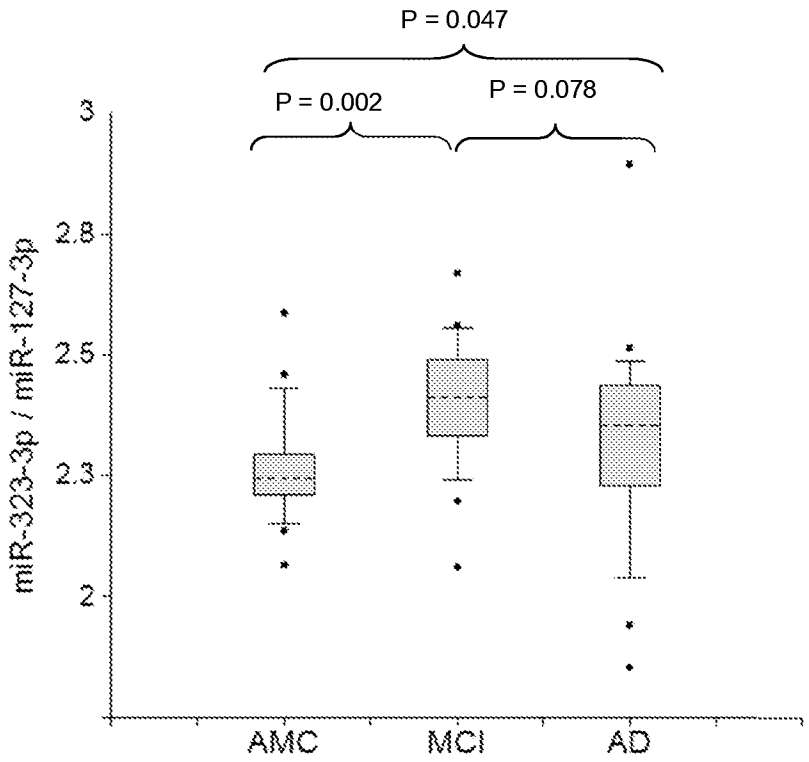


Figure 11C

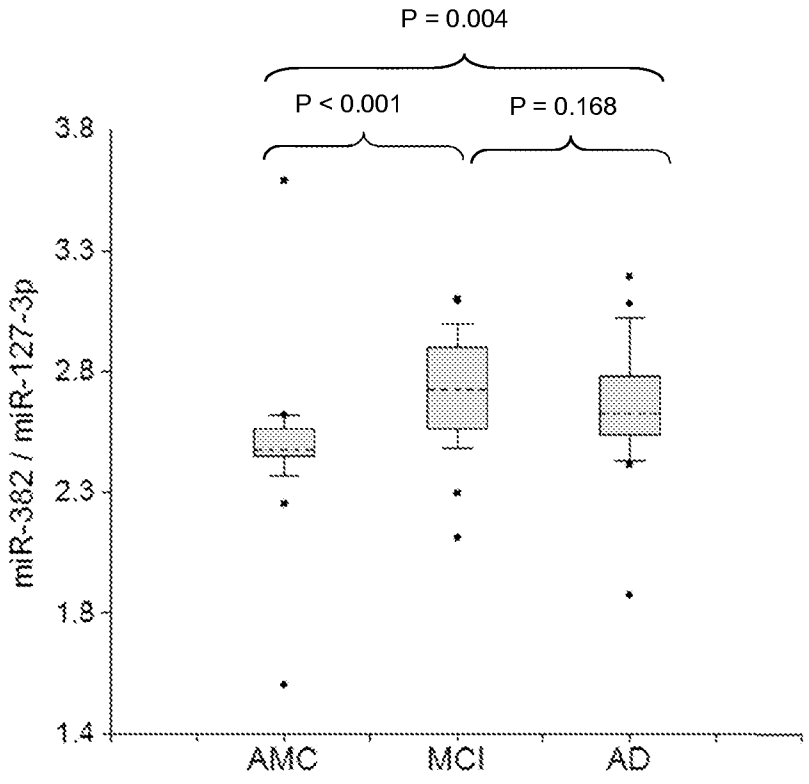


Figure 11D

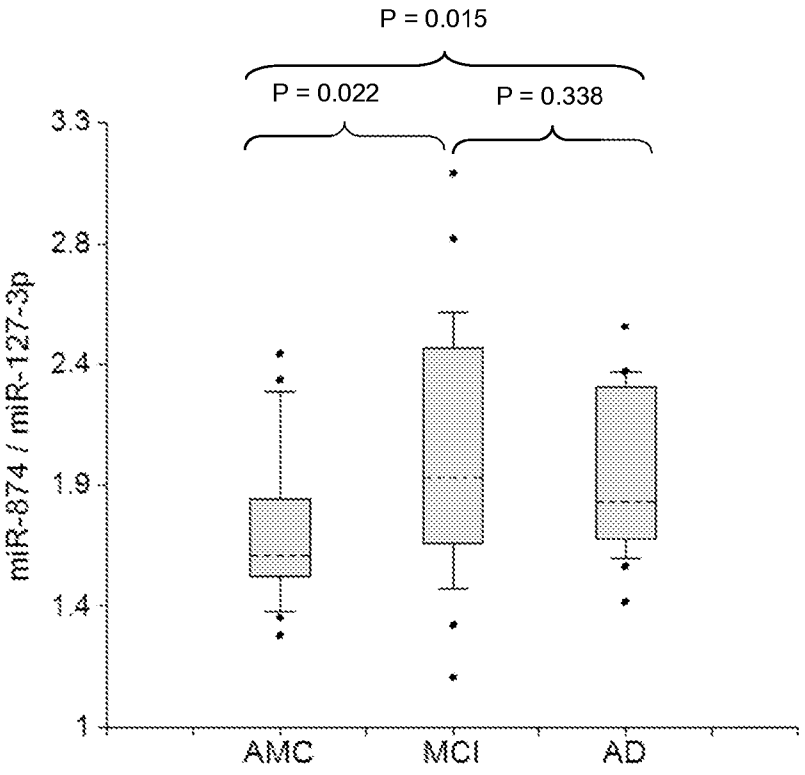


Figure 11E

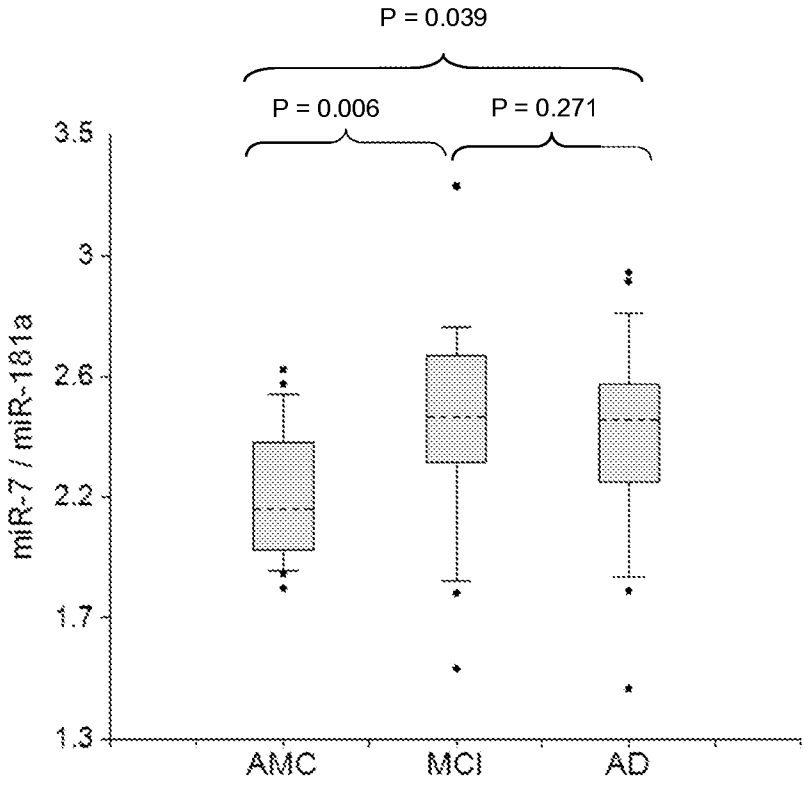


Figure 12A

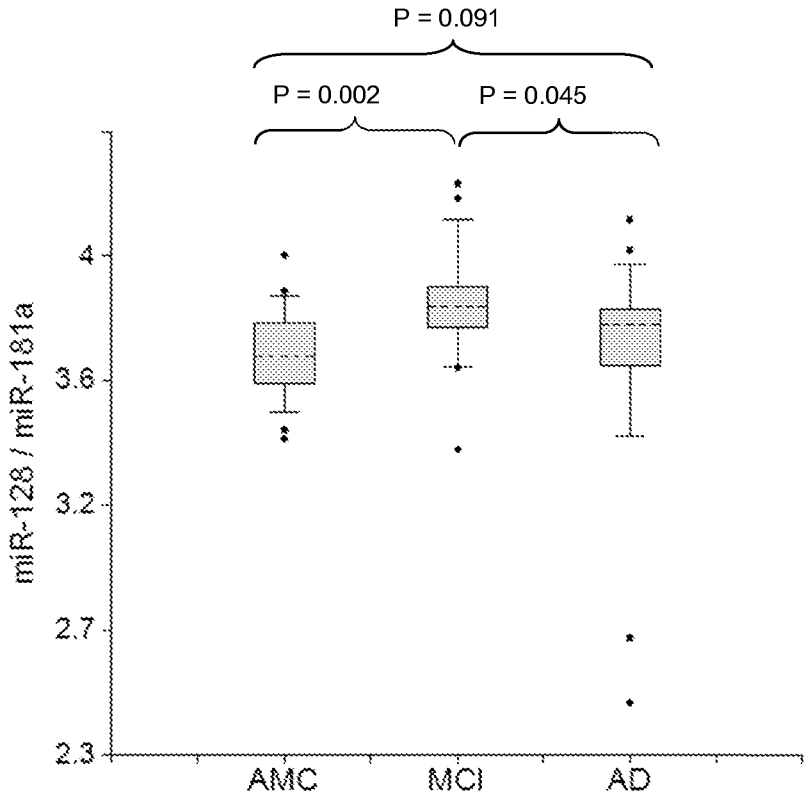


Figure 12B

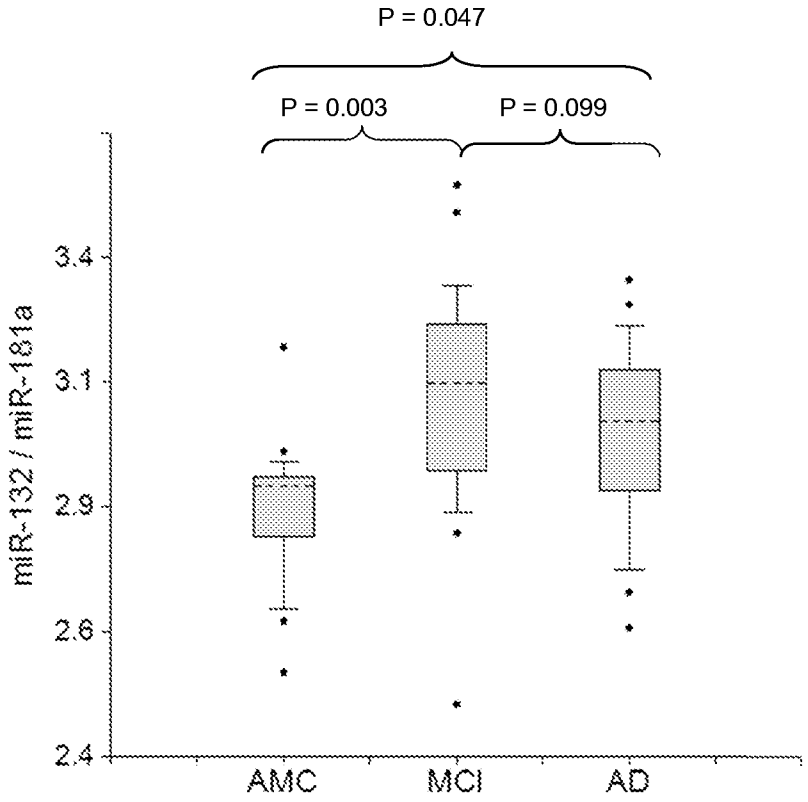


Figure 12C

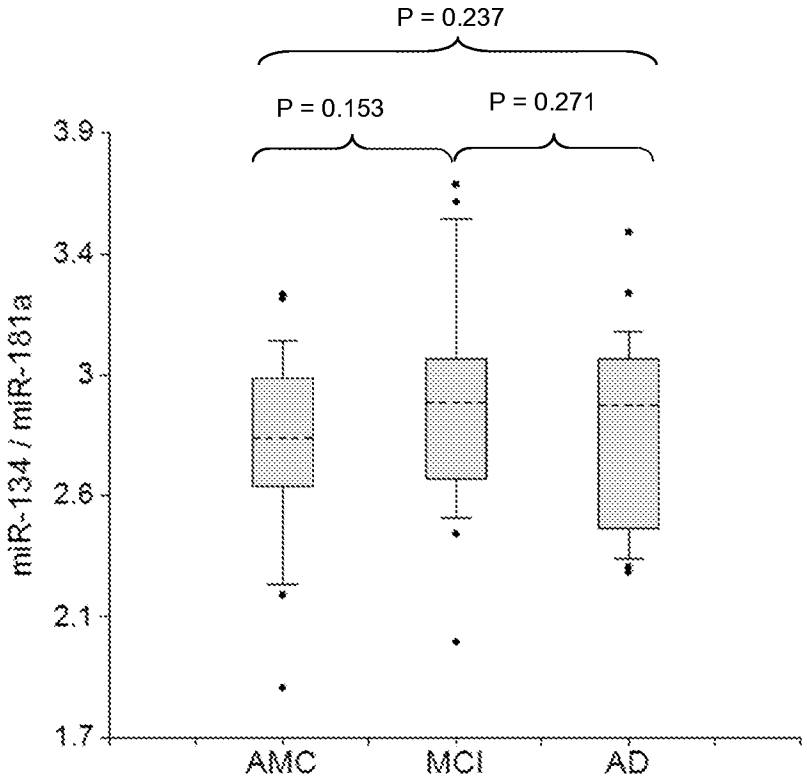


Figure 12D

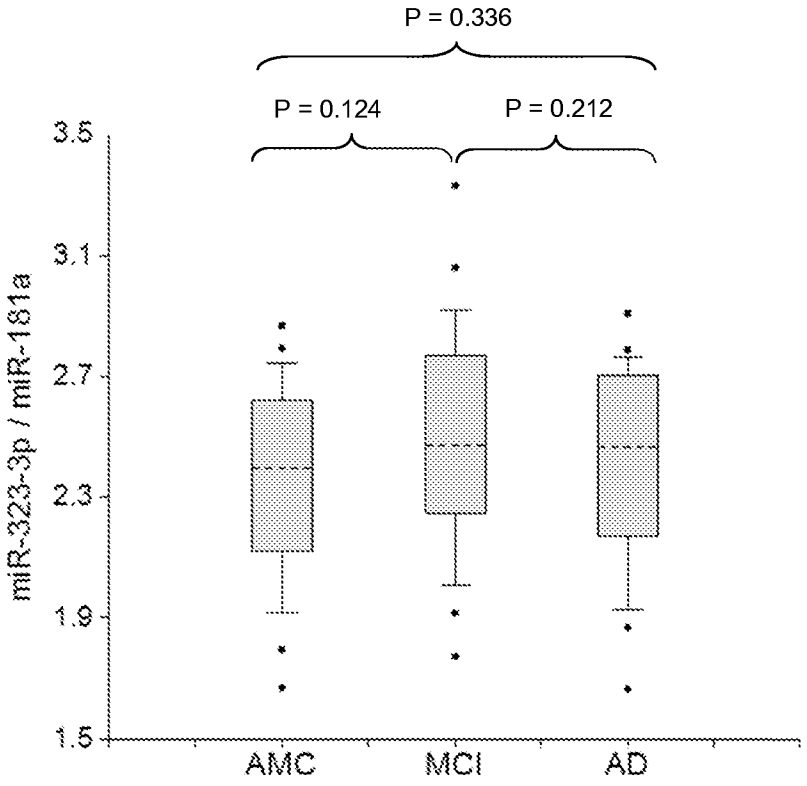


Figure 12E

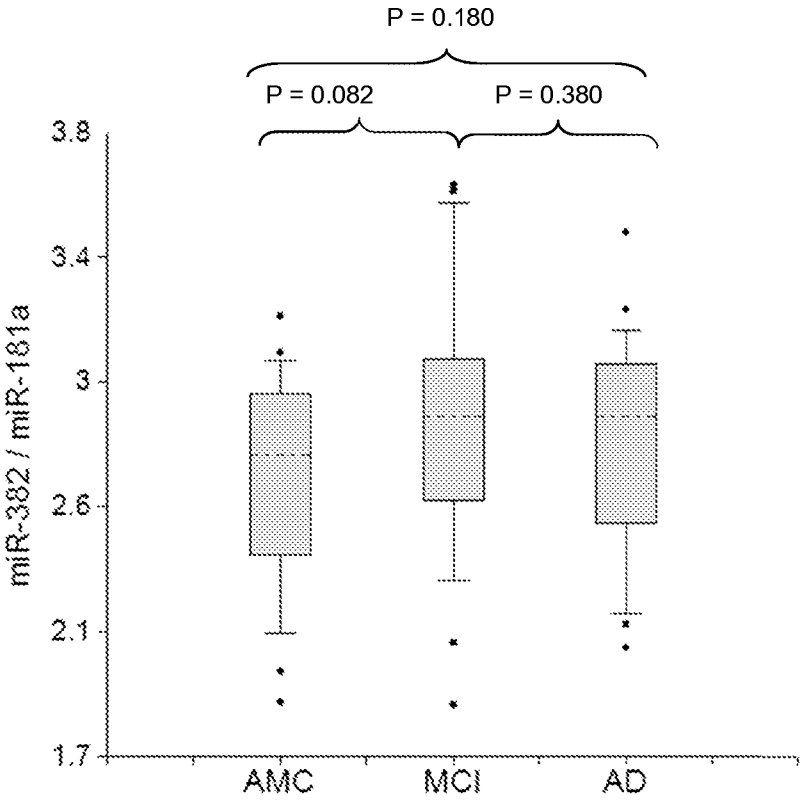


Figure 12F

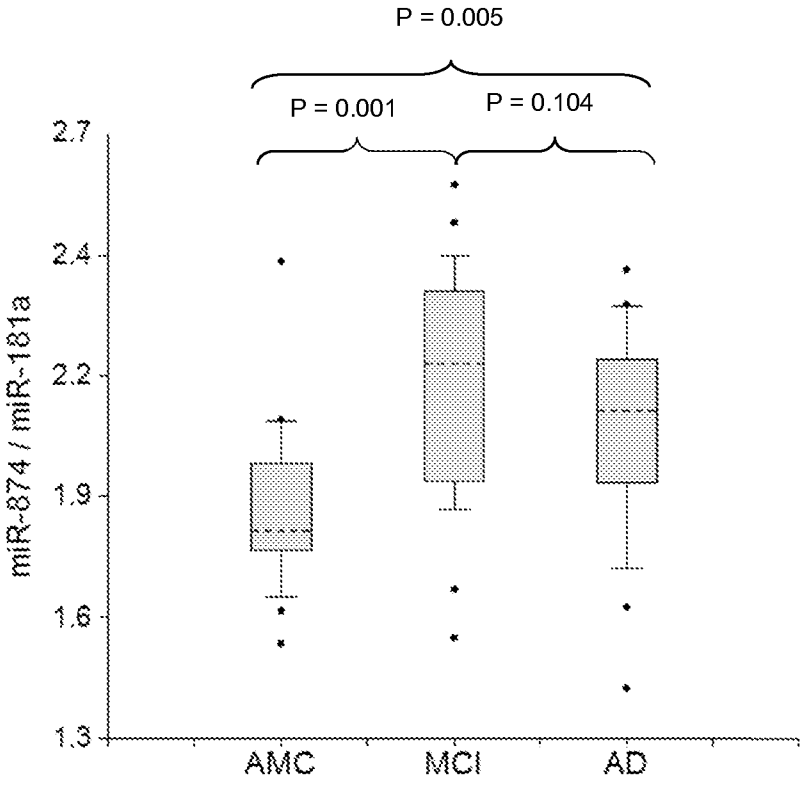


Figure 12G

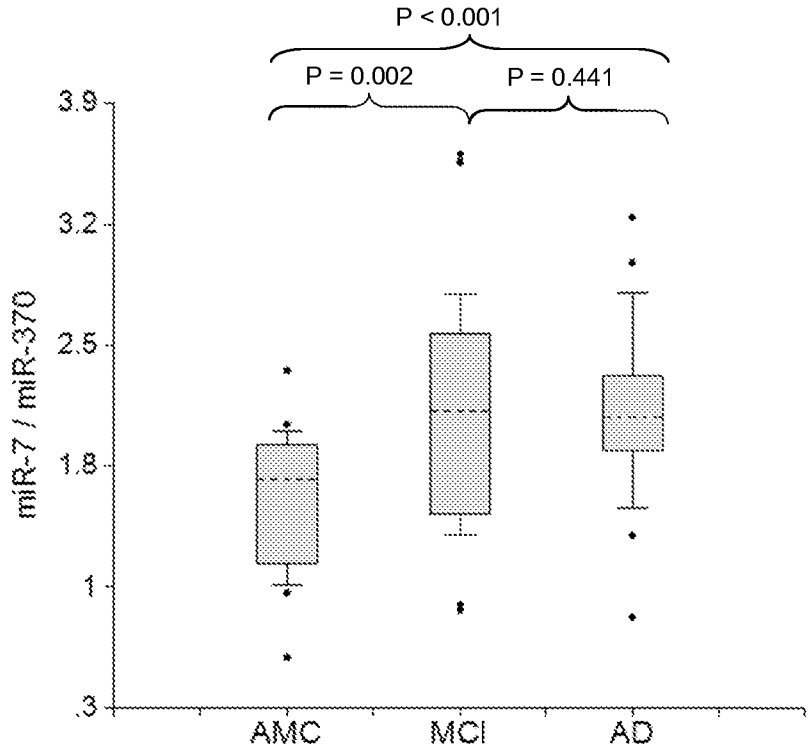


Figure 13A

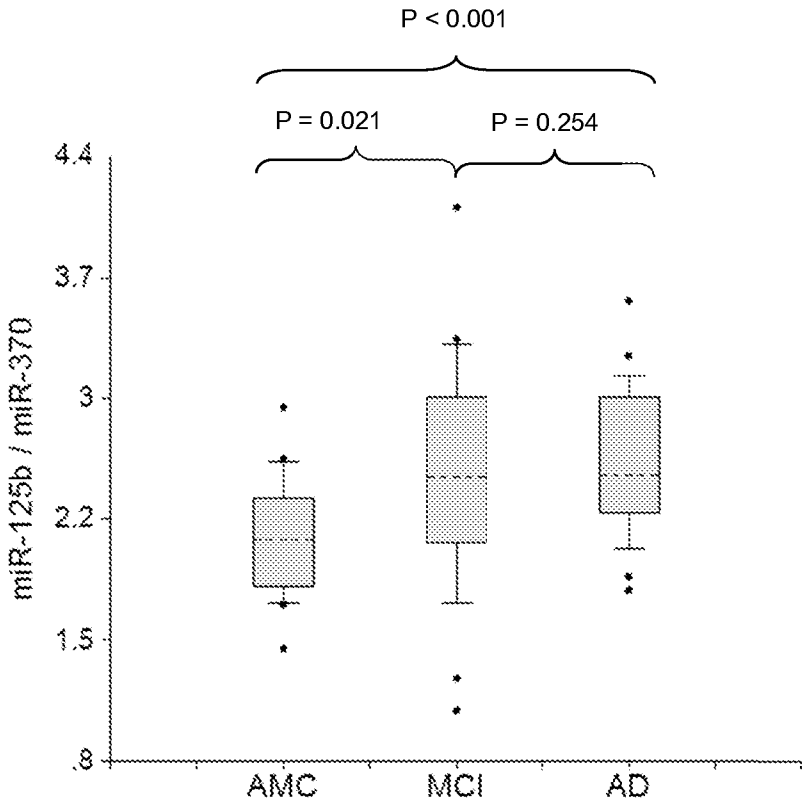


Figure 13B

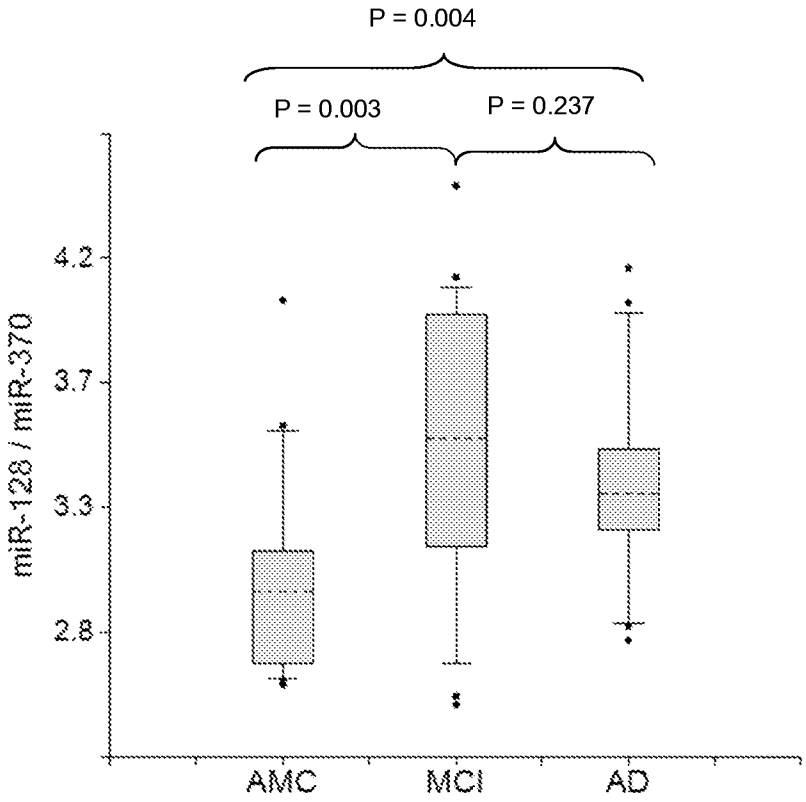


Figure 13C

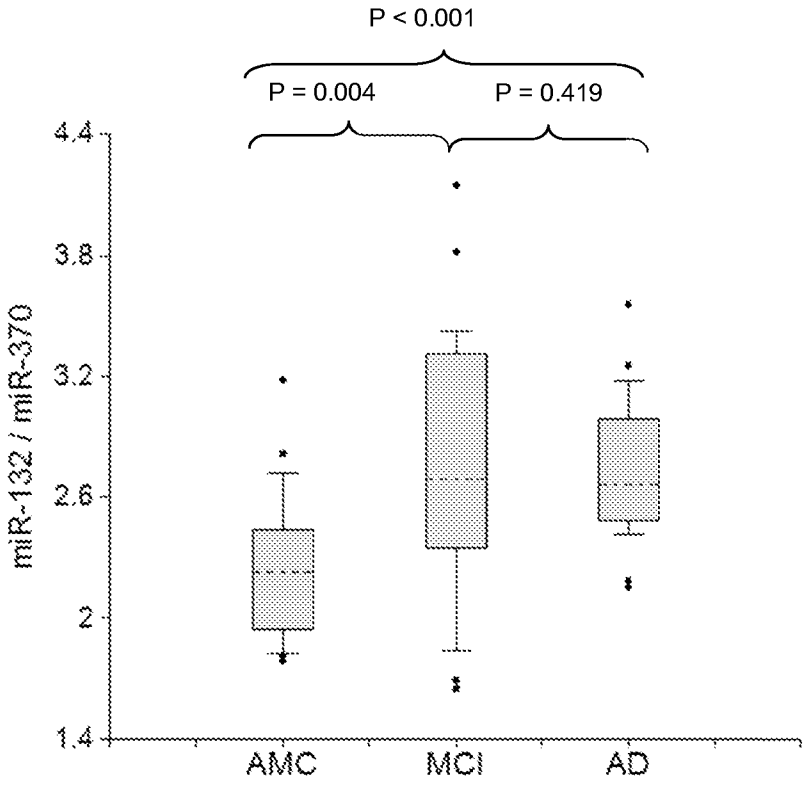


Figure 13D

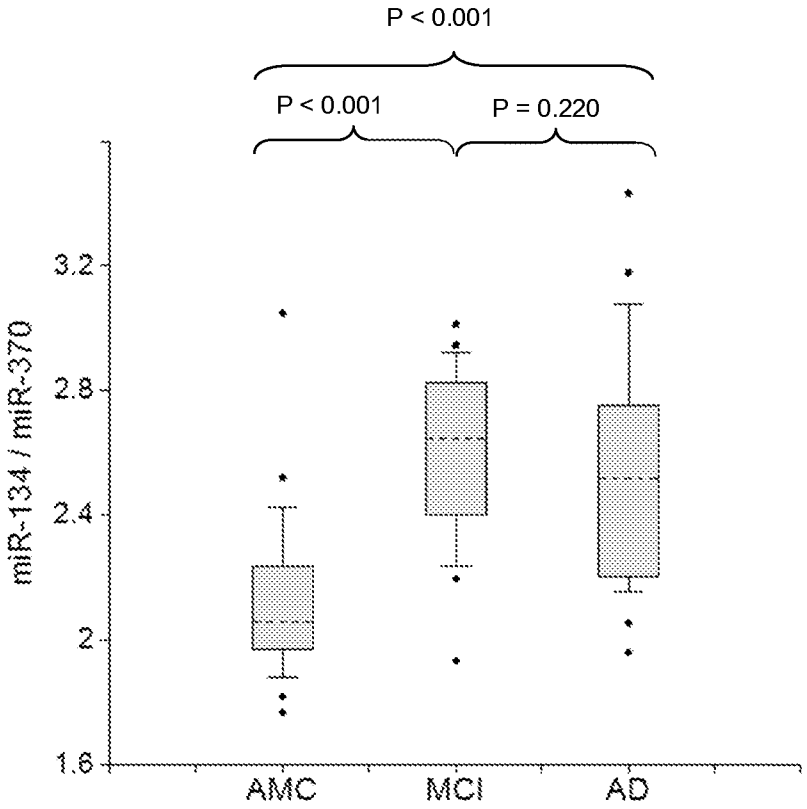


Figure 13E

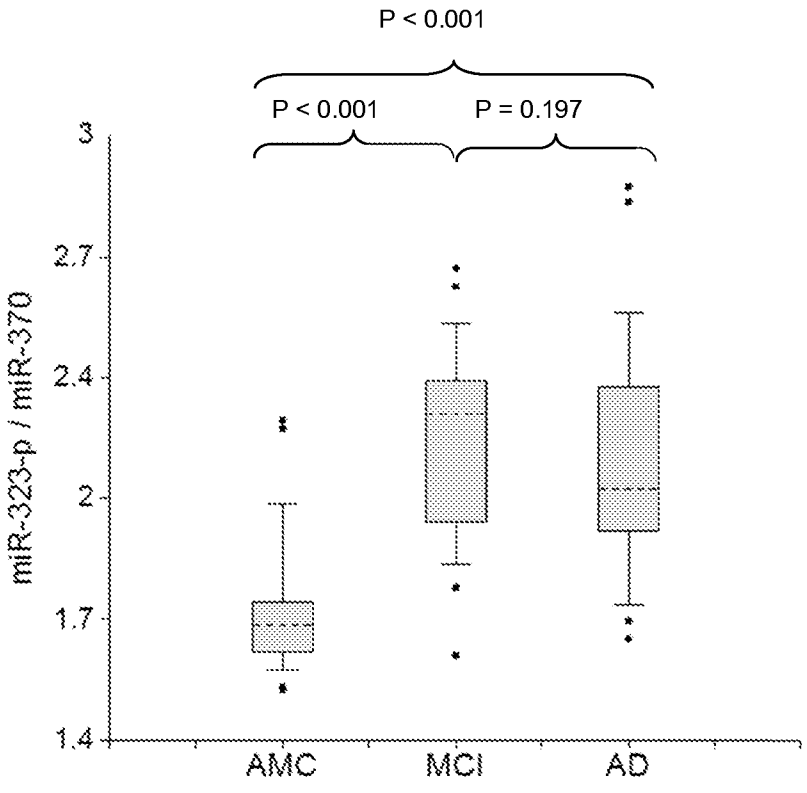


Figure 13F

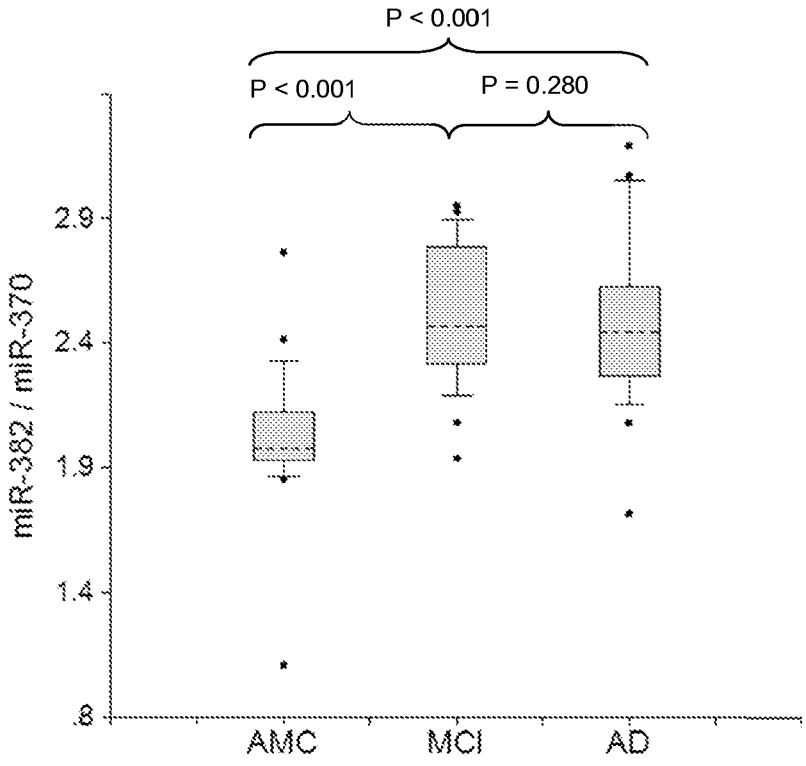


Figure 13G

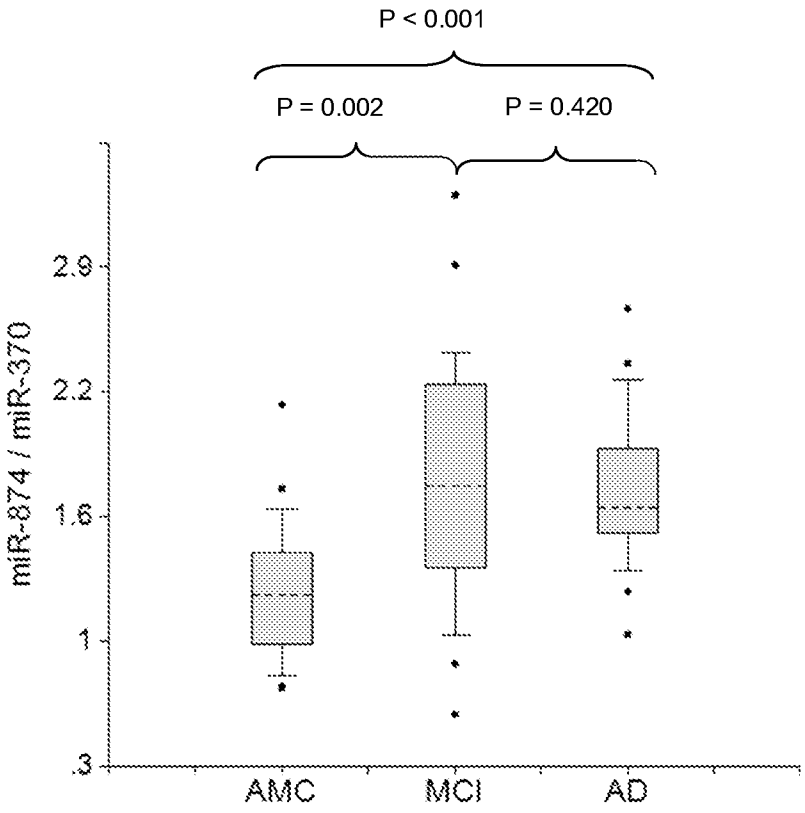


Figure 13H

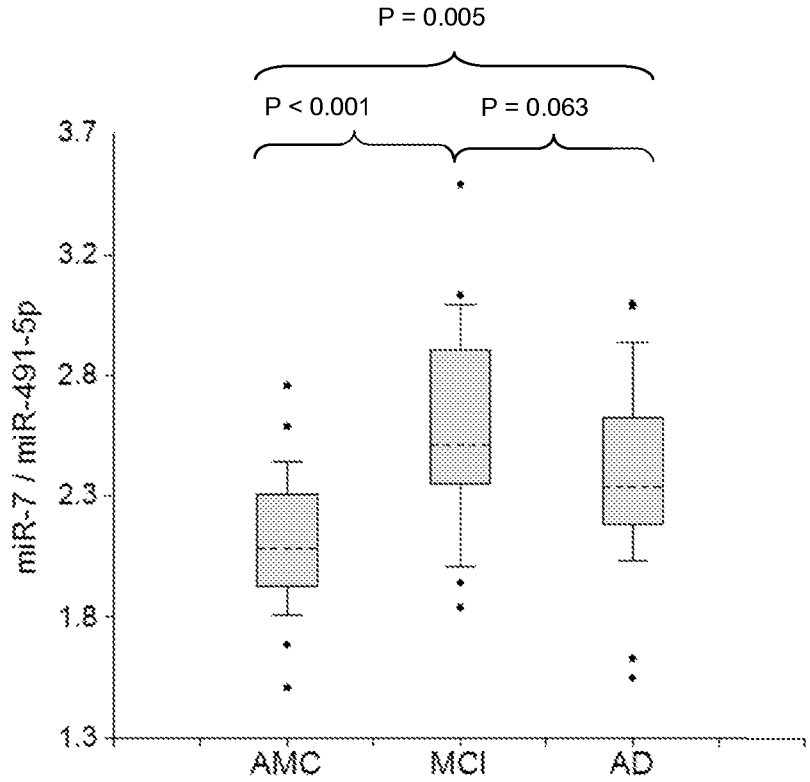


Figure 14A

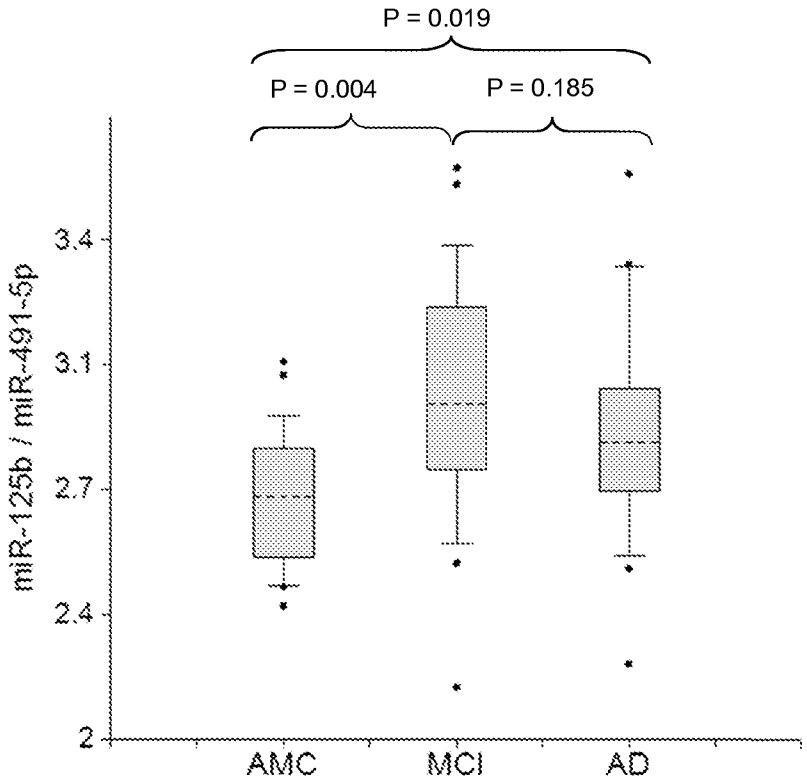


Figure 14B

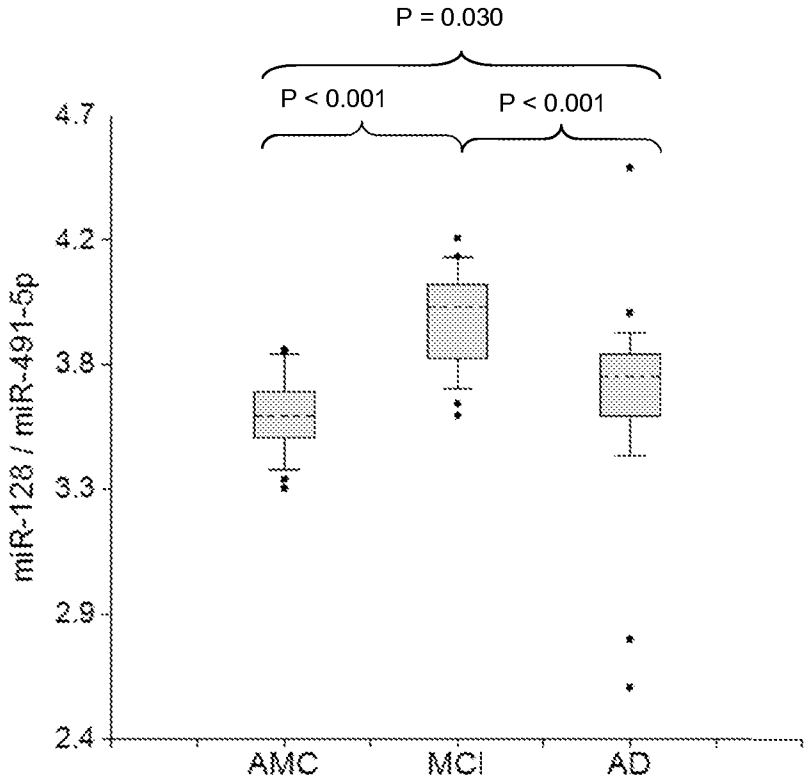
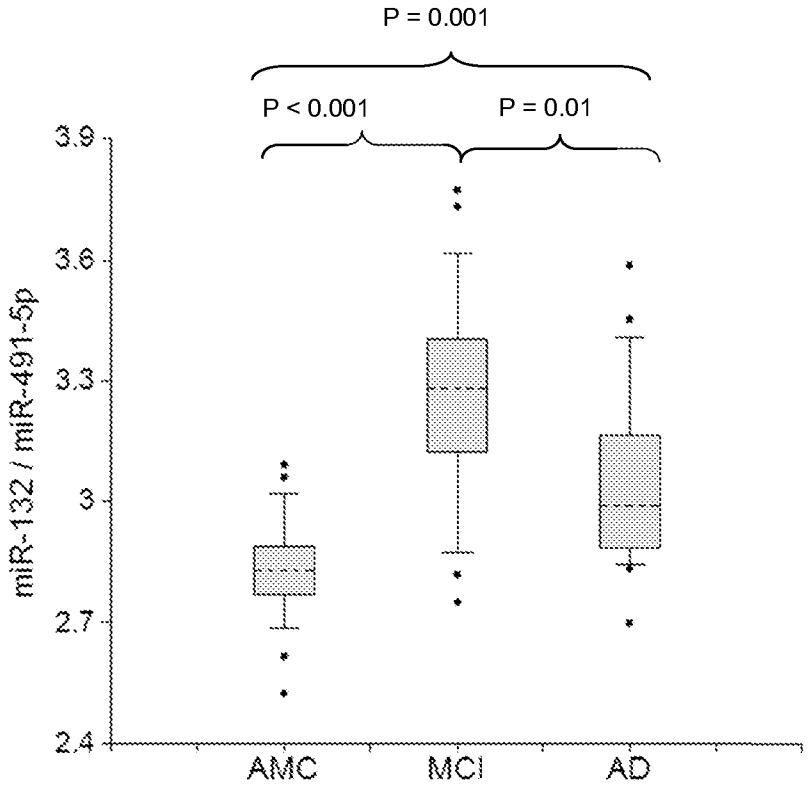


Figure 14C



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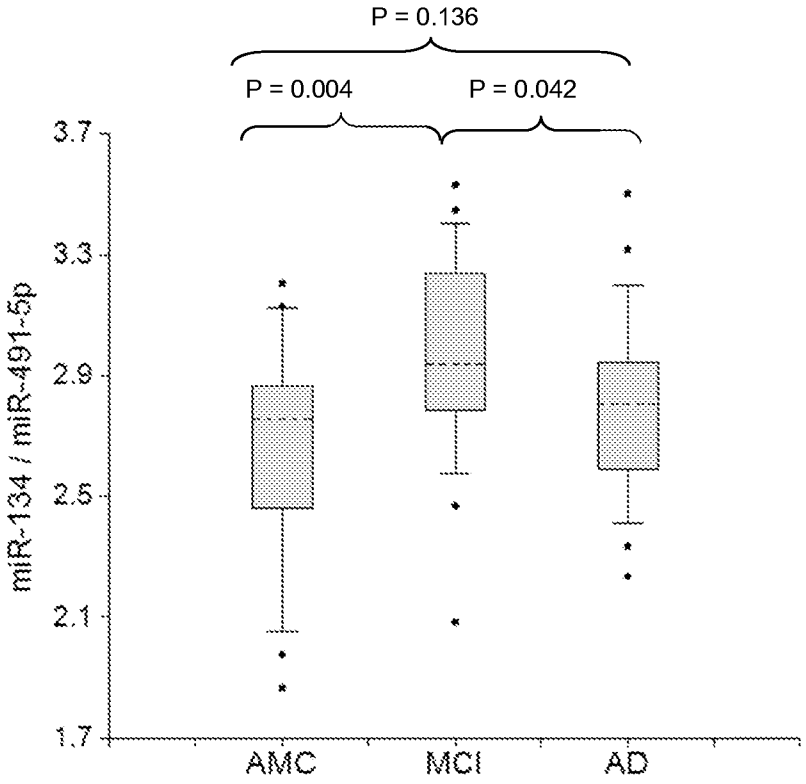

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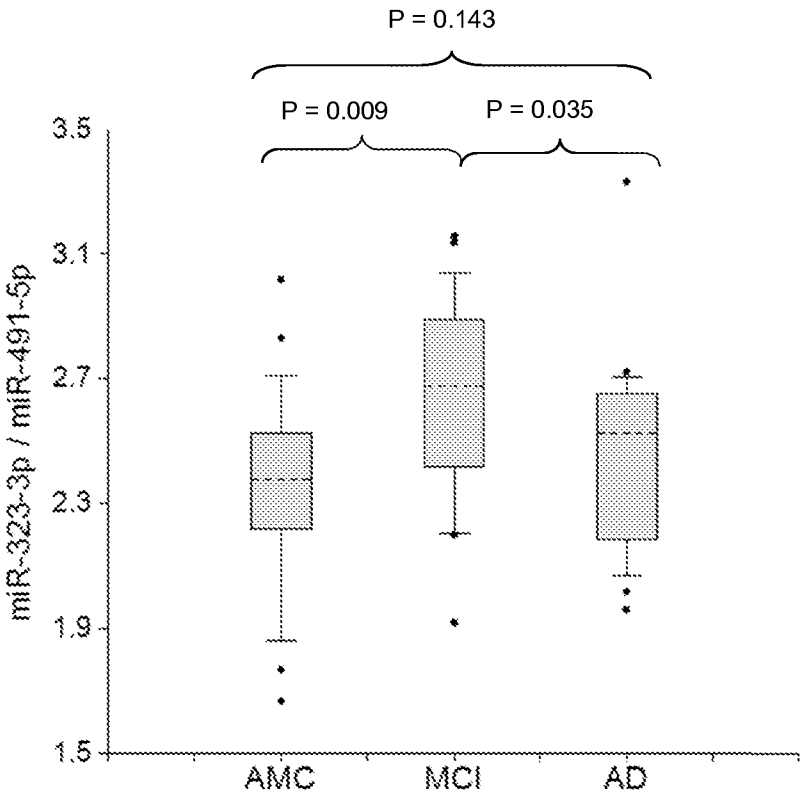

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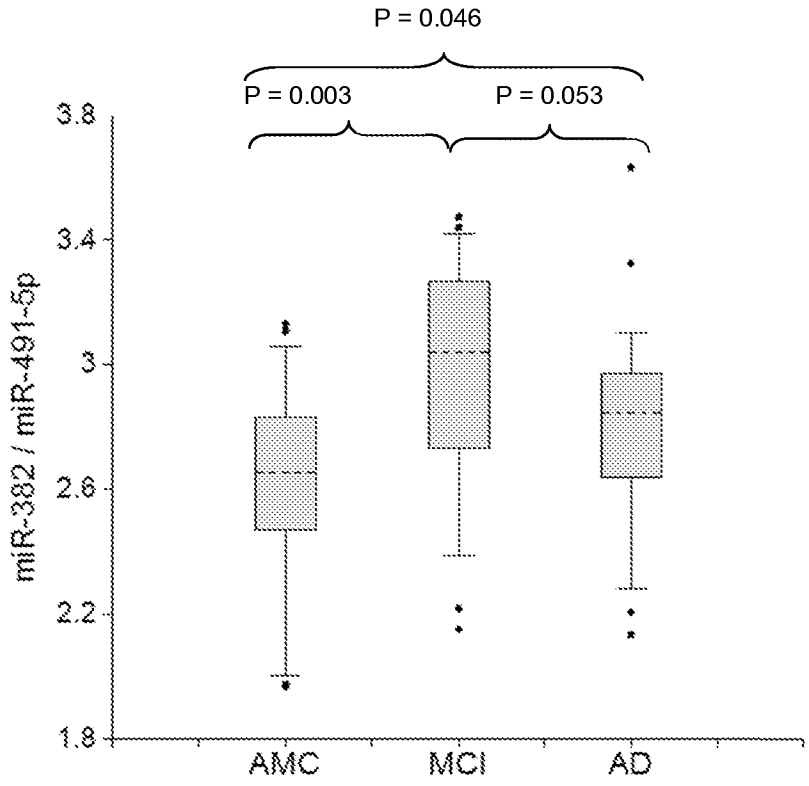


Figure 14G

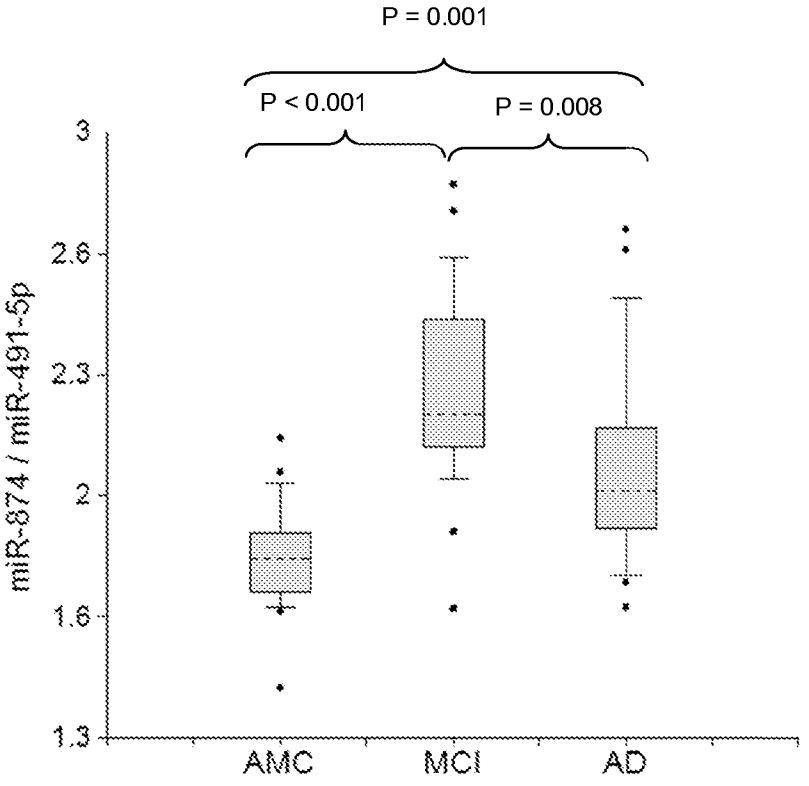


Figure 14H

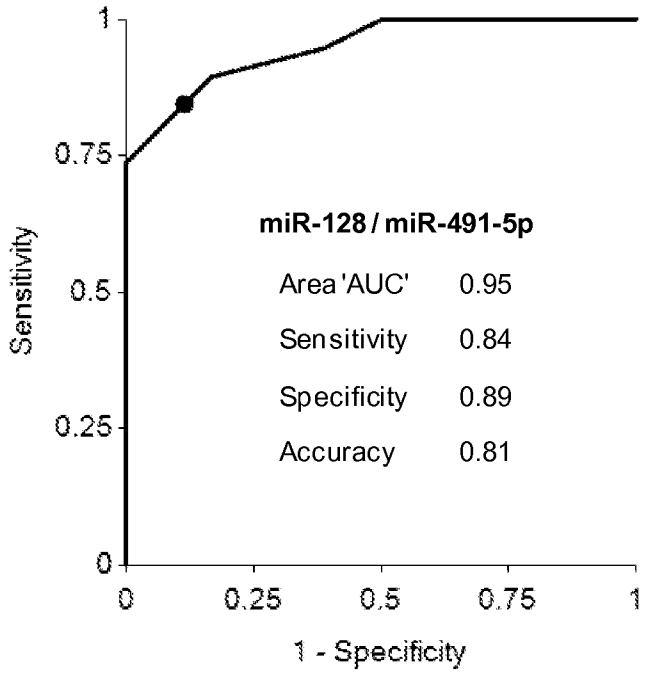


Figure 15A

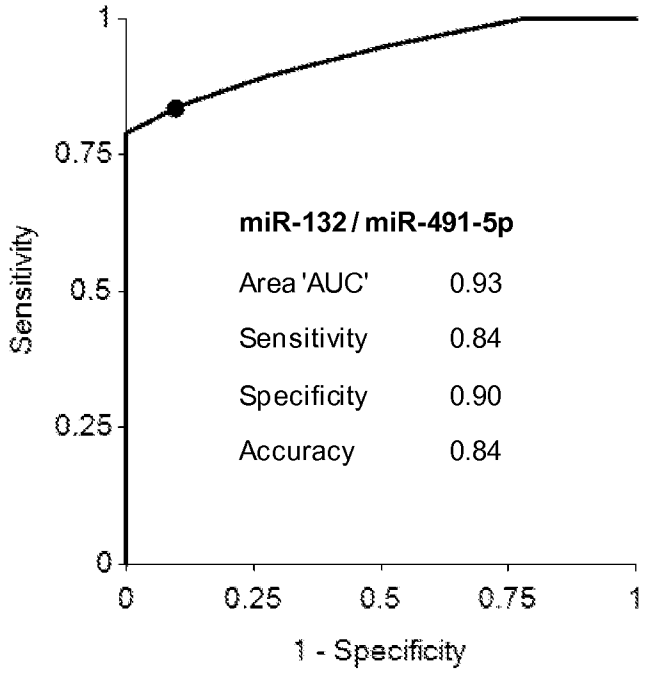


Figure 15B

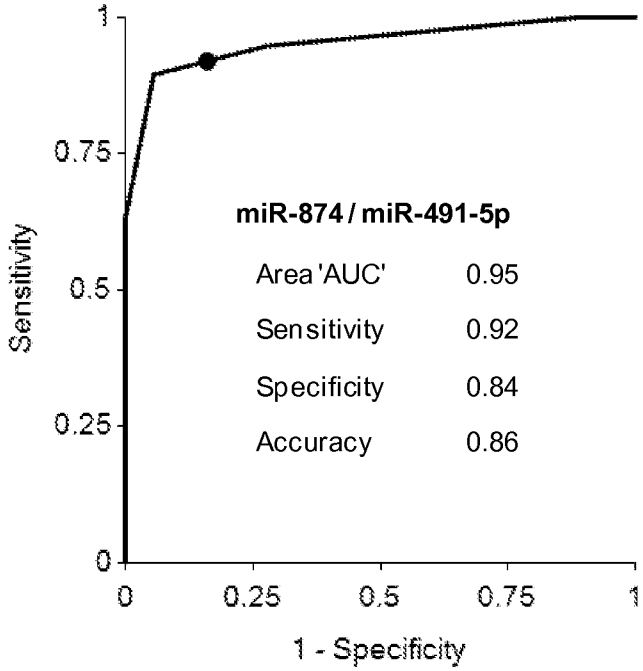


Figure 15C

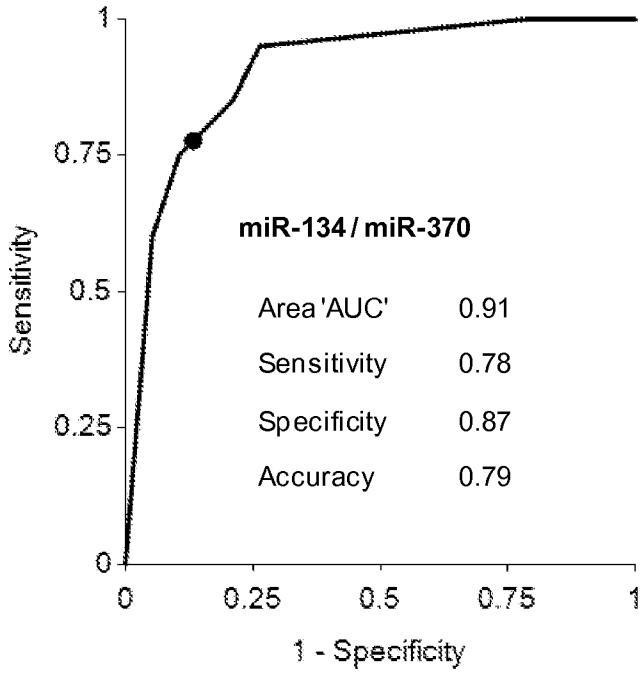


Figure 16A

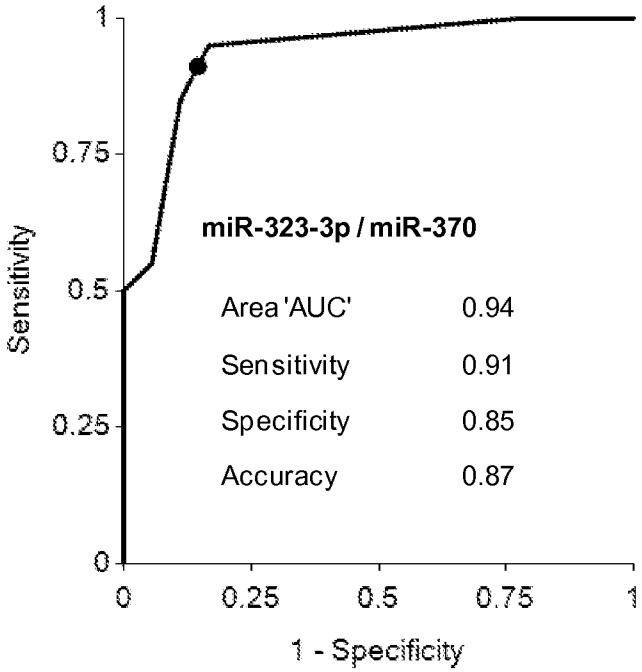


Figure 16B

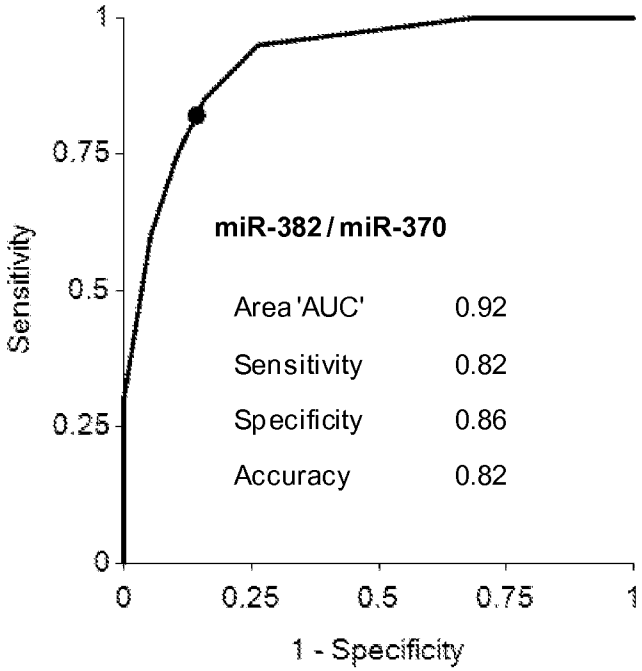


Figure 16C

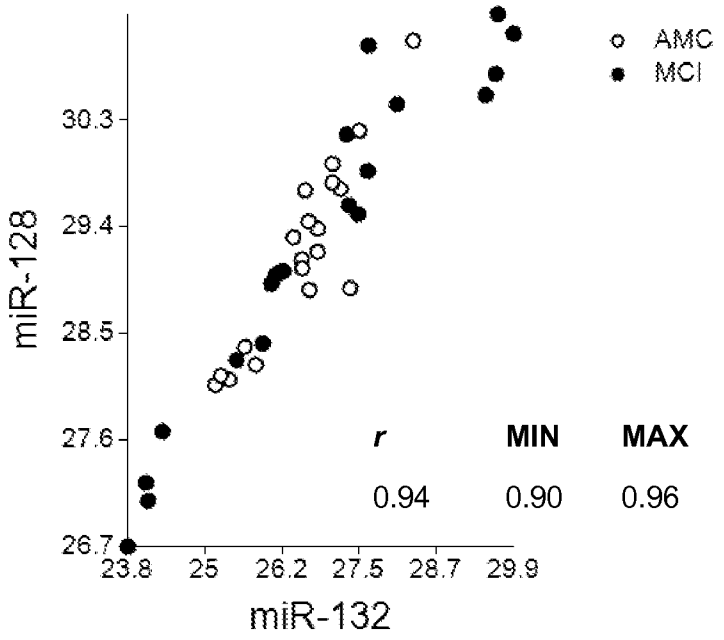


Figure 17A

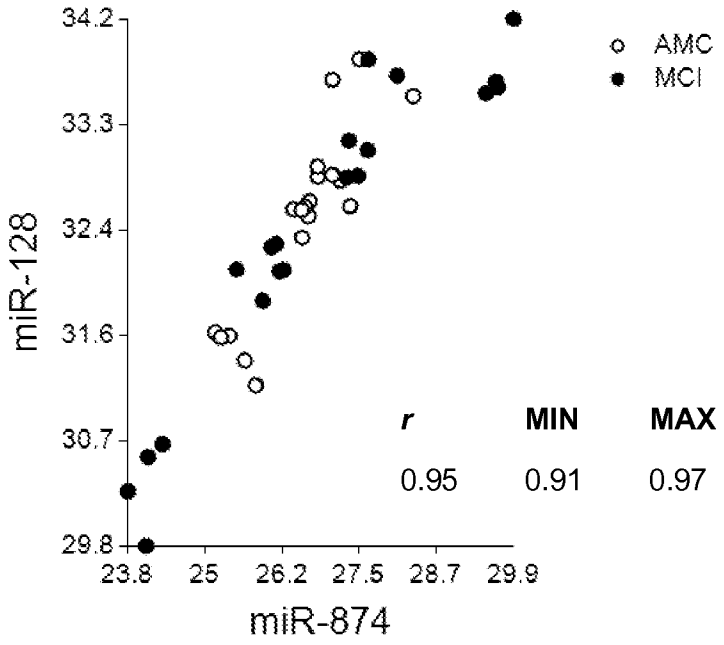


Figure 17B

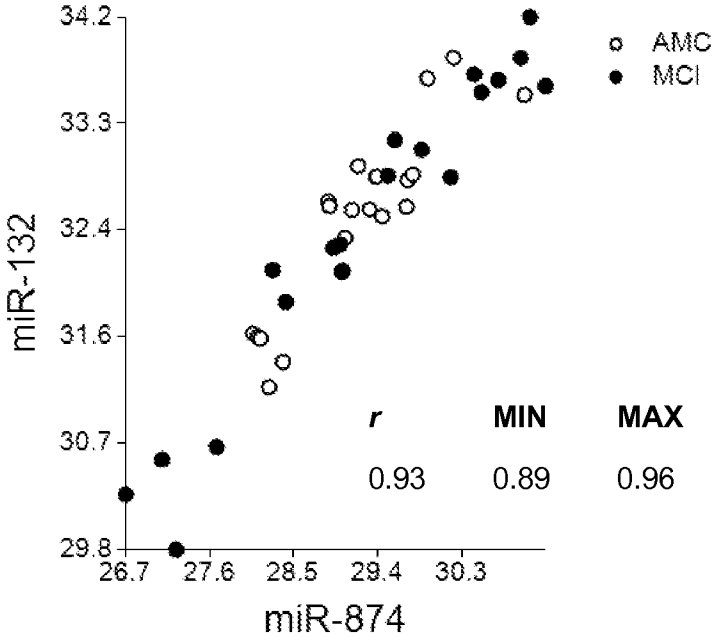


Figure 17C

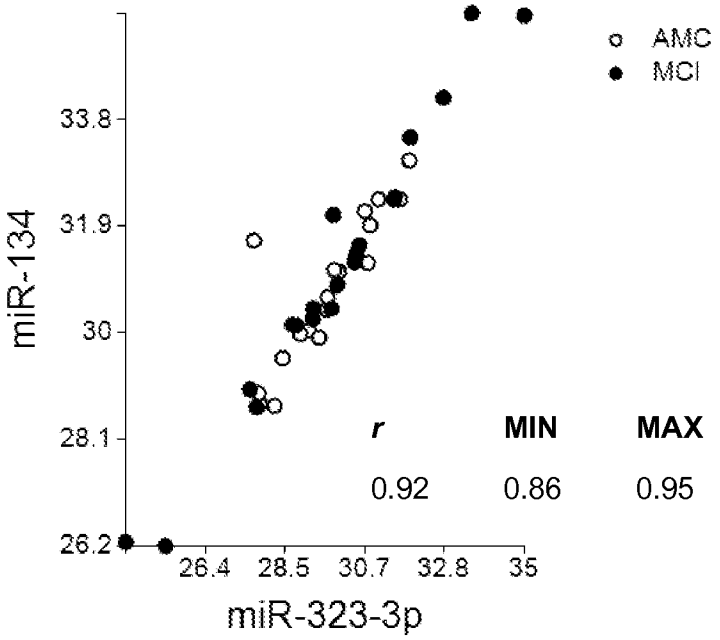


Figure 17D

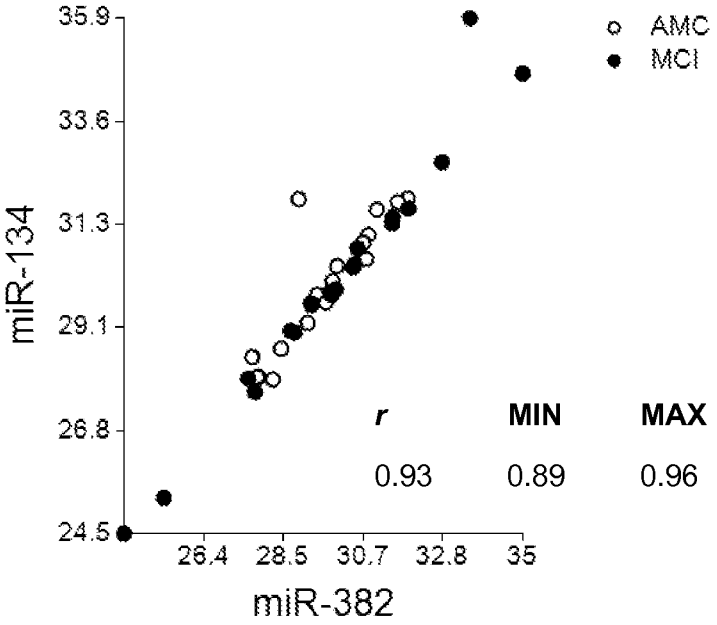


Figure 17E

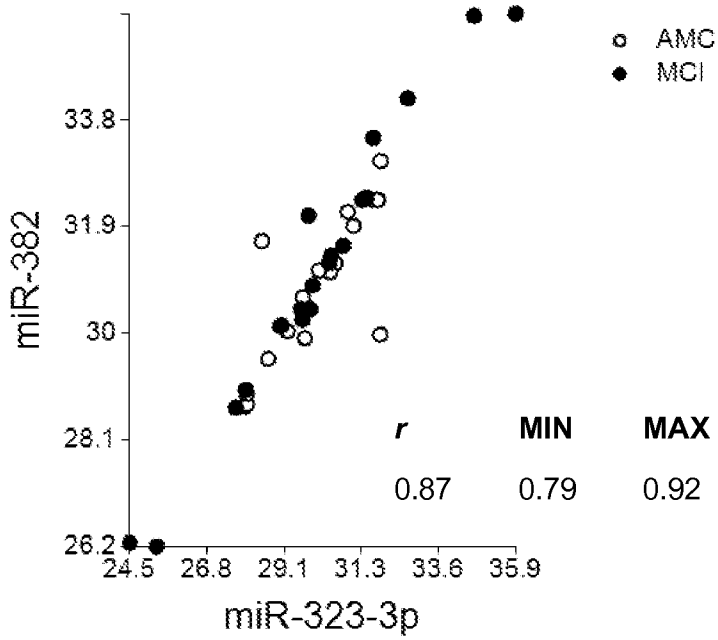


Figure 17F

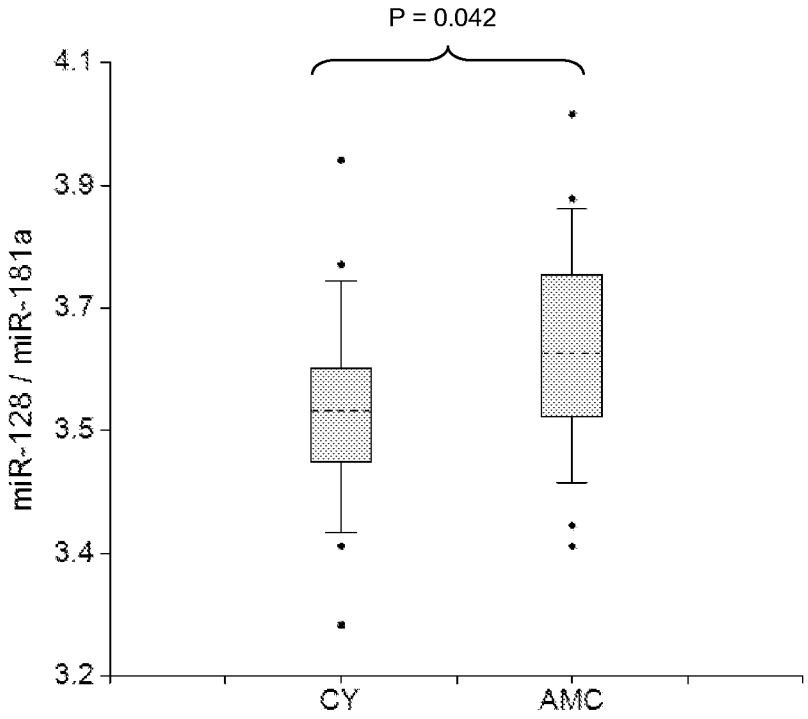


Figure 18A

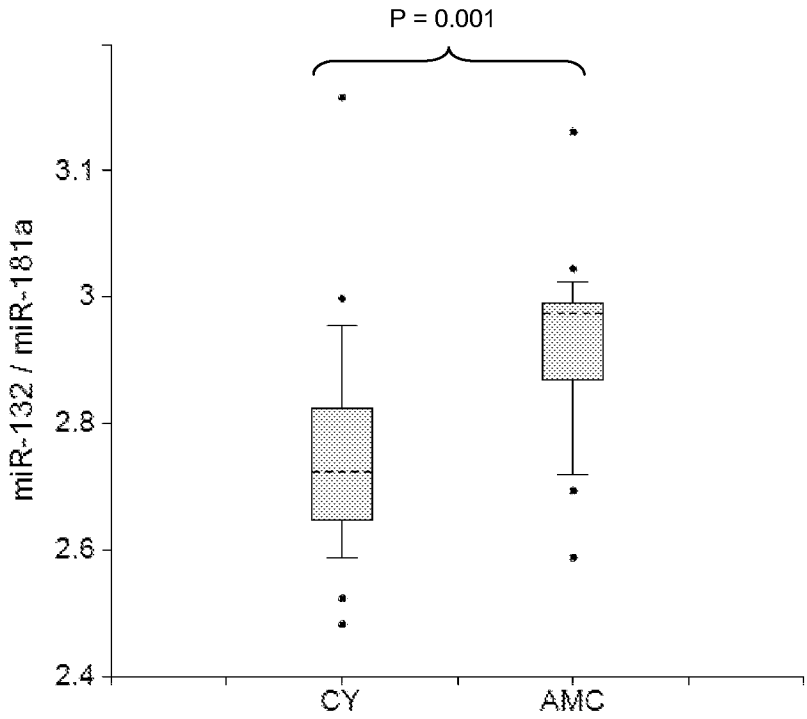


Figure 18B

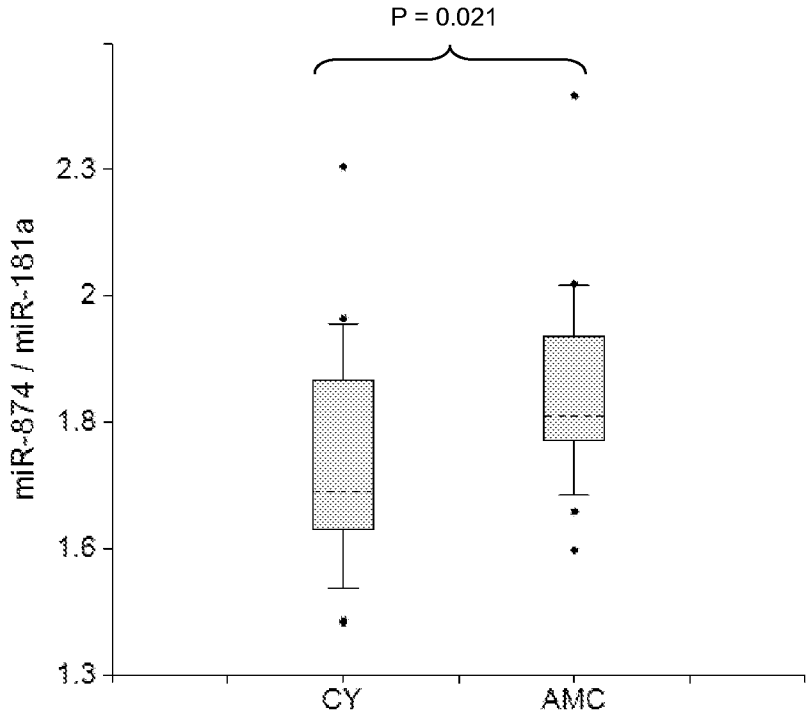


Figure 18C

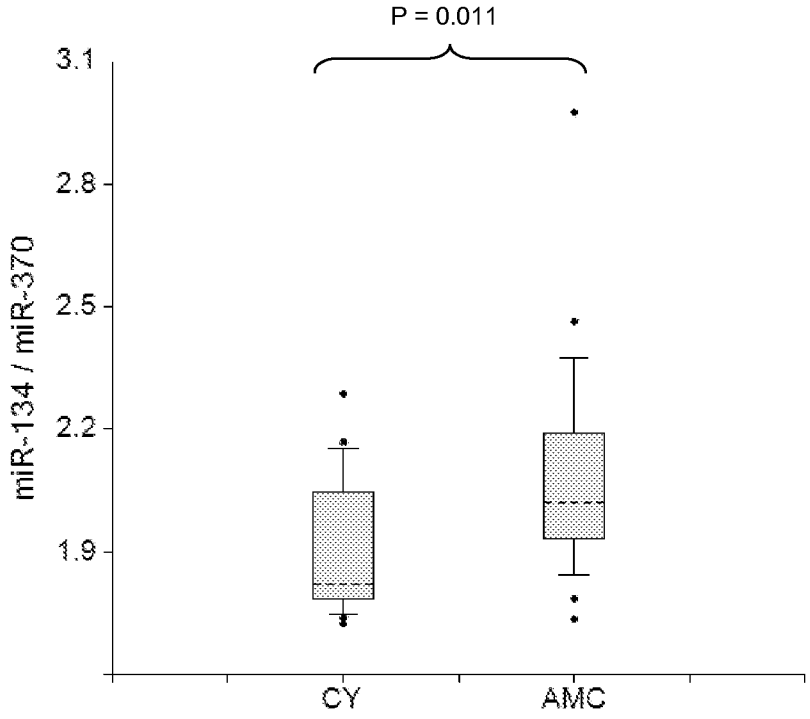
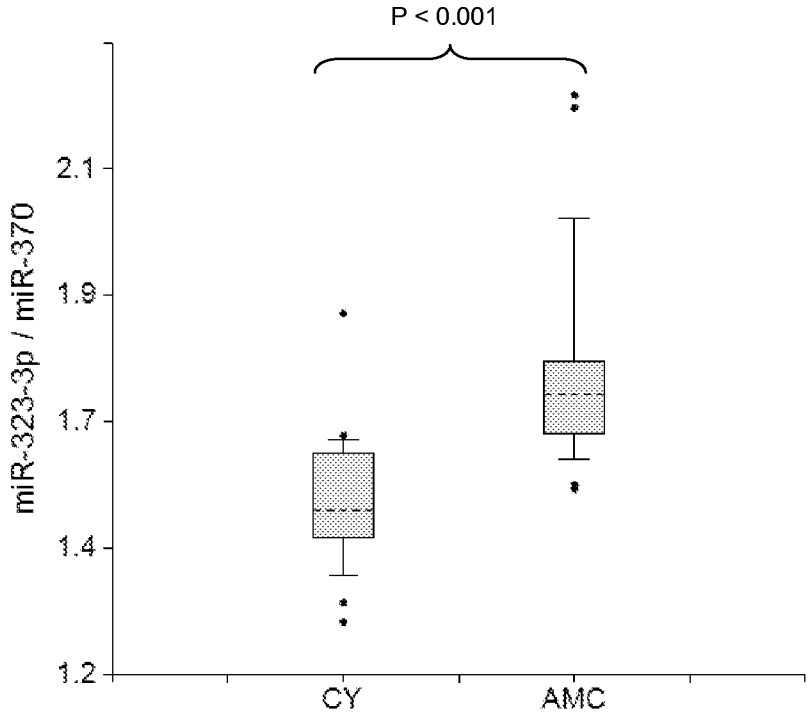


Figure 18D



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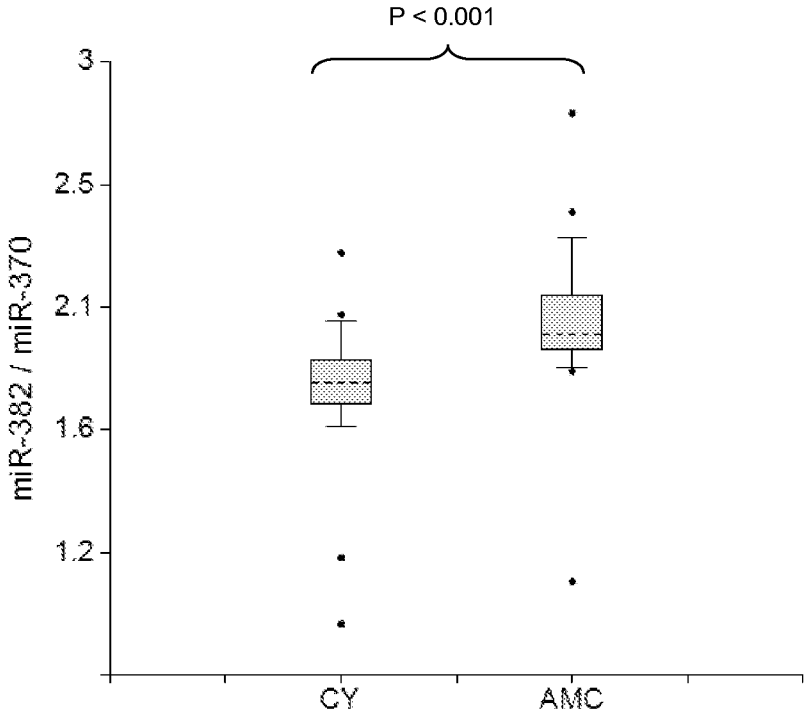

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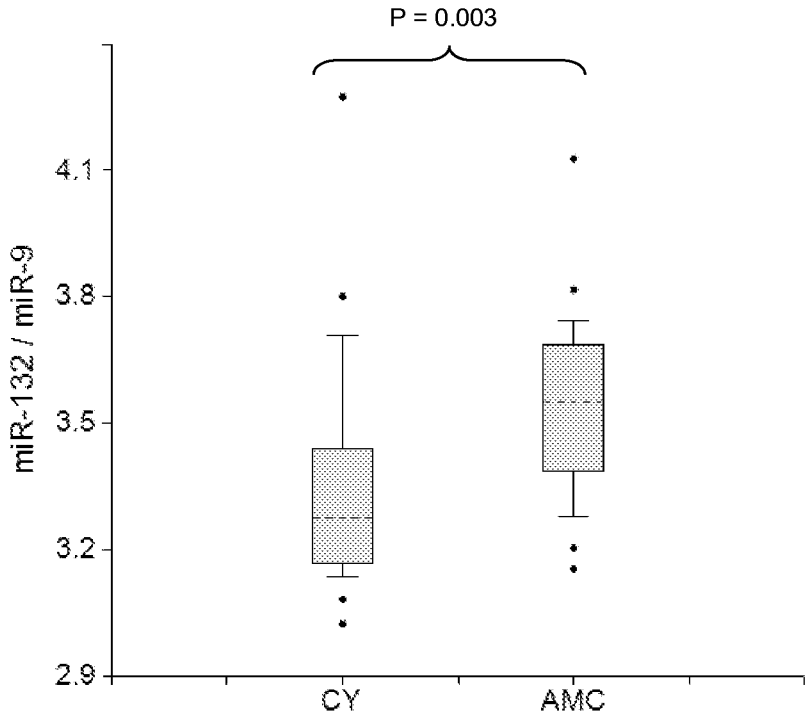

Figure 18G

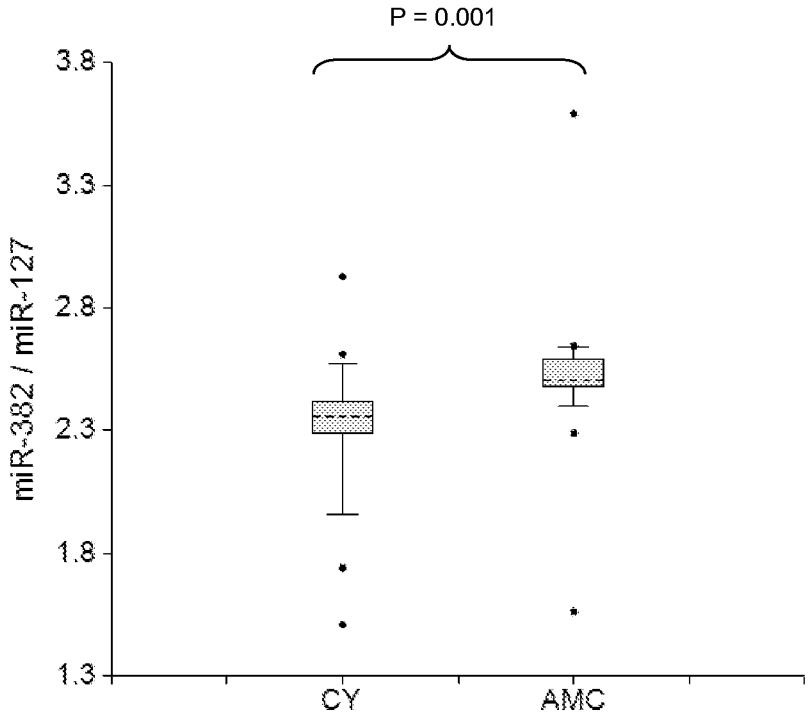

Figure 18H

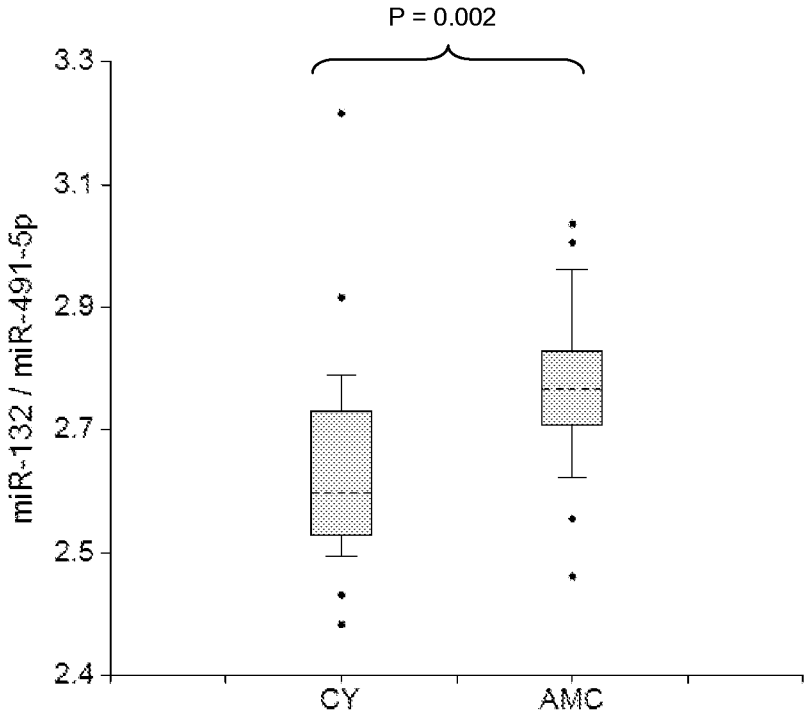

Figure 18I

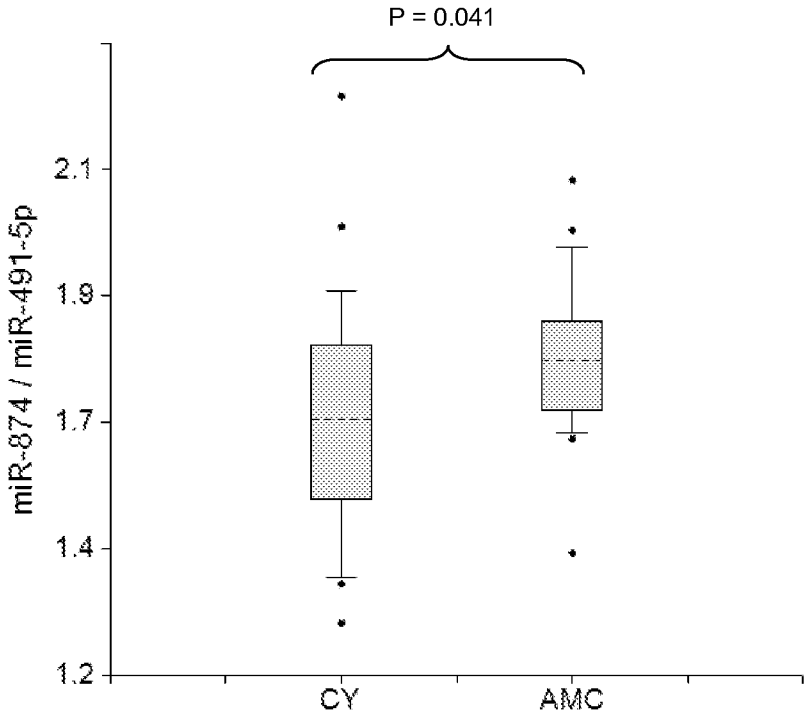

Figure 18J

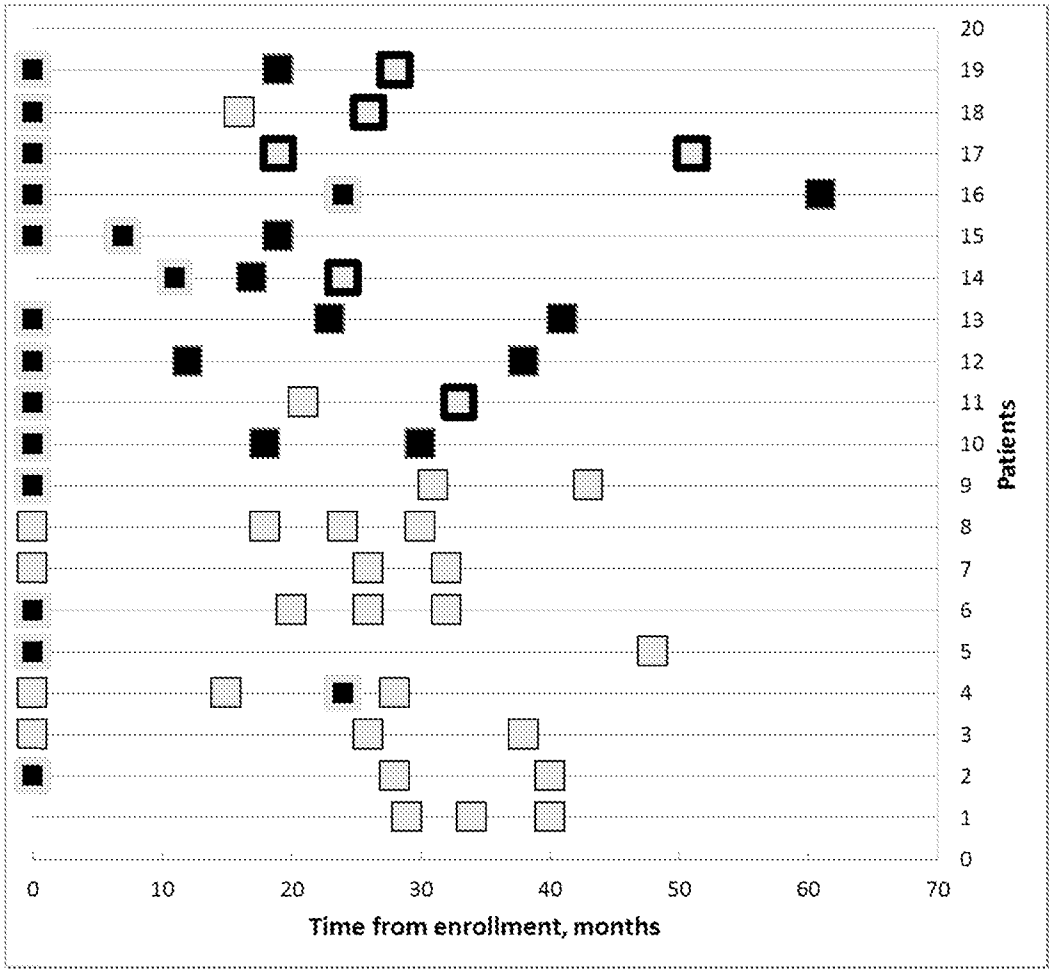
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Patients
0
10
20
30
40
50
60
70
Time from enrollment, months

Figure 19

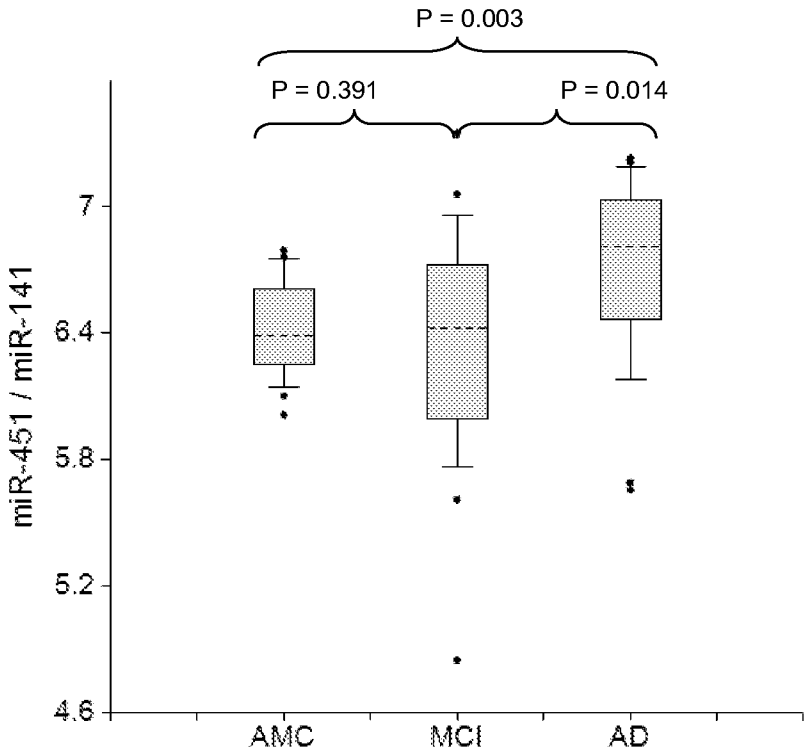

Figure 20A

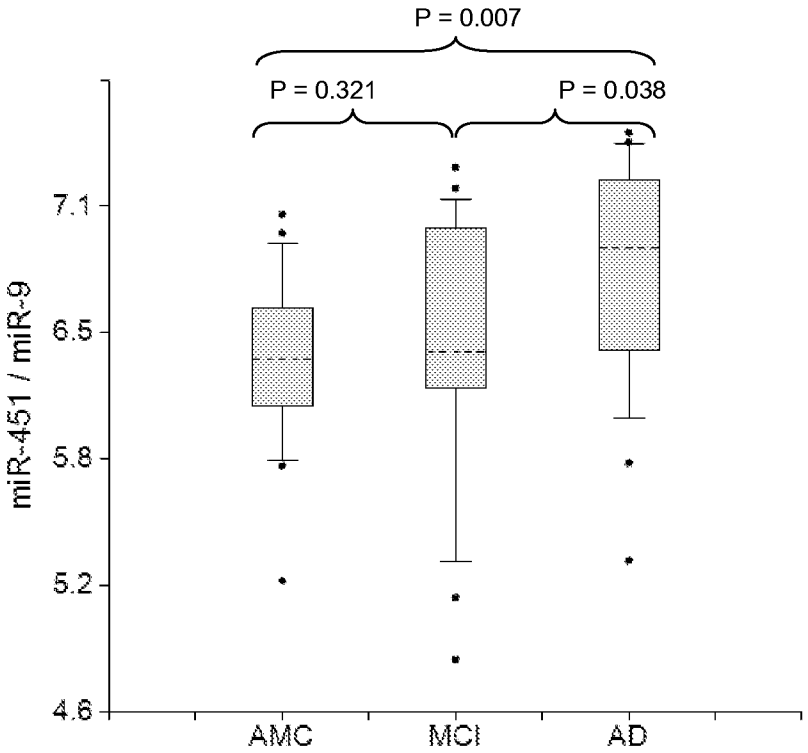

Figure 20B

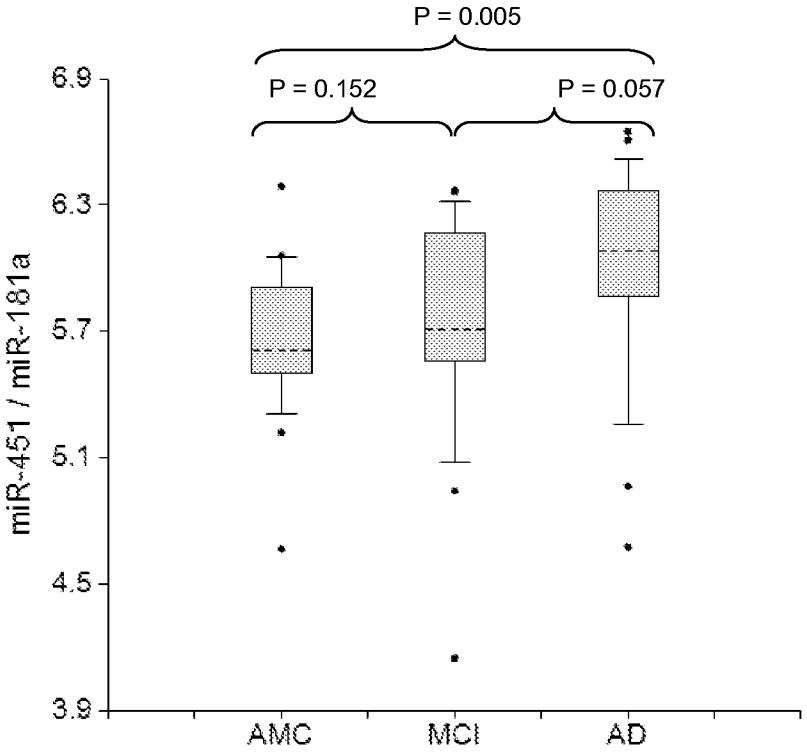

Figure 20C

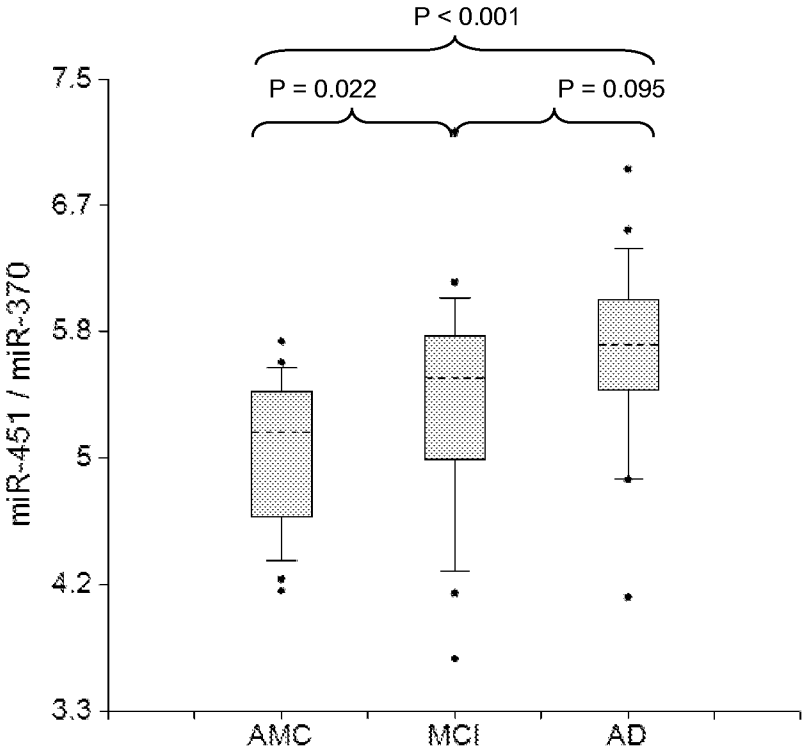

Figure 20D

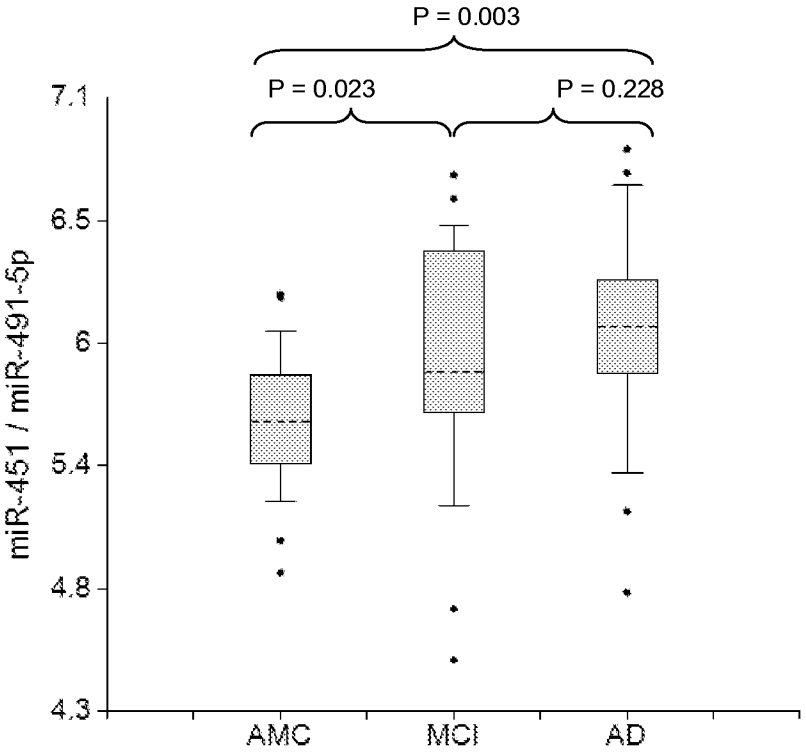

Figure 20E

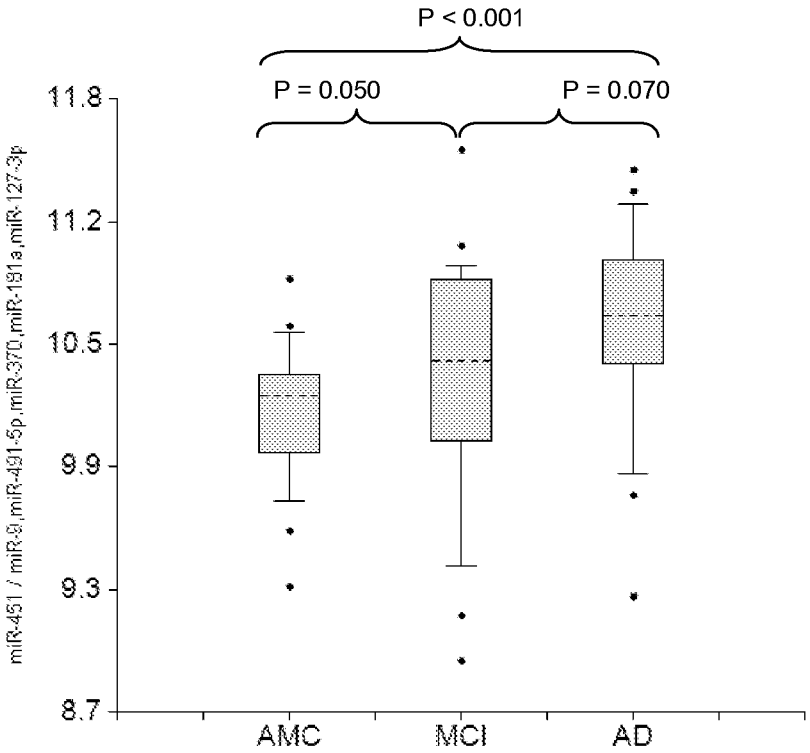

Figure 20F

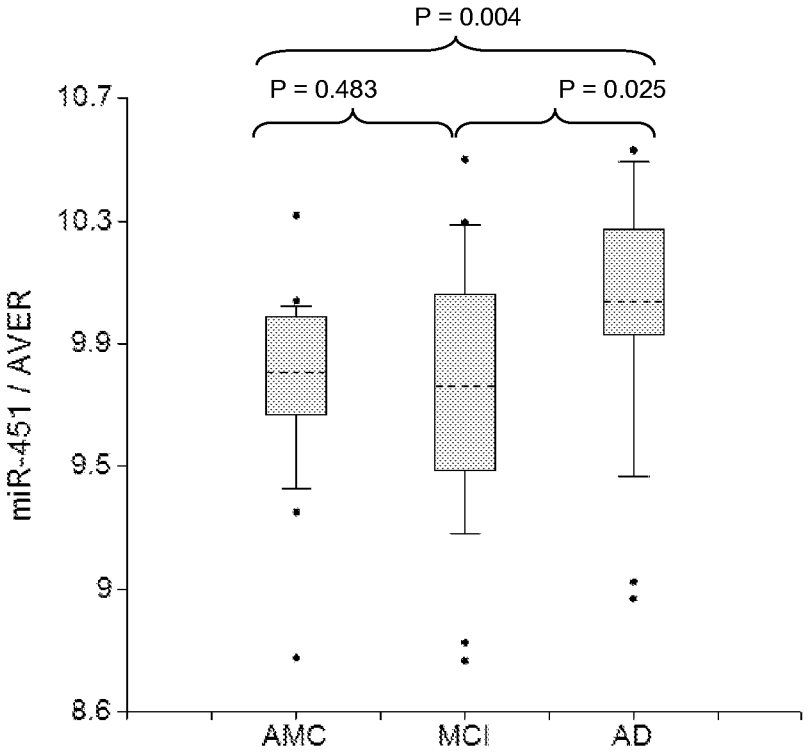

Figure 20G

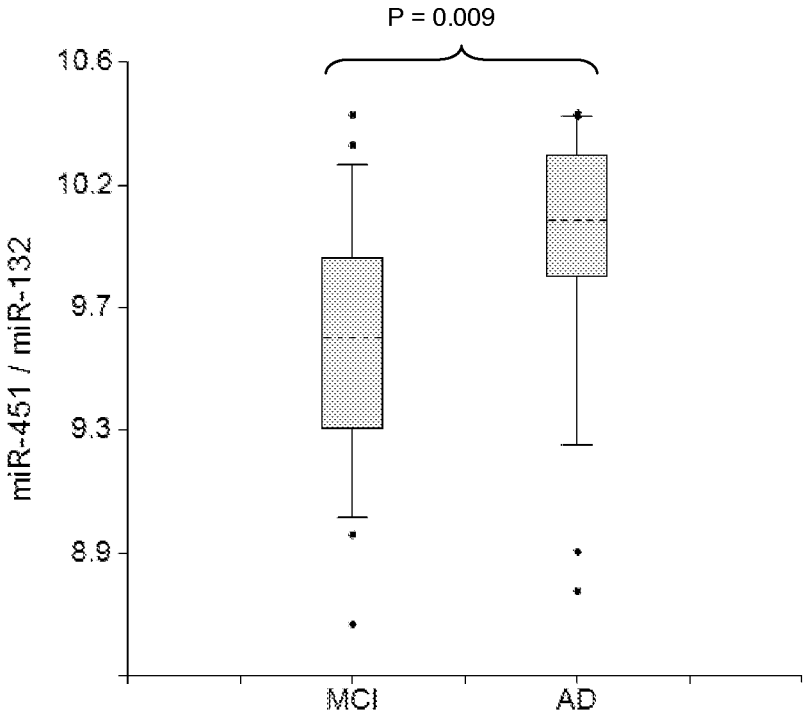

Figure 21A

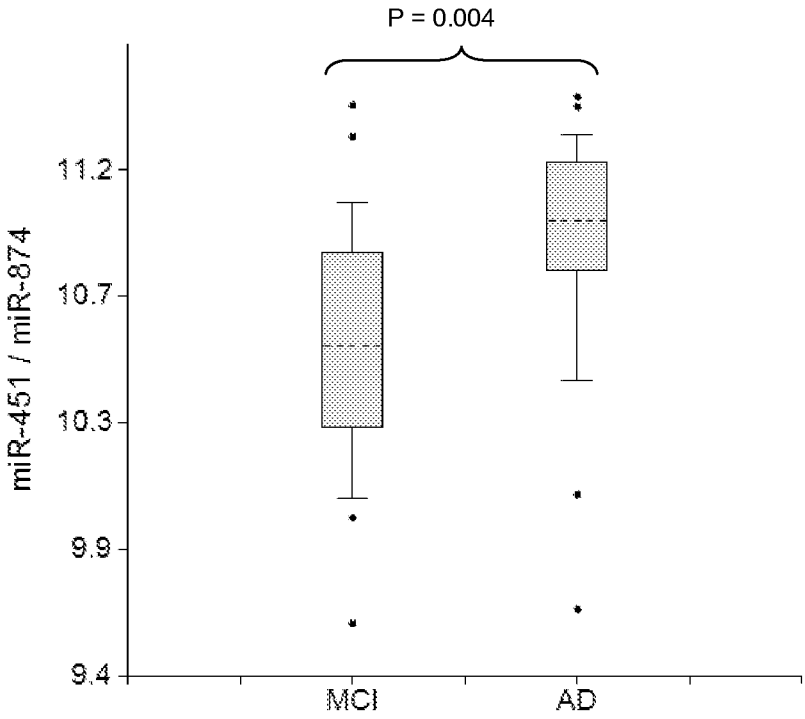

Figure 21B

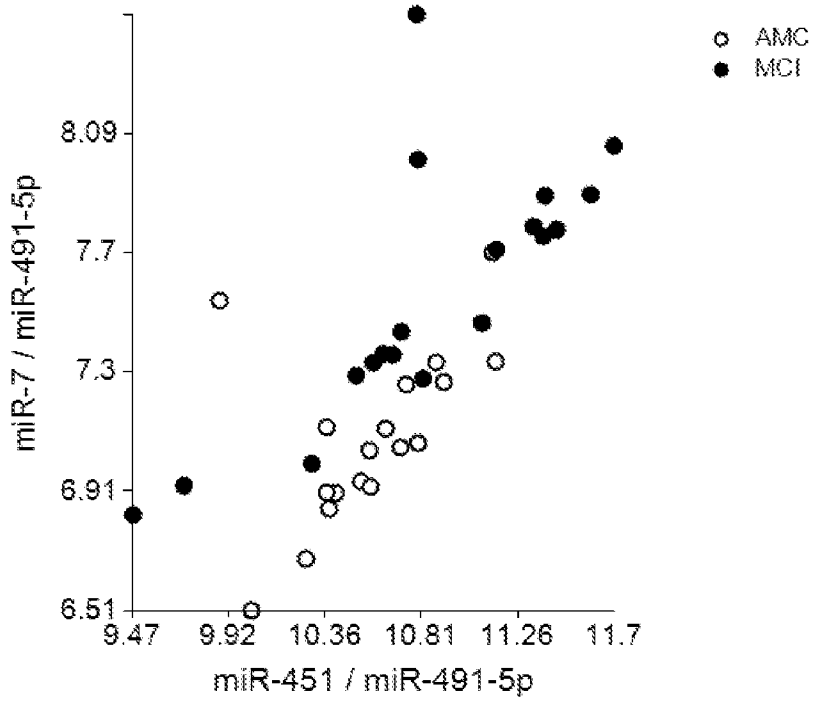

Figure 22A

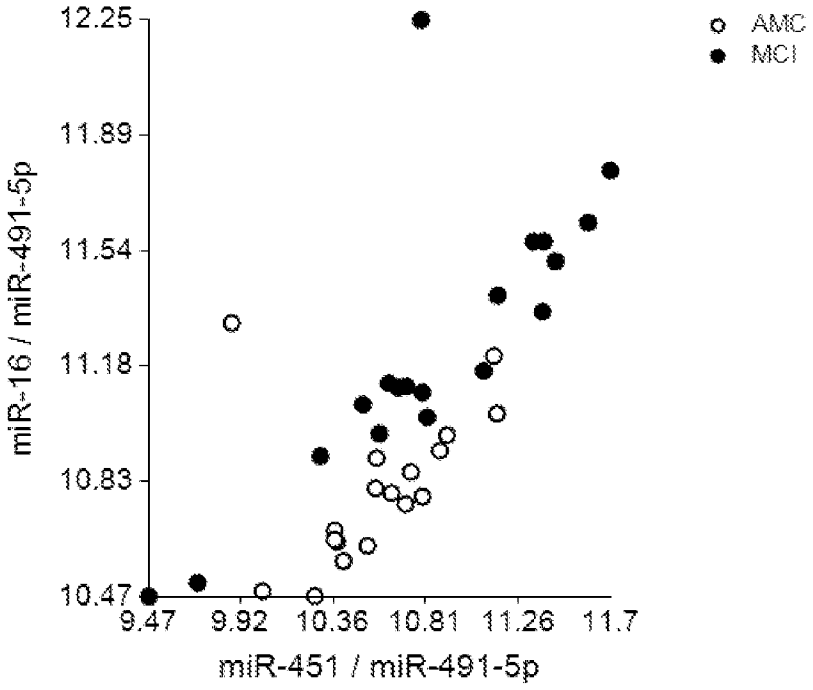

Figure 22B

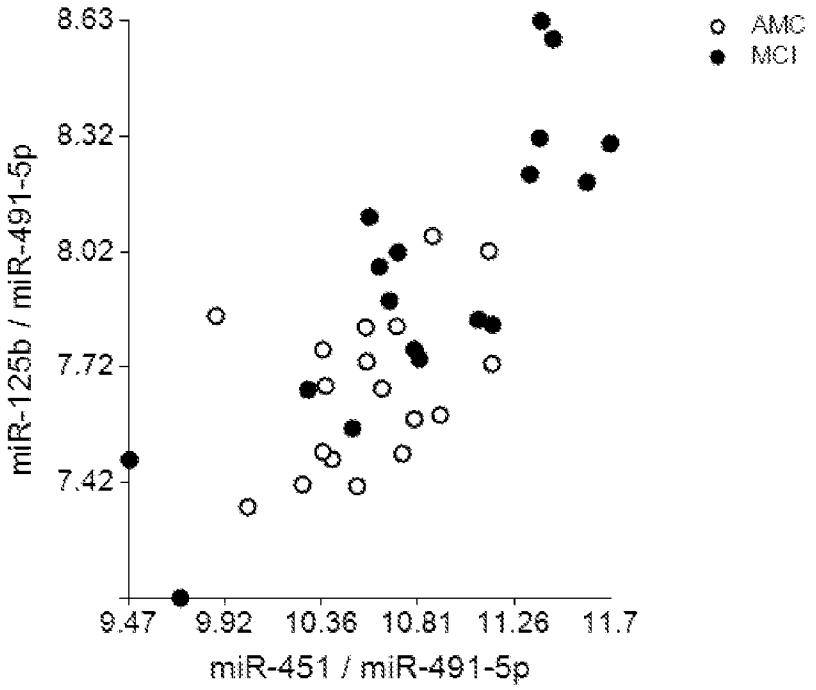

Figure 22C

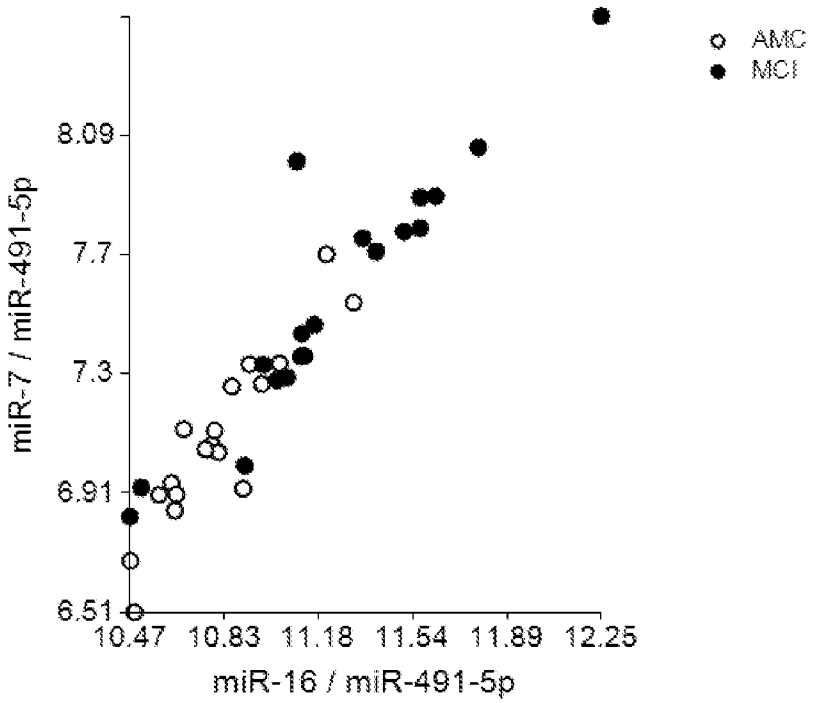

Figure 22D

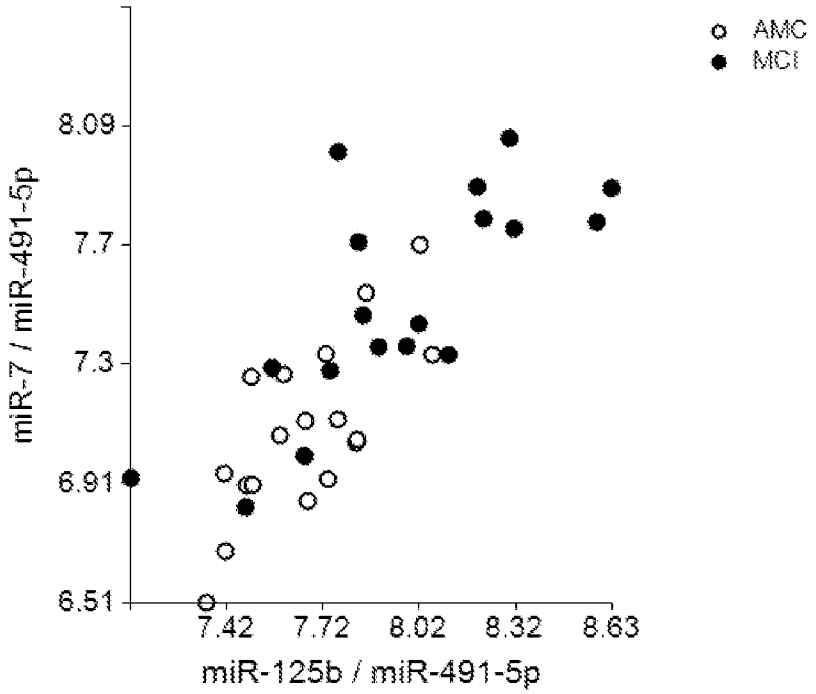

Figure 22E

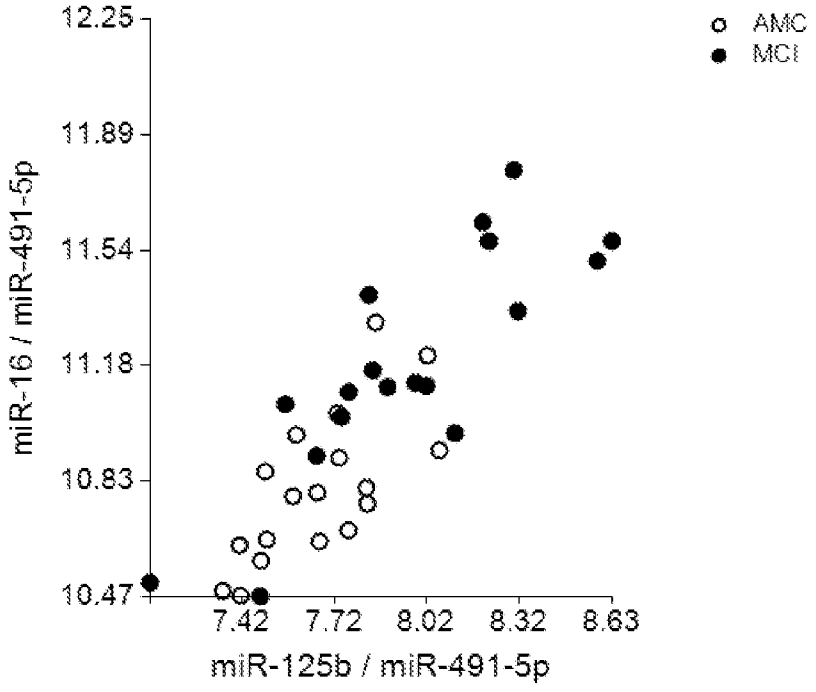

Figure 22F

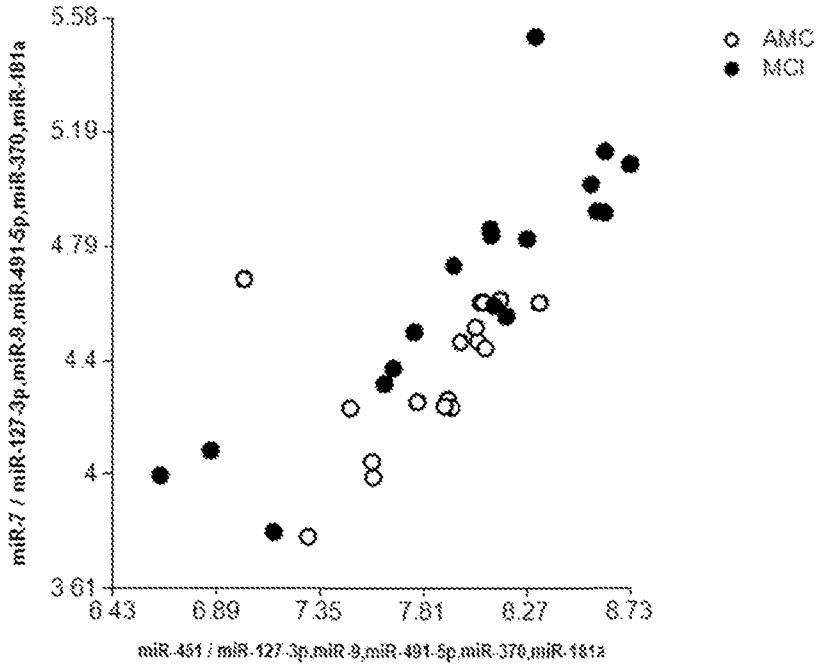

Figure 23A

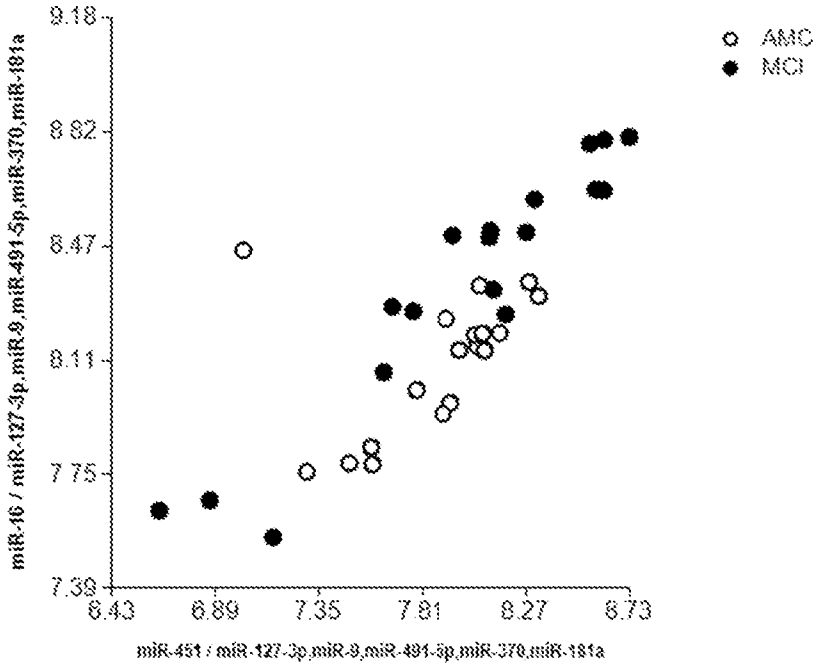

Figure 23B

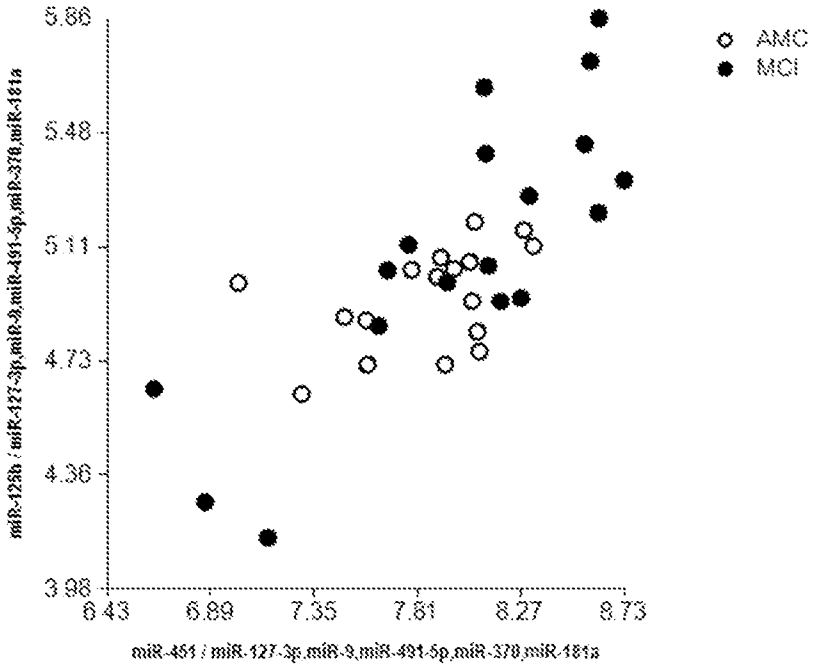

Figure 23C

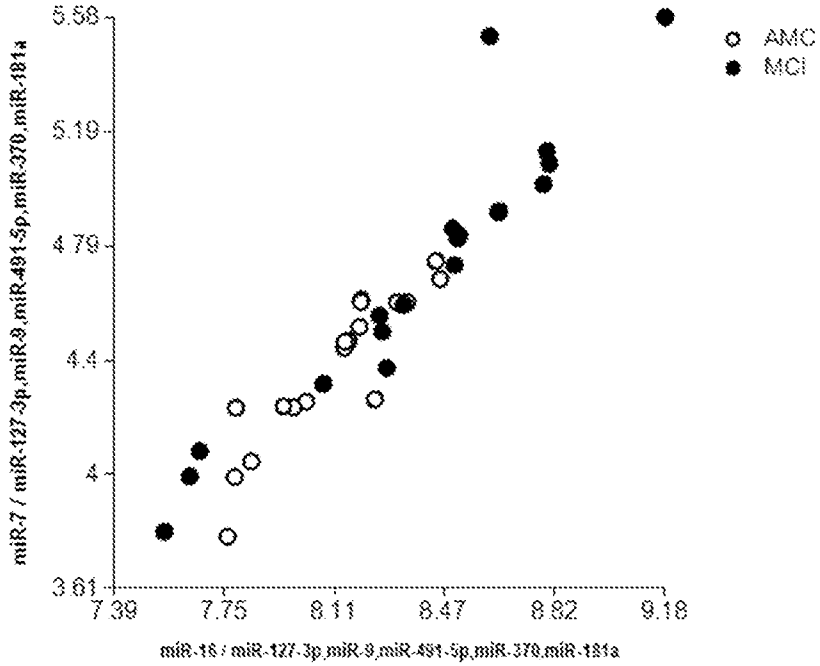

Figure 23D

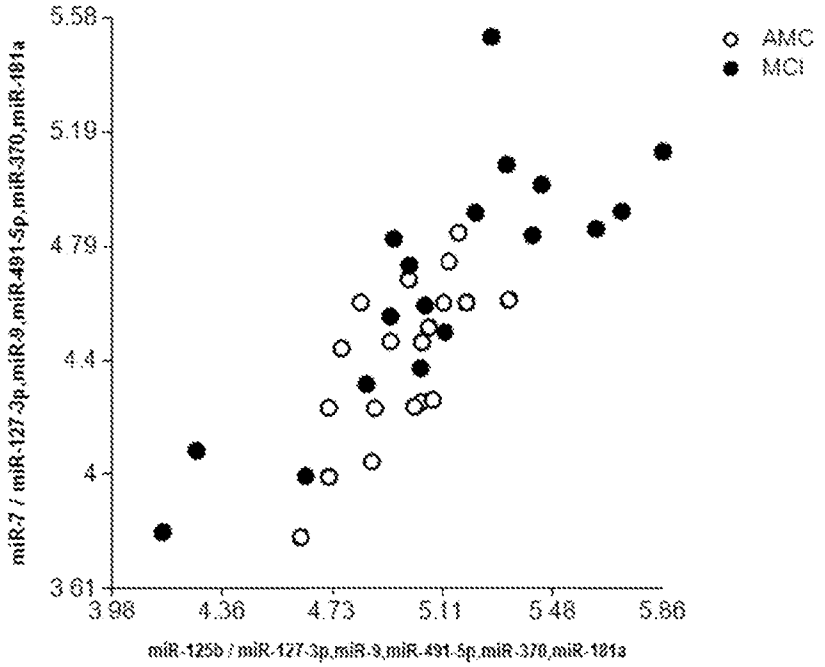

Figure 23E

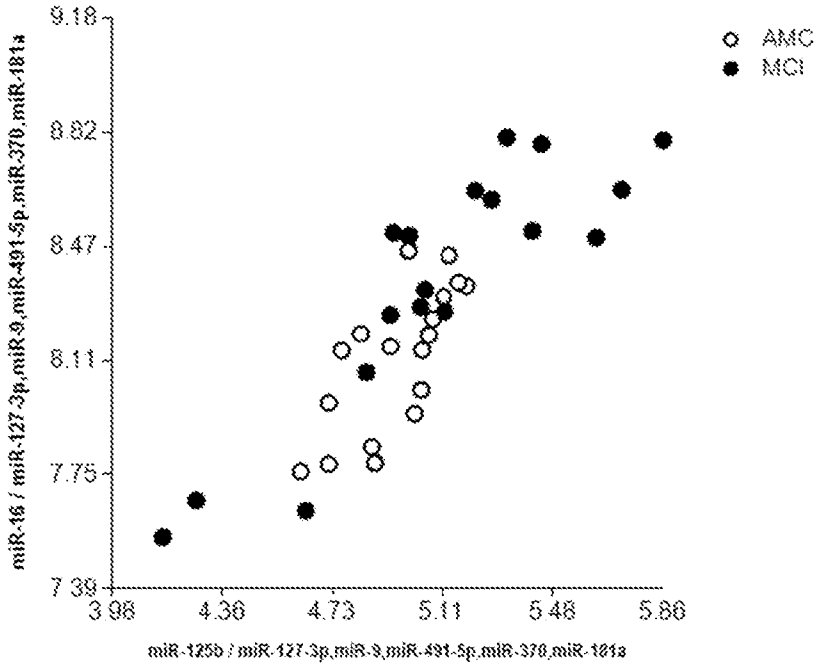

Figure 23F

MCI Patient Number	MCI compared to AD		MCI compared to AMC		
	mir-451 / mir-132	mir-451 / mir-874	miR-451 / miR-491-5p	miR-7 / miR-491-5p	miR-16 / miR-491-5p
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

Figure 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/34025

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; A61K 48/00 (2012.01)
USPC - 435/6.11; 435/6.12; 514/44A
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC- 435/6.11; 435/6.12; 514/44A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 514/44R; 536/24.5

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google, Google Scholar: Mild Cognitive Impairment, MCI, Alzheimer's, asymptomatic, early, Braak, miRNA, miR-7, miRNA-451, miR-25, miR-26, miR-98, miR-124, miR-125, miR-128, miR-132, miR-134, miR-137, miR-138, miR-146, miR-154, miR-182, miR-183, miR-200, miR-218, miR-292, miR-297, miR-322, miR-323, miR-329, miR-325

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Cogswell, et al. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis. 2008,14(1):27-41; Abstract, pg 28-30, pg 32, col 1; pg 34, pg 35, col 2 to pg 37, Table 1, 2, 4	1, 23, 36, 38, 41-44, 53 ---------- 40
Y	Kemppainen, et al. MicroRNAs as biomarkers in blood and other biofluids. 2007. [Retrieved from the Internet 08 September 2012:<http://www.asuragen.com/pdfs/posters/biomarkers.pdf>]; in entirety	40
X,P	WO 2011/057003 A2 (Umansky, et al.) 12 May 2011 (12.05.2011) Abstract	1, 23, 36, 38, 40-44, 53
A	Braak, et al. Neuropathological staging of Alzheimer's related changes. Acta Neuropathol 1991, 82:239-259; see pg 241, Table 1	1, 23, 36, 38, 40-44, 53
A	Geekiyanage, et al. Blood serum miRNA: Non-invasive biomarkers for Alzheimer's disease. Exp Neurol. ePub 01 December 2011, 235(2):491-6	1, 23, 36, 38, 40-44, 53
A	Hebert, et al. Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Proc Natl Acad Sci USA. 2008, 105(17):6415-20	1, 23, 36, 38, 40-44, 53
A	McDonald, et al. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem. 12 April 2011, 57(6):833-40	1, 23, 36, 38, 40-44, 53
A	Satoh. Molecular network of microRNA targets in Alzheimer's disease brains. Exp Neurol. ePub 16 September 2011, 235(2):436-46	1, 23, 36, 38, 40-44, 53

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 September 2012 (07.09.2012)	28 SEP 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/34025

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Schipper, et al. MicroRNA expression in Alzheimer blood mononuclear cells. Gene Regul. Syst. Bio. 2007, 1: 263-274.	1, 23, 36, 38, 40-44, 53
A	US 2010/0227908 A1 (Cairns, et al.) 09 September 2010 (09.09.2010) para [0011], [0014], [0015]	20
A	WO 2008/153692 A2 (Krischevsky, et al.) 18 December 2008 (18.12.2008) Abstract, claims 1, 4, 6, 7	20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/34025

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: claims 1, 20-23, 36, 38, 40-44, 46-53, drawn to a method for detection ofpre-MCI or MCI in a subject, by
a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;
b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with an age-matched control level of said miRNA, and
c. (i) identifying the subject as being afflicted with pre-MCI or Mcl when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the age-matched control or
(ii) identifying the subject as not being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is not increased as compared to the age matched control. The first invention (claims 1, 20, 23, 36, 38, 40-44, 53) is restricted to miR-7. Should an additional fee(s) be paid, Applicant is invited to elect an additional miRNA(s) to be searched.
********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 20, 23, 36, 38, 40-44, 53, restricted to miR-7

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/34025

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

Group II: claims 2, 20-23, 26-30, 32-33, 37-44, 52-53, drawn to a method for detection of pre-MCI or MCI in a subject, by
a. measuring the level of a synapse or neurite miRNA in a bodily fluid sample collected from the subject;
b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject;
c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);
d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and
e. (i) identifying the subject as being afflicted with pre-MCI or MCI when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio or
(ii) identifying the subject as not being afflicted with pre-MCI or MCI when ratio of the levels of the miRNAs calculated in step (c) is not higher than the corresponding age-matched control ratio.

Group III: claims 3-4, 20-22, 24-30, 32-33, 36-44, 46-53, drawn to a method for predicting likelihood of progression from pre-MCI to MCI in a subject, by
a. measuring the level of at least one synapse or neurite miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been obtained at spaced apart time points;
b. comparing the level of said miRNA in each of the bodily fluid samples collected from the subject with an age-matched control level of the said miRNA, and
c. predicting that the disease in the subject will progress from pre-MCI to MCI if the level of said miRNA is increased compared to the age-matched control in two or more consequently obtained bodily fluid samples collected from the subject.

Group IV: claims 5-6, 20-23, 26-30, 32-33, 38, 40-44, 46-53, drawn to a method for detection of brain aging in a subject, by
a. measuring the level of at least one synapse or neurite miRNA in a bodily fluid sample collected from the subject;
b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with
(i) a control level of said miRNA obtained from the same subject in the past or with
(ii) a predetermined young age standard, and
c. identifying the subject as being subject to brain aging when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the control (i) or as compared to the predetermined young age standard (ii).

Group V: claims 7-10, 20-30, 32, 33, 38, 40-53, drawn to a method for determining the effectiveness of pre-MCI or MCI treatment in a subject, by
a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from the subject obtained prior to initiation of the treatment;
b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained in the course of or following the treatment;
c. comparing the levels of the miRNA measured in steps (a) and (b), and
d. (i) determining that the treatment is effective if the level of the miRNA has decreased in the course of or following the treatment;
(ii) determining that the treatment is not effective if the level of miRNA has not decreased in the course of or following the treatment.

Group VI: claims 11-14, 20-22, 24-30, 32-33, 38-53, drawn to a method for identifying a compound useful for slowing down the progression or treating pre-MCI or MCI by
a. measuring the level of at least one synapse or neurite miRNA in one or more bodily fluid samples collected from a subject having pre-MCI or MCI, wherein said bodily fluid sample(s) is obtained prior to test compound administration;
b. measuring the level of said miRNA in one or more bodily fluid samples collected from the subject obtained following administration of a test compound;
c. comparing the levels of the miRNA measured in steps (a) and (b), and
d. (i) identifying that the test compound is useful for slowing down the progression or treating pre-MCI or MCI if the level of the miRNA has decreased after the compound administration;
(ii) identifying that the test compound is not useful for slowing down the progression or treating pre-Mel or Mel if the level of miRNA has not decreased after the compound administration.

Group VII: claims 15-31, 34-35, 38, 40-44, 46-53, drawn to a method for predicting progression from Mel to dementia stage of AD in a subject which had been diagnosed with MCI by
a. measuring the level of miR-451 in a bodily fluid sample collected from the subject;
b. measuring the level of at least one synapse or neurite miRNA in the same bodily fluid sample;
c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b);
d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and
e. determining that the disease in the subject will progress from MCI to dementia stage of AD if the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio.

Group VIII+: claims 54-71, drawn to a kit comprising primers or probes specific for at least one miRNA. The first invention (claims 54, 60, 66, 70-71) is restricted to miR-7. Should an additional fee(s) be paid, Applicant is invited to elect an additional miRNA(s) to be searched.

The inventions listed as Groups I+ through VIII+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Groups I+ through VII do not include the inventive concept of a kit comprising primers or probes specific for at least one miRNA, as required by Group VIII+.

The inventions of Groups I+ through IV and VI-VII do not include the inventive concept of a method for determining the effectiveness of pre-MCI or MCI treatment in a subject, as required by Group V.

********** See Supplemental Sheet to continue **********

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking and the preceding Supplemental Sheet:

The inventions of Groups I+ through V and VII do not include the inventive concept of a a method for identifying a compound useful for slowing down the progression or treating pre-Mel or MCIt, as required by Group VI.
The inventions of Groups I+ through III and VII do not include the inventive concept of a method for detection of brain aging in a subject , as required by Group IV.
The inventions of Group I+ through V do not include the inventive concept of a a method for predicting progression from MCI to dementia stage of AD in a subject which had been diagnosed with MCI, as required by Group VII.

The inventions of Groups I+ and II do not include the inventive concept of a method for predicting likelihood of progression from pre-MCI to MCI in a subject, as required by Group III.

The inventions of Group I+ do not include the inventive concept of a method for detection of pre-MCI or MCI in a subject, by b. measuring the level of a normalizer miRNA in the same bodily fluid sample collected from the subject; c. calculating the ratio of the levels of the miRNAs measured in steps (a) and (b); d. comparing the ratio of the levels of the miRNAs calculated in step (c) with a corresponding age-matched control ratio, and e. (i) identifying the subject as being afflicted with pre-MCI or MCI when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding age-matched control ratio or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when ratio of the levels of the miRNAs calculated in step (c) is not higher than the corresponding age -matched control ratio, as required by Group II.

The inventions of Group I+ share the technical feature of a method of claim 1. However, this shared technical feature does not represent a contribution over prior art as being anticipated by an article titled "Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways" by Cogswell, et al. (J Alzheimers Dis. 2008, 14(1):27-41) (hereinafter "Cogswell" that discloses a method for detection of Alzheimer's Disease in a subject (Abstract, "We additionally recovered miRNAs from cerebrospinal fluid and discovered AD-specific miRNA changes consistent with their role as potential biomarkers of disease"), which method comprises:
a. measuring the level of at least one miRNA bodily fluid sample collected from the subject (pg 35, col 2, "MiRNA expression is altered in Alzheimer's CSF. Since miRNA expression appears to be more stable than mRNAs and can be detected in human fluids [31] we set out to examine whether miRNAs were: 1) detectable in cerebrospinal fluid; and 2) were differentially expressed between AD and non-affected patients. ... To detect miRNAs in CSF we employed a sensitive pre-amplification procedure that permitted the detection of 201 out of the 242 miRNAs tested"; pg 37, Table 4, "Directional fold changes for miRNAs altered in CSF. Data shown is significant ($p < 0.05$)");
b. comparing the level of said miRNA in the bodily fluid sample collected from the subject with an age-matched control level of said miRNA (pg 35, col 2, "MiRNAs were isolated from frozen cerebrospinal fluid from 10 Braak 5 and 10 Braak 1 patients, none of whom were used for the AD brain tissue study", wherein Braak 5 patients constitute Alzheimer's Disease and Braak1 constitute age mathed non-demented control, as evidenced by pg 29, Table 2), and
c. (i) identifying the subject as being afflicted with Alzheimer's disease when the level of said miRNA in the bodily fluid sample collected from the subject is increased as compared to the age-matched control or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is not increased as compared to the age matched control (pg 35, col 2 to col 36, col 1, "Sixty miRNAs were detected as significantly different ($p < 0.05$) between the Braak stage 5 and Braak stage 1 samples. The distribution of fold changes was evenly distributed in both up and down directions (Table 4). Notably all of the members of the miR-30 family were induced...".
Cogswell does not expressly disclose that said miRNAs are synapse or neurite miRNAs. However, this limitation is inherently present in Cogswell, because miR-125a, miR-138, miR-146b, miR-154, miR- 345, miR-429 disclosed by Cogswell (see pg 37, Table 4) are in fact synapse or neurite miRNA (see instant application, pg 20, ln 14-23, and claims 20, 28).
Cogswell further discloses a method for detection of with pre-MCI or MCI in a subject (Abstract, "Using a sensitive qRT-PCR platform we identified regional and stage-specific deregulation of miRNA expression in AD patient brains. We used experimental validation in addition to literature to reveal how the deregulated brain microRNAs are biomarkers for known and novel pathways in AD pathogenesis related to amyloid processing, neurogenesis, insulin resistance, and innate immunity"; pg 29, Table 1, Brain sample data, postmortem, wherein Braaks 1-3 correspond to pre-MCI or MCI, as evidenced by a paper titled "Neuropathological staging of Alzheimer's related changes" by Braak, et al. (Acta Neuropathol 1991, 82:239-259) (hereinafter "Braak") (see pg 241, Table 1), which method comprises:
a. measuring the level of at least one synapse or neurite miRNA in a brain sample collected from the subject (pg 28, col 2, "Brain miRNA isolation and detection");
b. comparing the level of said miRNA in the brain sample collected from the subject with an age-matched control level of said miRNA (pg 28, col 2, "Alzheimer's disease samples and patient data. Age matched hippocampus, medial frontal gyrus, and cerebellum samples from different Braak stages were obtained from the Netherlands Brain Bank (Table 1). Braak stage 5 and Braak stage 1 CSF was obtained from the Kathleen Price Bryan Brain Bank (Table 2); pg 32, col 1), and
c. (i) identifying the subject as being afflicted with pre-MCI or MCI when the level of said miRNA in the brain sample collected from the subject is increased as compared to the age-matched control or (ii) identifying the subject as not being afflicted with pre-MCI or MCI when the level of said miRNA in the bodily fluid sample collected from the subject is not increased as compared to the age matched control (pg 30, col 1 to col 2, "Statistical analysis of miRNA expression data. Normalized miRNA log2 abundances were analyzed using a t-test for each pairing of Braak 5,6 versus Braak 0,1 and Braak 3,4 versus Braak 0,1, for each tissue, cerebellum, hippocampus, and medial frontal"; pg 32, col 2, "As hippocampus and medial frontal gyrus are regions primarily affected by AD pathology, the data were queried for miRNAs whose expression was significantly ($p < 0.05$) altered in both regions as well as either or both regions in the early (B3,4) and late (B5,6) stages of the disease. Expression of twelve miRNAs (miR-200c, -212, -26a, -27a, -30c, -30e-5p, -34a, -381, -422a, -423, -9, -92) was altered in both hippocampus and medial frontal gyrus in either B3,4 or B5,6 groups (Fig. 1A). Expression of nine miRNAs (miR-100, -125b, -132, -145, -146b, -148a, -210, -27b, -425) was shared between B3,4 and B5,6 in either or both hippocampus)").

********** See Supplemental Sheet to continue **********

********** Supplemental Sheet **********

In Continuation of Box III and the preceding Supplemental Sheet:

Although Cogswell discloses detecting AD in a subject by measuring the level of at least one miRNA bodily fluid sample collected from the subject, Cogswell does not disclose a method for detection of with pre-MCI or MCI in a subject by measuring the level of at least one miRNA bodily fluid sample collected from the subject. However, one of ordinary skill in the art would have been motivated to refine, in the course of routine experimentation and with a reasonable expectation of success, the method of Cogswell by using CSF miRNA measurements not only for diagnosing of AD but also for diagnosing its earlier stages, such as Braaks 1-4 that correspond the claimed pre-MCI or MCI stages. As said method would have been obvious to one of ordinary skill in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

The inventions of Group VII+ share the technical feature of a kit comprising primers or probes specific for at least one miRNA. However, this shared technical feature does not represent a contribution over prior art as being anticipated by US 2011/0086348 A1 (14 April 2011) to Prasad, et al. (hereinafter "Prasad") that discloses such a kit (para [0051]-[0054], "a kit for generating a miRNA profile for a sample is provided. The kit includes two of more miRNA probes having two or more miRNA coding sequences corresponding to sequences identified in TABLE 1 and TABLE 2, and reagents for labeling miRNA in the sample in a suitable container means"). As said kit was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another special technical feature of the inventions listed as Groups I+ and VII+ is the specific miRNA(s) recited therein. As said miRNAs were known in the art at the time of the invention (see Cogswell, Suppelemental Table 4) and no significant structural similarities can readily be ascertained among the claimed miRNAs, the inventions do not share a special technical feature. Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ through VIII+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.