Abstract:
The invention is directed to methods for early diagnosis, progression and treatment monitoring of Parkinson's disease (PD) and its differentiation from other neurodegenerative diseases by quantifying brain-enriched miRNA in bodily fluids.
METHODS OF USING miRNAs FROM BODILY FLUIDS FOR DETECTION AND MONITORING OF PARKINSON'S DISEASE (PD)

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/905,703, filed on November 18, 2013 and U.S. Provisional Application No. 61/935,806, filed on February 4, 2014, the disclosures of which are herein incorporated by reference in their entirety.

TECHNICAL FIELD OF THE INVENTION

The present invention is directed to methods for early diagnosis, progression and treatment monitoring of Parkinson's disease (PD) and its differentiation from other neurodegenerative diseases by quantifying brain-enriched miRNAs in bodily fluids.

BACKGROUND OF THE INVENTION

Parkinson's disease (PD) is the second most common neurodegenerative disease. Approximately 60,000 Americans are diagnosed with Parkinson's disease each year, and an estimated 0.5 and 7-10 million people in USA and worldwide, respectively, are living with Parkinson's disease (http://www.parkinson.org/parkinson-s-disease.aspx ; http://www.pdf.org/en/parkinson_statistics ).

The annual economic impact of Parkinson's disease in the United States is estimated to be around $10.8 billion and growing (http://www.parkinsoninfo.org/about-parkinsons-disease/economic-impact/ ).

The combined direct and indirect cost of Parkinson's, including treatment, social security payments and lost income from inability to work, is estimated to be nearly $25 billion per year in the United States alone (http://www.pdf.org/en/parkinson_statistics ).

Mechanisms of PD development are not well understood although many underlying processes have been described (Walter and Schulz-Schaeffer. Acta Neuropathol. 2010; 120: 131-143; David Sulzer. Trends in Neurosciences. 2007; 30: 244-250). PD is initiated by metabolic disorders, including abnormal processing of alpha-synuclein, which aggregates form so called Levy bodies, followed by synapse dysfunction, destruction and finally by neuronal death (Kazantsev AG, Kolchinsky AM. Arch Neurol. 2008; 65:1577-1581). Neurons of the substantia nigra in midbrain are maximally suffering in PD (Walter and Schulz-Schaeffer. Acta
Neuropathol. 2010; 120: 131-143) but other brain areas, such as frontal cortex (Braak et al. Neurobiology of Aging. 2003; 24: 197-211; Hou et al. J. Clin. Neuroscience. 2010; 17: 628-633), are also involved in the pathology at different stages of PD development. The substantia nigra in midbrain produces dopamine, which transmits signals to the corpus striatum involved in movement regulation and significant decrease in dopamine production causes numerous movement disorders characteristic of PD. The latter (tremor, postural instability and parkinsonian gait, rigidity, masked face, and bradykinesia) are currently the major symptoms used for PD diagnosis (Parkinson's Disease: Diagnosis and Clinical Management. Editors: Factor and Weiner, 2nd edition. 2008, Demos Medical Publishing, LLC, New York, NY). There are other symptoms, which are developed by some PD patients, such as cognitive problems, which can lead to PD dementia, depression, skin problems, olfactory dysfunction and others (Aarsland et al. Mov. Disord. 2005; 20: 1255-1263; Song et al. Eur. Neurol. 2008; 59: 49-55; Mollenhauer et al. Neurology. 2013; 81:1226-1234). Some data indicate involvement of inflammatory processes in PD development (Tansey and Goldberg. Neurobiol. Dis. 2010; 37:510-518; Amor et al. Immunology. 2013 Dec 16. [Epub ahead of print]).

Currently, there is no effective cure for PD, although medication and surgery can improve some symptoms of PD (Gazewood et al. Am. Fam. Physician. 2013; 87: 267-273). For example, dopamine replacement drugs such as L-DOPA and its combination with dopa decarboxylase inhibitors are widely used. Dopamine receptor agonists, monoamine oxidase and catechol o-methyltransferase inhibitors are other classes of drugs used to treat PD (Kansara et al. J. Neural Transm. 2013; 120: 197-210). Changes in lifestyle and physical therapy help to slow down PD progression.


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) and in different brain areas, such as hippocampus, midbrain, frontal cortex, pituitary gland, and in different cell types, such as neurons and glial cells (Sempere et al. Genome Biol. 2004; 5: R13; Deo et al. Dev. Din. 2006; 235:2538-2548; Bak et al. RNA. 2008; 14: 432-444; Trivedi and Ramakrishna


For the use of miRNA in diagnostics, it is also important that miRNA secretion varies depending on cellular physiology (Palma et al. Nucleic Acids Res. 2012; 40:9125-9138; Pigati et al. PLoS One. 2010; 5: e13515). In addition to miRNA release into extracellular space and subsequent appearance in the bodily fluids due to cell death, miRNA appear in circulation due to blebbing of apoptotic bodies, budding and shedding of microvesicles, active secretion in the form of exosomes and of miRNA complexes with proteins (AG02, NPM1 and others) and high density lipoproteins (HDL) (reviews: Sun et al. Clin. Chem. Lab. Med. 2012; 50: 2121-2126; Zandberga et al. Genes Chromosomes Cancer. 2013; 52: 356-369). All these forms of cell-free miRNA are highly stable in the bloodstream and other bodily fluids. The secretion of miRNA is selective and can be significantly changed by various pathological processes. For example, changes in the spectrum of miRNA secreted in exosomes from prion-infected neuronal cells, as compared to uninfected cells, have been demonstrated (Belingham et al. Nucleic Acids Res. 2012; 40: 10937-10949).

Two approaches are widely used for searching miRNA biomarkers of various diseases in bodily fluids:

1. Measurement of hundreds of different miRNA in a bodily fluid from patients with a pathology of interest and from control subjects using miRNA array or next generation sequencing (NGS) (Qin et al. Cancer Inform. 2013; 12: 83-101). While this approach allows to analyze a huge numbers of various miRNA, currently the miRNA array-based and sequencing techniques are not sufficiently sensitive to detect many miRNA whose concentration in bodily fluids is relatively low. As a consequence, most of the miRNA detectable in bodily fluids by arrays and NGS are ubiquitous miRNA expressed in all or many tissues, and many of them derive from blood cells (Pritchard et al. Cancer Prev. Res. (Phila). 2012; 5:492-497; Leidner and Thompson. PLoS One. 2013; 8: 57841). The detection of changes in the concentrations of such ubiquitous miRNA in patients with one pathology does not mean that the same miRNA cannot be involved in other diseases of different organs. Many miRNA are associated with a particular pathology type, such as cancer, inflammation, hypoxia, etc., and changes in their concentration in bodily fluids can be associated with diseases of different organs. For example, changes of miR-155 concentrations were found in the bloodstream of patients with breast, esophageal, lung, pancreatic cancers and lymphomas (Blair and Yan. DNA Cell Biol. 2012; 31 Suppl. 1: S49-61; Xie et al. Bioinformatics. 2013; 29: 638-644). Level of miR-21 increases in plasma/serum of
patients with osteosarcoma, bladder, esophageal, gastric, lung, breast, colorectal cancers, neck squamous cell carcinoma and other tumors (Blair and Yan. DNA Cell Biol. 2012; 31 Suppl. 1: S49-61; Farazi et al. J. Pathol. 2011; 223: 102-115; Xie et al, Bioinformatics. 2013; 29: 638-644). It follows that the potential biomarkers found by miRNA arrays should be also tested in other pathologies, not only in healthy control subjects.

2. The second approach is based on analysis of disease-specific miRNAs identified by comparison of miRNAs isolated from pathologic and normal tissue, organ or cell type. Here, subsequent to identification of disease-specific miRNAs (e.g., by an array followed by RT-PCR), their presence in bodily fluids is analyzed. In this strategy, since a limited number of circulating miRNAs is tested, the use of individual RT-PCR is possible which allows to increase sensitivity and reproducibility of the analysis. However, in many cases when this method was applied, no correlation was detected between miRNA concentration and pathology-induced changes in the tissue and in bodily fluids (Boeri et al., Proc. Natl. Acad. Sci. USA. 2011; 108: 3713-3718; Cuk et al. Int. J. Cancer. 2013; 132: 1602-1612).

**SUMMARY OF THE INVENTION**

As outlined in the Background section, above, despite recent advances, there is a great need in the art in sensitive methods of early detection of neurodegenerative diseases such as Parkinson’s disease (PD). The present invention addresses this and other needs by providing methods for early diagnosis, progression and treatment monitoring of PD and its differentiation from other neurodegenerative diseases by quantifying brain-enriched miRNAs in bodily fluids.

In one aspect, the invention provides a method for detecting a first neurodegenerative disease in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the first neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the first neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the first neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than
expression and/or secretion of the first miRNA during development of the first neurodegenerative disease;
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 control ratio, and
e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In a related aspect, the invention provides a method for detecting a first neurodegenerative disease in a subject, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
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 control ratio, and
e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In one embodiment of the above two methods, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (d)-(e) of the above two methods:
f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease, and

g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of the above two methods, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (a)-(e) of the above two methods:

f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease;

g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is (i) enriched in a brain area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;

h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);

i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;

j) (i) identifying the subject as being afflicted with the second neurodegenerative disease in addition to the first neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.
In one embodiment of any of the above methods, said first neurodegenerative disease is Parkinson's disease (PD).

In one embodiment of the above disease differentiation methods, said first neurodegenerative disease is Parkinson's disease (PD) and said second neurodegenerative disease is selected from the group consisting of Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, and olivopontocerebellar atrophy (OPCA).

In one aspect, the invention provides a method for detecting Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is neuronal miRNA enriched in midbrain and/or frontal cortex;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA is (i) enriched in a brain area(s) which is not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;

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

d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and

e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In a related aspect, the invention provides a method for detecting Parkinson's disease (PD) in a subject, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and
e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In one embodiment of the above two methods for detecting PD, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and with the steps (d)-(e) of the above two methods for detecting PD:

f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease different from PD;
g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of the above two methods for detecting PD, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and with the steps (a)-(e) of the above two methods for detecting PD:
f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease and is a pre-identified numerator in biomarker miRNA pair for said second neurodegenerative disease;
g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is and is a pre-identified denominator in biomarker miRNA pair for said second neurodegenerative disease and is (i) enriched in a brain area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease and is different from the cell type where the third miRNA is enriched, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;
h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);
i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;
j) (i) identifying the subject as being afflicted with the second neurodegenerative disease in addition to PD if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of any of the above methods of disease detection, the control ratio is a predetermined value which represents a statistically validated threshold ratio of the levels of said first and second miRNAs (a single 'cut-off' value) equal to the highest possible value within the range of corresponding values in age-matched healthy subjects. In another embodiment of any of the above methods of disease detection, the control ratio is the ratio of the levels of said first and second miRNAs in a similarly processed bodily fluid sample from the same subject collected in the past.

In one embodiment of any of the above methods of disease differentiation, the standard range of ratios of miRNAs characteristic of the second neurodegenerative disease is a
statistically validated predetermined range of values established by determining the ratios of the same miRNAs in a large cohort of subjects diagnosed with the second neurodegenerative disease. In one specific embodiment, the cohort of subjects diagnosed with the second neurodegenerative disease represents a full range of development stages of said second neurodegenerative disease. In another specific embodiment, the cohort of subjects diagnosed with the second neurodegenerative disease represents one or more development stages of said second neurodegenerative disease.

In one embodiment of any of the above methods of disease differentiation, said second neurodegenerative disease is selected from the group consisting of Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, and olivopontocerebellar atrophy (OPCA).

In another aspect, the invention provides a method for monitoring changes in development of a neurodegenerative disease in a subject (e.g., a subject who had been previously diagnosed with said neurodegenerative disease), which method comprises:

a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluids samples as in step (a), wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
(i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a related aspect, the invention provides a method for monitoring changes in development of a neurodegenerative disease in a subject (e.g., a subject who had been previously diagnosed with said neurodegenerative disease), which method comprises:

a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluids samples as in step (a), wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a related aspect, the invention provides a method for monitoring changes in development of Parkinson's disease (PD) in a subject (e.g., a subject who had been previously diagnosed with PD), which method comprises:
a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first brain-enriched miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid samples as in step (a), wherein said second brain-enriched miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first brain-enriched miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first brain-enriched miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In another related aspect, the invention provides a method for monitoring changes in development of Parkinson’s disease (PD) in a subject (e.g., a subject who had been previously diagnosed with PD), which method comprises:
a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid samples as in step (a), wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a separate aspect, the invention provides a method for monitoring the effect of a treatment on development of a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;

g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the treatment is effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s)
calculated in step (f), or (ii) determining that the treatment is not effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In a related aspect, the invention provides a method for monitoring the effect of a treatment on development of a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;

g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the treatment is effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the treatment is not effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In another related aspect, the invention provides a method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and
h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In yet another related aspect, the invention provides a method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first
miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and
h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In a separate aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In a related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;

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

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

In another related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is enriched in midbrain and/or frontal cortex;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

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 following administration of the test compound;

g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h)  (i) identifying that the test compound is useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In yet another related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:

a)  measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;

b)  measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d)  measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;

e)  measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

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 following administration of the test compound;

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

h)  (i) identifying that the test compound is useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).
In one embodiment of any of the above methods, the neurodegenerative disease is Parkinson's disease (PD).

In one embodiment of any of the above methods, the first brain-enriched miRNA is a neuronal miRNA. In one embodiment of any of the above methods, the first brain-enriched miRNA is synapse and/or neurite miRNA.

In one embodiment of any of the above methods, the second miRNA is a brain-enriched miRNA, which (1) is enriched in a brain area(s) which is not affected by the first neurodegenerative disease (e.g., PD) or (2) is enriched in a brain cell type which is not affected by the first neurodegenerative disease (e.g., PD). In one embodiment of any of the above methods, the second miRNA is a brain-enriched miRNA selected from the group consisting of miRNAs which are mainly expressed in brain areas not involved in PD, miRNAs which are mainly expressed in glial cells, and brain-enriched miRNAs downregulated in PD.

In one embodiment of any of the above methods involving the first and second miRNAs which are brain-enriched, the first brain-enriched miRNA is enriched in neurons and the second brain-enriched miRNA is enriched in glial cells.

In one embodiment of any of the above methods involving the first and second miRNAs which are brain-enriched, the pair of the first miRNA and the second miRNA is selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-323-3p, miR-107/miR-323-3p, miR-127/miR-323-3p, miR-181b/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-411, miR-107/miR-411, and miR-491-5p/miR-411.

In one embodiment of any of the above methods involving the first and second miRNAs one of which is brain-enriched and another inflammation-associated, the pair of the first miRNA and the second miRNA is selected from the group consisting of: miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-146a, miR-155/miR-16, miR-155/miR-132, miR-155/miR-323-3p, miR-155/miR-411, miR-491-5p/miR-146b, miR-155/miR-146a, and miR-155/miR-146b.

In one embodiment of any of the above disease differentiation methods involving the first and second miRNAs which are brain-enriched, the pair of the first miRNA and the second
miRNA is selected from the group consisting of: let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-335, miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-134, miR-181b/miR-132, let-7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-491-5p/miR-132, miR-107/miR-323-3p, miR-127/miR-134, miR-491-5p/miR-874, miR-491-5p/miR-323-3p, miR-127/miR-432, let-7e/miR-134, let-7e/miR-411, miR-107/miR-411, miR-491-5p/miR-411, miR-107/miR-411, miR-181b/miR-335, miR-99b/miR-335, miR-107/miR-210, miR-127/487b, miR-181a/miR-874, miR-9/miR-9*, miR-107/miR-487b, miR-107/miR-432, miR-9/miR-335, miR-181a/miR-132, miR-181b/miR-210, and miR-99b/miR-132. In another embodiment of any of the above disease differentiation methods involving the first and second miRNAs which are brain-enriched, the pair of the first miRNA and the second miRNA is selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-411, miR-99b/miR-335, miR-491-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-411, miR-107/miR-132, miR-181b/miR-132, miR-9/miR-9*, miR-107/miR-134, miR-181b/miR-874, miR-107/miR-132, miR-181b/miR-210, miR-9/miR-335, miR-181a/miR-9*, miR-491-5p/miR-9*, miR-181a/miR-874, let-7e/miR-132, miR-491-5p/miR-132, miR-181b/miR-9*, miR-107/miR-134, miR-491-5p/miR-146a, miR-107/miR-146a, miR-491-5p/miR-146a, miR-491-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, miR-155/miR-411 and miR-744/miR-146a. In another embodiment of the above disease differentiation methods involving the first and second miRNAs one of which is brain-enriched and another inflammation-associated, the pair of the first miRNA and the second miRNA is selected from the group consisting of: let-7e/miR-146a, miR-107/miR-146a, miR-491-5p/miR-146a, miR-107/miR-146b, miR-491-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, miR-155/miR-411 and miR-744/miR-146a. In another embodiment of the above disease differentiation methods involving the first and second miRNAs one of which is brain-enriched and another inflammation-associated, the pair of the first miRNA and the second miRNA is selected from the group consisting of: miR-107/miR-146a, miR-491-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, let-7e/miR-146b,
miR-9/miR-146b, let-7e/miR-146a, miR-155/miR-874, miR-155/miR-9*, miR-155/miR-411, miR-744/miR-146a, miR-155/miR-210, miR-155/miR-146b, miR-744/miR-146b, miR-155/miR-146a, miR-155/miR-134, and miR-181b/miR-146b.

In one embodiment of any of the above methods, the method comprises measuring the level and calculating the ratios of the levels for two or more different pairs of miRNA. In one specific embodiment, the method comprises measuring the level and calculating the ratios of the levels for one or more pair combinations selected from the group consisting of:
(a) miR-181b/miR-323-3p and miR-99b/miR-9*,
(b) miR-491-5p/miR-487b plus miR-9/miR-146a, and
(c) miR-491-5p/miR-210 plus miR-181a/miR-146b.

In one embodiment of any of the above disease detection or differentiation methods, the method comprises measuring the levels of the miRNAs in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points.

In one embodiment of any of the above methods involving bodily fluid samples which have been collected at spaced apart time points, the bodily fluid samples are obtained several months apart (e.g., 3-6 months apart).

In one embodiment of any of the above methods, the method further comprises normalizing the levels of the first and second miRNAs to the level of a normalizer miRNA. In one specific embodiment, the normalizer miRNA is miRNA which is expressed in numerous tissues but is not significantly expressed in brain.

In one embodiment of any of the above methods, the subject is human. In another embodiment of any of the above methods, the subject is an experimental animal (e.g., an animal model of a neurodegenerative disease such as, e.g., PD).

In one embodiment of any of the above methods, the bodily fluid is selected from the group consisting of blood plasma, serum, urine, cerebrospinal fluid (CSF), and saliva.

In one embodiment of any of the above methods, the method comprises the step of collecting the bodily fluid sample(s) from the subject (e.g., prior to step (a)).

In one embodiment of any of the above methods, the level of the miRNAs is determined using a method selected from the group consisting of hybridization, RT-PCR, and sequencing.

In one embodiment of any of the above methods, prior to measuring miRNA level, the miRNA is purified from the bodily fluid sample.
In one embodiment of any of the above methods, the method further comprises the step of reducing or eliminating degradation of the miRNAs.

In one embodiment of any of the above disease detection or disease monitoring methods, the method further comprises administering a therapeutic or preventive treatment to the subject that has been diagnosed as having the neurodegenerative disease (e.g., PD) or as being at risk of progression to a more severe form of the neurodegenerative disease (e.g., PD). In case of PD, non-limiting examples of useful drug treatments include, for example, administration of dopamine-replenishing or dopamine mimicking drugs such as, e.g., levodopa or levodopa combination treatments, which may include administration with dopa decarboxylase inhibitors (e.g., carbidopa, benzerazide); dopamine enhancers, such as catechol o-methyltransferase (COMT) inhibitors (e.g., entacapone, tolcapone); dopamine receptor agonists (e.g., ropinirole, pramipexole, rotigotine, apomorphine, pergolide, bromocriptine); monoamine oxidase (MAOIs) inhibitors, which can be used alone or with levodopa (e.g., selegiline, rasagiline, zydis selegiline HC1 salt); amantadine (used to combat tremor and side effects of levodopa administration); anticholinergens (e.g., trihexyphenidyl, benztrapine). Other potentially useful drugs currently under study for treatment of PD include antiglutamatergics (e.g., memantine, safinomide); neurturin therapies; anti-apoptotics (e.g., omigapil, CEP-1347); promitochondrials (e.g., Coenzyme Qio, creatine); calcium channel blockers, including isradipine, and growth factors such as GDNF; as well as drugs or vaccines targeting alpha-synuclein. Non-limiting examples of useful surgical therapies include, for example, deep brain stimulation (DBS), involving implantation of a battery-powered electrode in the brain; operations directly on neural tissue (e.g., thalamotomy, pallidotomy, subthalmotomy); and dopamnergic cell transplant. Diet and physical exercise regimen may also be used (separately or in combination with other treatments) to alleviate PD symptoms. Non-limiting examples of useful food supplements include, for example, antioxidants such as vitamins C and E, calcium, ginger root, green tea and green tea extracts, St. John's Wort, Ginkgo biloba, milk thistle, vitamin B12, and folic acid. Currently it is not clear if PD can be reversed. Effective treatment can mean PD improvement (decrease of a biomarker miRNA ratio) or prevention/inhibition of further development of PD (biomarker miRNA ratio stays the same or increases slower).

In one embodiment of any of the above disease detection or disease progression monitoring methods, the method further comprises recruiting the subject in a clinical trial.
In conjunction with the above methods of the invention, the invention also provides various kits. Non-limiting examples of the kits of the invention include:

1. A kit for detecting PD comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-323-3p, miR-107/miR-323-3p, miR-99b/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-41, miR-107/miR-41, and miR-491-5p/miR-41; miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-146a, miR-155/miR-16, miR-155/miR-335, miR-155/miR-323-3p, miR-155/miR-41, miR-491-5p/miR-146b, miR-155/miR-146a, and miR-155/miR-146b.

2. A kit for differentiating PD from MCI comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-335, miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-134, miR-181b/miR-132, let-7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-491-5p/miR-132, miR-107/miR-323-3p, miR-127/miR-134, miR-491-5p/miR-874, miR-491-5p/miR-323-3p, miR-127/miR-323-3p, let-7e/miR-41, miR-107/miR-41, miR-491-5p/miR-41, miR-107/miR-874, miR-181a/miR-9*, miR-491-5p/miR-210, miR-181b/miR-335, miR-99b/miR-335, miR-107/miR-210, miR-127/miR-323-3p, miR-491-5p/miR-323-3p, miR-127/miR-323-3p, miR-181a/miR-335, miR-9/miR-9*, miR-107/miR-323-3p, miR-491-5p/miR-323-3p, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-41, miR-181a/miR-335, let-7e/miR-9*, miR-491-5p/miR-41, let-7e/miR-132, miR-181b/miR-132, miR-127/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-41, miR-107/miR-41, and miR-491-5p/miR-41.

3. A kit for differentiating PD from AD comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-41, miR-99b/miR-335, miR-491-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-41, miR-181a/miR-335, let-7e/miR-9*, miR-491-5p/miR-41, let-7e/miR-132, miR-181b/miR-132, miR-127/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-41, miR-107/miR-41, and miR-491-5p/miR-41.
9/miR-9*, miR-9/miR-134, miR-107/miR-134, miR-181b/miR-874, let-7e/miR-134, miR-107/miR-132, miR-107/miR-9*, miR-127/miR-335, miR-9/miR-132, miR-181b/miR-9*, miR-491-5p/miR-132, let-7e/miR-210, miR-491-5p/miR-9*, miR-107/miR-323-3p, miR-491-5p/miR-323-3p, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-210, miR-9/miR-874, miR-9/miR-323-3p, miR-491-5p/miR-9*, miR-491-5p/miR-9*, miR-107/miR-132, miR-107/miR-323-3p, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-744/miR-146a, miR-181b/miR-485-3p, miR-181b/miR-323-3p; miR-107/miR-146a, miR-491-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, let-7e/miR-146b, miR-9/miR-146b, let-7e/miR-146b, miR-155/miR-146a, miR-155/miR-146b, miR-155/miR-146b, miR-155/miR-146a, miR-155/miR-134, and miR-181b/miR-146b.

4. A kit comprising primers and/or probes specific for one or more combinations of pairs of miRNAs selected from the group consisting of:
   (a) miR-181b/miR-323-3p and miR-99b/miR-9*,
   (b) miR-491-5p/miR-487b and miR-9/miR-146a, and
   (c) miR-491-5p/miR-2 10 and miR-181a/miR-146b.

Any of the above kits can further comprise miRNA isolation and/or purification means and/or instructions for use.

In a separate aspect, the invention provides a method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
   (a) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;
   (b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
   (c) calculating the mean level of each miRNA measured in step (b);
   (d) calculating the difference between the mean miRNA levels calculated in step (c);
   (e) comparing the differences between the mean miRNA levels calculated in step (d) between all studied miRNAs and selecting as potential biomarker pairs those miRNA pairs for which the difference calculated in step (d) for one miRNA is at least 1.5 times the difference calculated for the other miRNA;
   (f) calculating Spearman's rank correlation coefficient (r) for each potential biomarker miRNA pair selected in step (e), and
identifying the miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if its \((r)\) value calculated in step \((f)\) is at least 0.8.

In a related aspect, the invention provides a method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

\(\text{(a)}\) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;

\(\text{(b)}\) measuring the level of each miRNA selected in step \((a)\) in bodily fluid samples from two subject cohorts;

\(\text{(c)}\) calculating Spearman's rank correlation coefficient \((r)\) for all possible pairs of individual miRNAs measured in step \((b)\);

\(\text{(d)}\) selecting as potential biomarker pairs those miRNA pairs which have the \((r)\) value calculated in step \((c)\) of at least 0.8;

\(\text{(e)}\) calculating the mean level of each miRNA selected in step \((d)\);

\(\text{(f)}\) calculating the difference between the mean miRNA levels calculated in step \((e)\);

\(\text{(g)}\) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if the difference calculated in step \((f)\) for one miRNA is at least 1.5 times the difference calculated for the other miRNA.

In another related aspect, the invention provides a method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

\(\text{(a)}\) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;

\(\text{(b)}\) measuring the level of each miRNA selected in step \((a)\) in bodily fluid samples from two subject cohorts;

\(\text{(c)}\) calculating Spearman's rank correlation coefficient \((r)\) for all possible pairs of individual miRNAs measured in step \((b)\);

\(\text{(d)}\) selecting as potential biomarker pairs those miRNA pairs which have the \((r)\) value calculated in step \((c)\) of at least 0.8;

\(\text{(e)}\) calculating P-value of two subject cohorts separation for each miRNA pair selected in step \((d)\), and

(g) identifying the miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if its \((r)\) value calculated in step \((f)\) is at least 0.8.
(f) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this pair differentiates two subject cohorts with a statistically significant P-value.

In yet another related aspect, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the mean level of each miRNA measured in step (b);
(d) electronically calculating a difference between the mean miRNA levels calculated in step (c);
(e) selecting from the group of measured miRNAs a set of potential miRNA pairs each comprising a first miRNA and a second miRNA, wherein the calculated difference in the mean level in step (d) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA;
(f) electronically calculating the Spearman's rank correlation coefficient (r) for each potential miRNA pair selected in (e);
(g) selecting from the set of potential miRNA pairs those miRNA pairs, which are suitable for the diagnosis and/or monitoring of the pathology, wherein the (r) value calculated in step (f) is at least 0.8, and
(h) displaying all or part of the miRNA pairs selected in step (g).

In a further related aspect, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the Spearman's rank correlation coefficient \( r \) for all possible pairs of individual miRNAs measured in step (b);

(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the \( r \) value calculated in step (c) is at least 0.8;

(e) electronically calculating the mean level of each miRNA selected in step (d);

(f) electronically calculating the difference between the mean miRNA levels calculated in step (e);

(g) selecting from the group of measured miRNAs a set of suitable miRNA biomarker pairs each comprising a first miRNA and a second miRNA, wherein for each suitable biomarker miRNA pair, the calculated difference in the mean level in step (f) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA, and

(h) displaying all or part of the suitable biomarker miRNA pairs selected in step (g).

In an additional related aspect, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;

(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;

(c) electronically calculating the Spearman's rank correlation coefficient \( r \) of the levels measured in step (b) for all possible pairs of individual miRNAs;

(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the \( r \) value calculated in step (c) is at least 0.8;

(e) electronically calculating P-value of two subject cohorts separation for each miRNA pair selected in step (d);

(f) selecting a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this miRNA pair differentiates two subject cohorts with a statistically significant P-value, and

(g) displaying all or part of the suitable biomarker miRNA pairs selected in step (f).
In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the miRNA level is measured by a method selected from the group consisting of RT-PCR-based methods, miRNA array-based methods, new generation sequencing, and hybridization.

In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the two subject cohorts are (1) subjects having the pathology and (2) age, gender and ethnicity-matched healthy subjects. In another embodiment of any of the above methods for selecting a biomarker miRNA pair, the two subject cohorts are subjects having two different pathologies of the organ. In yet another embodiment of any of the above methods for selecting a biomarker miRNA pair, the two subject cohorts are (1) younger subjects and (2) older subjects. In a further embodiment of any of the above methods for selecting a biomarker miRNA pair, the two subject cohorts are age and ethnicity-matched (1) males and (2) females.

In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the bodily fluid is selected from the group consisting of plasma, serum, cerebrospinal fluid (CSF), urine, and saliva.

In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the subject is human. In another embodiment of any of the above methods for selecting a biomarker miRNA pair, the subject is an experimental animal.

In one embodiment of any of the above methods for selecting a biomarker miRNA pair involving P-value, the P-value is calculated in step (e) using Mann-Whitney test. In one embodiment of any of the above methods for selecting a biomarker miRNA pair involving P-value, the statistically significant P-value is at least 0.05.

In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the pathology is a neurodegenerative disease (e.g., Parkinson's disease (PD), Alzheimer's disease (AD) and Mild Cognitive Impairment (MCI)).

In one embodiment of any of the above methods for selecting a biomarker miRNA pair, the organ affected by the pathology is brain.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in the following description, claims and drawings.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A-F are graphs showing ratios of miRNA levels (biomarker pairs) in plasma of age-matched controls (AMC) and Parkinson's disease (PD) patients. A, C and E present box and
whisker plots in the Log10 scale. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. The points indicate assay values located outside of 80% data. B, D and F present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between PD patients and AMC. Sensitivity, specificity and accuracy for each biomarker/normalizer pair are calculated for the "cutoff" point (indicated as a dot on each plot) - the value of the ratio of paired miRNA where the accuracy of predictions is the highest.

Figures 2A-F are graphs showing ratios of miRNA levels (biomarker pairs) in plasma of MCI and PD patients. A, C and E present box and whisker plots in the Log10 scale. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. The points indicate assay values located outside of 80% data. B, D and F present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between PD patients and MCI. Sensitivity, specificity and accuracy for each biomarker/normalizer pair are calculated for the "cutoff" point (indicated as a dot on each plot) - the value of the ratio of paired miRNA where the accuracy of predictions is the highest.

Figures 3A-F are graphs showing ratios of miRNA levels (biomarker pairs) in plasma of AD and PD patients. A, C and E present box and whisker plots in the Log10 scale. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. The points indicate assay values located outside of 80% data. B, D and F present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between PD patients and AMC. Sensitivity, specificity and accuracy for each biomarker/normalizer pair are calculated for the "cutoff" point (indicated as a dot on each plot) - the value of the ratio of paired miRNA where the accuracy of predictions is the highest.

Figures 4A-C present Receiver-Operating Characteristic (ROC) curve analysis of differentiation between PD patients and AMC (A), PD and MCI patients (B) and PD and AD patients (C). Sensitivity, specificity and accuracy for each biomarker/normalizer pair and their combination are calculated for the "cutoff" point (indicated as a dot on each plot) - the value of the ratio of paired miRNA where the accuracy of predictions is the highest.
DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the following ideas and findings made by the present inventors:

(1) changes in concentrations of circulating miRNAs enriched in the brain, and more specifically in brain areas involved in a particular pathology, are more likely to reflect associated pathologic processes in the brain than ubiquitous or other brain-enriched miRNAs;

(2) miRNAs present in neurites and synapses should be analyzed, because dysfunction and destruction of neurites and synapses is characteristic of early stages of neurodegeneration, and therefore, can affect expression and secretion of these miRNAs;

(3) to compensate for processes unrelated directly to a particular pathology, e.g., changes in blood-brain barrier permeability, the present inventors used the "biomarker pair" approach normalizing miRNAs enriched in neurons of damaged brain area(s) by other brain-enriched miRNAs, which could be expressed in brain areas or cell types not involved in early stages of a specific neurodegenerative disease which is being diagnosed, as well as miRNAs with plasma levels changing differently when compared with miRNAs enriched in the area of interest;

(4) high correlation of plasma concentrations of miRNAs used as numerator and denominator in a biomarker miRNA pair is very important for its sensitivity and specificity.

The present invention is based on analysis of the ratios of the levels for pairs of circulating cell-free miRNA in bodily fluids, wherein both miRNA in the pair are brain-enriched, and either (i) are enriched in certain brain areas, which are (for one miRNA in the pair) or are not (for the other miRNA in the pair) involved in PD development, or (ii) are enriched in different cell types (e.g., neurons and glial cells), or (iii) are enriched in the same brain area but whose expression and/or secretion change differently due to PD development. Brain-enriched miRNAs which are particularly useful as numerators in the miRNA pairs of the invention include neuronal miRNAs present in neurites and synapses (i.e., synapse and/or neurite miRNAs), whose normal functioning suffers on early stages of PD, AD and other neurodegenerative diseases. The present invention also encompasses the analysis of miRNAs involved in inflammatory processes (e.g., miR-155) and their use as potential biomarkers in combination with miRNAs enriched in brain area(s) involved in pathology. Since various neurodegenerative diseases are characterized by neuronal pathology in different brain areas such biomarker miRNA pairs can be used for differentiating those pathologies from each other independent of their clinical symptoms, if any.
In addition, discovered biomarkers, reflecting important events in pathology development, could be used for disease and treatment monitoring as well as drug screening.

Use of brain-enriched miRNA significantly increases chances that changes of their levels in plasma are caused by brain pathology, and changes in bodily fluid concentration of miRNA enriched in a particular brain area should be indicative of pathology in that brain part. For example, changes in levels of hippocampus- or midbrain-enriched miRNA would be respectively associated with AD and PD, reflecting synapse and neuronal dysfunctions in these brain areas. In addition, concentrations of organ-enriched miRNA in blood cells are low, which decreases contamination of plasma and serum by miRNA leakage during purification of these bodily fluids.

The concentrations of miRNAs detected in bodily fluids depend on many biological and technical factors. Biological factors include miRNA levels in various tissues, intensity of secretion and excretion into extracellular space, forms of circulating miRNAs (exosomes and other vesicles, complexes with proteins and lipids) affecting their ability to cross various barriers, e.g. blood-brain, placental, and kidney barriers, and miRNA stability and half-life in the bloodstream. Technical factors include variability in methods of bodily fluid collection and storage, methods used for miRNA extraction, and presence in bodily fluids of various factors affecting miRNA purification and quantitation. As a consequence, the importance of miRNA normalization is broadly recognized (Meyer et al., Biotechnol. Lett. 2010; 32: 1777-1788). At the same time, no single normalization method is commonly accepted.

The present invention is based on the use of brain-enriched miRNA pairs instead of (or in addition to) normalization per ubiquitous RNA or an average of numerous miRNA. The use of brain-enriched miRNA pairs (one as a numerator and another one as a denominator in a ratio) has several advantages. First, any pathology is usually associated with up-regulation of some miRNAs and down-regulation of other miRNAs, thus considering miRNA pairs of up- and down-regulated miRNAs may increase test sensitivity and specificity. Second, the use of a pair of brain-enriched miRNAs, rather than one brain-enriched miRNA, decreases potential overlap with pathologies of other organs. Third, one can expect that changes unrelated to or non-specific for a pathology of interest, such as, e.g., changes in blood supply, blood-brain permeability and others, will be better compensated for by using the pair of miRNAs enriched in the same organ. For example, changes in relative concentrations of miRNAs enriched in different brain areas or
different cell types (e.g., neurons and glial cells) may be an indicator of disease progression, and so on.

In the present invention, since various miRNA are involved in regulation of different processes, combination of several miRNA pairs were also tested to find out the groups of miRNA pairs providing the highest test accuracy. Non-limiting examples of such groups include miR-181b/miR-323-3p plus miR-99b/miR-9*; miR-491-5p/miR-487b plus miR-9/miR-146a; miR-491-5p/miR-210 plus miR-181a/miR-146b (see Figure 4).

**Definitions**

As used herein in connection with enrichment in brain, the term "enriched" means that miRNA concentration in brain is at least 4-5 times higher than in other organs.

As used herein in connection with enrichment in a certain area of the brain, the term "enriched" means that miRNA concentration in said area of the brain is higher (preferably, at least 2-fold higher, more preferably at least 5-fold higher, most preferably at least 10-fold higher) than in brain in general. The term refers to the difference in concentrations within the brain areas (e.g., as measured using qRT-PCR).

Within the meaning of the present invention, the term "synapse and/or neurite miRNA" refers to miRNA which (i) is "brain-enriched" and (ii) is present in a synapse and/or neurite (i.e., axon and/or dendrite and/or spine). To be useful in the methods of the present invention, 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 "Mild Cognitive Impairment" or "MCI" refers to an intermediate stage between the expected cognitive decline of normal aging and the more serious decline of dementia. It can involve problems with memory, language, thinking and judgment that are greater than normal age-related changes (see, e.g., Jack et al., Alzheimer's and Dementia. 2011, Epub April 19).

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 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.

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

The term "neurodegenerative disease" is used herein to refer to brain pathologies, such as, e.g., Parkinson's disease (PD), Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), Chronic Traumatic Encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, olivopontocerebellar atrophy (OPCA), and other pathologies characterized by metabolic changes, followed by synapse dysfunction, neurite and synapse destruction and ultimately by neuronal death.
As used herein, the term "development stage of a neurodegenerative disease" refers to a specific extent of severity of a neurodegenerative disease (e.g., asymptomatic stage, early disease with some symptoms, late stages of disease, etc.).

The term "development of a neurodegenerative disease" 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 neurodegenerative disease" 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.

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

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: DI40-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; Barrel, 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). Unless otherwise noted, the name of a specific miRNA refers to a mature miRNA sequence. Under current nomenclature rules, human miRNAs are preceded with the prefix "hsa-" (i.e., an abbreviation for Homo sapiens). Throughout the specification and figures the hsa- prefix may be dropped for purposes of abbreviation, thus, for example, "hsa-miR-155" and "miR-155" would represent the same RNA sequence.

Examples of neurite and/or synapse miRNAs useful in the methods of the present invention include, without limitation, Let-7e, miR-7, miR-9, miR-98, miR-99, miR-124a, miR-125a, miR-125b, miR-128a, miR-132, miR-134, miR-135a, miR-137, miR-138, miR-154, miR-182, miR-183, miR-213, miR-218, miR-323-3p, miR-329, miR-337, miR-369-3, miR-369-5p,
miR-370, miR-381, miR-382, miR-409-3p, miR-425, miR-433-5p, miR-483-3p, miR-485-5p, miR-487b, miR-491-5p, miR-494, miR-495, miR-496, miR-541, miR-543, miR-656, miR-668, miR-874, miR-889, miR-935, miR-939, miR-9* and miR181a-1*. 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. In a preferred embodiment, the subject is a human.

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.


Methods for Identification of Diagnostic miRNA Pairs

To identify the most promising biomarker pairs, the present inventors used the following approach: selection of a numerator and a denominator for each pair from those circulating miRNAs, which significantly correlate (Spearman's rank correlation coefficient r>0.8) in a respective bodily fluid of different individuals. Concentrations of miRNAs in plasma depend on numerous factors, including (i) levels of miRNA expression in various organs and tissues; (ii) levels of miRNA secretion from different cell types; (iii) stability of miRNAs in extracellular space and their appearance in plasma in different forms, such as exosomes and other microvesicles, complexes with proteins, lipids and, possibly, other molecules; and (iv) blood-brain barrier permeability for brain-enriched miRNAs. A pathological process may affect some or all of these factors. The present inventors suggest that a nominator and a denominator of an effective biomarker miRNA pair should share some of these basic common factors (e.g., both are brain-enriched and secreted in exosomes) and would change differently in response to a pathology. This does not mean that any correlated miRNA will form a good biomarker pair, since if they are similarly changed by pathology their ratio will mask those changes.

The present invention provides a method of "promising" miRNA pair selection, which method comprises the following steps:

1. Concentrations of miRNAs pre-selected on the basis of their enrichment in an organ of interest (e.g., brain) are measured in a bodily fluid (e.g., plasma, serum, cerebrospinal fluid (CSF), saliva, urine) of at least two comparative cohorts (e.g., a disease and control for a diagnostic test, two diseases for a test capable of differentiating two pathologies, a disease at different stages of pathologic process development, or a disease before and after treatment for monitoring tests).

2. Means of each miRNA concentrations are calculated for comparative cohorts.
3. The difference between the means for each miRNA from two comparative cohorts is calculated and miRNAs are divided in two groups: (i) with high difference values; and (ii) with low or with opposite sign difference values.

4. miRNAs from different groups are combined as potential biomarker pairs if parameters determined in step 3 differ at least 1.5 times. One miRNA is used as a numerator and another miRNA is used as a denominator in a potential "promising" miRNA pair.

5. To further reduce an impact of individual variations of each particular miRNA concentration in plasma or other bodily fluid, miRNA with high positive correlation (Spearman's rank correlation coefficient r calculated for all samples in compared groups is >0.8) are selected as a numerator and a denominator for the biomarker pair. This step significantly decreases the number of potential biomarker miRNA pairs, reduces variance of selected biomarkers caused by factors unrelated to processes differentiating two comparative cohorts and significantly increases test sensitivity and specificity.

The order of steps 3-4 and step 5 can be switched as follows:

After step 1, calculate Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNA measured in step 1 in all bodily fluid samples. Then select as potential biomarker pairs of miRNA with a high positive correlation (r>0.8), compare a ratio of miRNA concentrations in two subject cohorts for each selected miRNA pair and determine a miRNA pair as a suitable biomarker if this pair differentiates two subject cohorts with a statistically significant P-value.

Selection of miRNAs for biomarker pairs is an important step in developing screening, diagnostic and monitoring tests based on analysis of cell-free circulating miRNAs in bodily fluids. The present invention addresses this issue by providing the following methods for selection of effective biomarker pairs.

In one embodiment, the invention provides a method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) calculating the mean level of each miRNA measured in step (b);
calculating the difference between the mean miRNA levels calculated in step (c);
(e) comparing the differences between the mean miRNA levels calculated in step (d)
between all studied miRNAs and selecting as potential biomarker pairs those miRNA pairs for
which the difference calculated in step (d) for one miRNA is at least 1.5 times the difference
calculated for the other miRNA;
(f) calculating Spearman's rank correlation coefficient (r) for each potential biomarker
miRNA pair selected in step (e), and
(g) identifying the miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring
of said pathology if its (r) value calculated in step (f) is at least 0.8.

In another embodiment, the invention provides a method for selecting a biomarker
miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the
following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the
pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two
subject cohorts;
(c) calculating Spearman's rank correlation coefficient (r) for all possible pairs of individual
miRNAs measured in step (b);
(d) selecting as potential biomarker pairs those miRNA pairs which have the (r) value
calculated in step (c) of at least 0.8;
(e) calculating the mean level of each miRNA selected in step (d);
(f) calculating the difference between the mean miRNA levels calculated in step (e);
(g) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of
said pathology if the difference calculated in step (f) for one miRNA is at least 1.5 times the
difference calculated for the other miRNA.

In a further embodiment, the invention provides a method for selecting a biomarker
miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the
following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the
pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) calculating Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNAs measured in step (b);
(d) selecting as potential biomarker pairs those miRNA pairs which have the (r) value calculated in step (c) of at least 0.8;
(e) calculating P-value of two subject cohorts separation for each miRNA pair selected in step (d), and
(f) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this pair differentiates two subject cohorts with a statistically significant P-value.

In another embodiment, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the mean level of each miRNA measured in step (b);
(d) electronically calculating a difference between the mean miRNA levels calculated in step (c);
(e) selecting from the group of measured miRNAs a set of potential miRNA pairs each comprising a first miRNA and a second miRNA, wherein the calculated difference in the mean level in step (d) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA;
(f) electronically calculating the Spearman's rank correlation coefficient (r) for each potential miRNA pair selected in (e);
(g) selecting from the set of potential miRNA pairs those miRNA pairs, which are suitable for the diagnosis and/or monitoring of the pathology, wherein the (r) value calculated in step (f) is at least 0.8, and
(h) displaying all or part of the miRNA pairs selected in step (g).
In yet another embodiment, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNAs measured in step (b);
(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the (r) value calculated in step (c) is at least 0.8;
(e) electronically calculating the mean level of each miRNA selected in step (d);
(f) electronically calculating the difference between the mean miRNA levels calculated in step (e);
(g) selecting from the group of measured miRNAs a set of suitable miRNA biomarker pairs each comprising a first miRNA and a second miRNA, wherein for each suitable biomarker miRNA pair, the calculated difference in the mean level in step (f) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA, and
(h) displaying all or part of the suitable biomarker miRNA pairs selected in step (g).

In a further embodiment, the invention provides a computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the Spearman's rank correlation coefficient (r) of the levels measured in step (b) for all possible pairs of individual miRNAs;
(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the (r) value calculated in step (c) is at least 0.8;
(e) electronically calculating P-value of two subject cohorts separation for each miRNA pair selected in step (d);

(f) selecting a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this miRNA pair differentiates two subject cohorts with a statistically significant P-value, and

(g) displaying all or part of the suitable biomarker miRNA pairs selected in step (f).

Non-limiting examples of the methods which can be used to measure miRNA level in any of the above methods of the invention include, e.g., RT-PCR-based methods, miRNA array-based methods, new generation sequencing, and hybridization.

Non-limiting examples of the bodily fluid samples which can be used in any of the above methods of the invention include, e.g., plasma, serum, cerebrospinal fluid (CSF), urine, and saliva.

In any of the above methods of the invention, the subjects can be, e.g., humans or experimental animals.

In any of the above methods of the invention, any two cohorts can be compared. Non-limiting examples of such cohorts include, e.g., pathology versus control [e.g., age, gender and ethnicity-matched healthy subjects], one pathology of the organ versus another pathology of the same organ, two age groups, [e.g., 20-50 years old versus 60-80 years old], males versus females [e.g., age and ethnicity-matched], two different ethnic or racial groups [e.g., age and gender-matched], etc.).

Spearman's correlation algorithm used in the methods of the invention:

\[
    r = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_i (x_i - \bar{x})^2 \sum_i (y_i - \bar{y})^2}}, \text{ wherein}
\]

\(x_i\) is values of numerator miRNA;

\(y_i\) is values of denominator miRNA;

\(\bar{x}\) is mean of numerator values, and

\(\bar{y}\) is mean of denominator values.
A minimal number of samples sufficient for obtaining a statistically significant difference between two cohorts in the above methods of the invention can be calculated by a standard formula for case-control study (see, e.g. Eng J. Radiology 2003, 227:309-313).

In the methods of the invention, a statistically significant P-value can be calculated using any method known in the art. Non-limiting examples of such methods are Student's t-test (for samples with normal distribution) and Mann-Whitney test (for samples with non-random distribution) (Mann and Whitney, Annals Math Stat. 1947, 18: 50-60). P-value >0.05 is usually accepted as statistically significant. If numerous potential biomarkers are tested Bonferroni correction can be applied.

**Diagnostic, Monitoring and Screening Methods of the Invention**

The present invention provides novel highly sensitive and noninvasive or minimally invasive methods for diagnosing and monitoring Parkinson's Disease (PD) in a subject, said methods comprising determining the ratio of the levels in a bodily fluid sample from the subject (e.g., blood plasma, serum, urine, cerebrospinal fluid (CSF), saliva) of two or more specific miRNAs.

The use of specific miRNA pairs in the diagnostic methods of the invention allows for very high sensitivity and reliability and make possible early diagnosis of PD and distinguishing it from other neurodegenerative diseases and conditions (e.g., Mild Cognitive Impairment (MCI) and Alzheimer's disease (AD)). This approach also provides detailed and comprehensive information for monitoring PD 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.

Non-limiting examples of miRNA pairs useful in the methods of the present invention include, e.g.:

**miRNA Pairs for detecting PD**

**Brain-enriched:**

let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-323-3p, miR-107/miR-323-3p, miR-127/miR-323-3p, miR-181b/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-411, miR-107/miR-411, miR-491-5p/miR-411.
Inflammatory:
miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-146a, miR-155/miR-16, miR-155/miR-132, miR-155/miR-323-3p, miR-155/miR-41 1, miR-491-5p/miR-146b, miR-155/miR-146a, miR-155/miR-146b.

miRNA Pairs for differentiating PD and MCI

Brain-enriched:
let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-335, miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-134, miR-181b/miR-132, let-7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-491-5p/miR-132, miR-107/miR-323-3p, miR-127/miR-134, miR-491-5p/miR-874, miR-491-5p/miR-323-3p, miR-127/miR-432, let-7e/miR-134, let-7e/miR-41 1, miR-107/miR-41 1, miR-491-5p/miR-41 1, miR-107/miR-874, miR-181a/miR-9*, miR-491-5p/miR-2 10, miR-181b/miR-335, miR-99b/miR-335, miR-107/miR-210, miR-127/487b, miR-181a/miR-874, miR-9/miR-9*, miR-107/miR-487b, miR-107/miR-432, miR-9/miR-335, miR-181a/miR-132, miR-181b/miR-210, miR-99b/miR-132.

Inflammatory:
let-7e/miR-146a, miR-107/miR-146a, miR-491-5p/miR-146a, miR-107/miR-146b, miR-491-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, miR-155/miR-41 1, miR-744/miR-146a.

miRNA Pairs for differentiating PD and AD

Brain-enriched:
let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-41 1, miR-99b/miR-335, miR-491-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, let-7e/miR-9*, miR-491-5p/miR-4 11, let-7e/miR-132, miR-181b/miR-132, miR-9/miR-9*, miR-9/miR-134, miR-107/miR-134, miR-181b/miR-874, let-7e/miR-134, miR-107/miR-132, miR-107/miR-9*, miR-127/miR-335, miR-9/miR-132, miR-181b/miR-9*, miR-491-5p/miR-132, let-7e/miR-210, miR-491-5p/miR-9*, miR-107/miR-323-3p, miR-491-5p/miR-323-3p, miR-491-5p/miR-9*, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-210, miR-9/miR-874, miR-9/miR-485-3p, miR-744/miR-134, miR-181b/miR-323-3p.

Inflammatory:
miR-107/miR-146a, miR-491-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, let-7e/miR-146b, miR-9/miR-146b, let-7e/miR-146a, miR-155/miR-874, miR-155/miR-9*, miR-744/miR-146a, miR-155/miR-210, miR-155/miR-146b, miR-744/miR-146b, miR-155/miR-146a, miR-155/miR-134, miR-155/miR-411, miR-181b/miR-146b.

In one aspect, the invention provides a method for detecting a first neurodegenerative disease in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the first neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the first neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the first neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the first neurodegenerative disease;

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 control ratio, and

e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In a related aspect, the invention provides a method for detecting a first neurodegenerative disease in a subject, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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 control ratio, and

e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In one embodiment of the above two methods, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (d)-(e) of the above two methods:

f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease, and

g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of the above two methods, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (a)-(e) of the above two methods:

f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease;

g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is (i) enriched in a brain
area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;

h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);

i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;

j) (i) identifying the subject as being afflicted with the second neurodegenerative disease in addition to the first neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of any of the above methods, said first neurodegenerative disease is Parkinson's disease (PD).

In one embodiment of the above disease differentiation methods, said first neurodegenerative disease is Parkinson's disease (PD) and said second neurodegenerative disease is selected from the group consisting of Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, and olivopontocerebellar atrophy (OPCA).

In one aspect, the invention provides a method for detecting Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is neuronal miRNA enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA is (i) enriched in a brain area(s) which is not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;

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

d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and

e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In a related aspect, the invention provides a method for detecting Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and

e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

In one embodiment of the above two methods for detecting PD, the method further comprises refining the diagnosis by the following steps, which steps can be performed
simultaneously or sequentially with each other and with the steps (d)-(e) of the above two methods for detecting PD:

f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease different from PD;

g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of the above two methods for detecting PD, the method further comprises refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and with the steps (a)-(e) of the above two methods for detecting PD:

f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease and is a pre-identified numerator in biomarker miRNA pair for said second neurodegenerative disease;

g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is and is a pre-identified denominator in biomarker miRNA pair for said second neurodegenerative disease and is (i) enriched in a brain area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease and is different from the cell type where the third miRNA is enriched, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;

h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);

i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;
identifying the subject as being afflicted with the second neurodegenerative disease in addition to PD if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

In one embodiment of any of the above methods of disease detection, the control ratio is a predetermined value which represents a statistically validated threshold ratio of the levels of said first and second miRNAs (a single "cut-off value) equal to the highest possible value within the range of corresponding values in age-matched (e.g., ± 2.5 years) healthy subjects. Such statistically validated threshold ("cut-off value) may be established by assaying a large cohort of healthy individuals in the select age-matched population and using a suitable statistical model (see, e.g., Knapp, R. G., and Miller, M.E. (1992) Clinical Epidemiology and Biostatistics, William and Wilkins, Harual Publishing Co. Malvern, Pa.). In another embodiment of any of the above methods of disease detection, the control ratio is the ratio of the levels of said first and second miRNAs in a similarly processed bodily fluid sample from the same subject collected in the past.

Since pathological processes characteristic of various neurodegenerative diseases are often observed in the brain of the same patient (Hu WT et al. Acta Neuropathol. 2010; 120: 385-399; Farlow MR et al. Dement. Geriatr. Cogn. Disord. Extra 2013; 3: 281-290; Stern RA et al. 2011; 3: S460-S467; Costanza A. et al. Neuropathol. 2011; 37: 570-584), the present invention also provides methods for differential diagnosis, which can involve the use of two or more different biomarker pairs and exclude the presence of an additional pathology or demonstrates simultaneous presence of such additional pathology (i.e., mixed pathology).

In the diagnostic methods of the invention, in order to further refine the diagnosis and differentiate between PD and other neurodegenerative diseases (such as, e.g., Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), Chronic Traumatic Encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, and olivopontocerebellar atrophy (OPCA)) the ratio of the levels of the miRNAs
within the biomarker pair in the subject's sample is being compared to the standard range of ratios of the levels of the same miRNAs found in subjects (preferably age-matched, e.g., ± 2.5 years) having PD or a different neurodegenerative disease. Such "refining" comparison can be performed either after the initial diagnosis (i.e., comparison with a healthy control threshold) or simultaneously with such initial diagnosis. Such "refining" comparison can be performed simultaneously for several different diseases or sequentially. The disease-specific standard range of ratios used in the "refining" comparison is usually a statistically validated predetermined range of values which may be established by assaying a large cohort of individuals diagnosed with a specific neurodegenerative disease in the select population (preferably age-matched, e.g., ± 2.5 years) and using a statistical model. A further "refining" to determine not only the specific disease but the extent of progression of such disease can be performed by using standard disease stage-specific ratio ranges for comparison (said standard ranges being established by assaying a large cohort of individuals diagnosed with a specific stage of a specific neurodegenerative disease).

In another aspect, the invention provides a method for monitoring changes in development of a neurodegenerative disease in a subject (e.g., a subject who had been previously diagnosed with said neurodegenerative disease), which method comprises:

a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluids samples as in step (a), wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
(i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a related aspect, the invention provides a method for monitoring changes in development of a neurodegenerative disease in a subject (e.g., a subject who had been previously diagnosed with said neurodegenerative disease), which method comprises:

a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluids samples as in step (a), wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a related aspect, the invention provides a method for monitoring changes in development of Parkinson's disease (PD) in a subject (e.g., a subject who had been previously diagnosed with PD), which method comprises:
a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first brain-enriched miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid samples as in step (a), wherein said second brain-enriched miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first brain-enriched miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first brain-enriched miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In another related aspect, the invention provides a method for monitoring changes in development of Parkinson’s disease (PD) in a subject (e.g., a subject who had been previously diagnosed with PD), which method comprises:
a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid samples as in step (a), wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

In a separate aspect, the invention provides a method for monitoring the effect of a treatment on development of a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;

g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the treatment is effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s)
calculated in step (f), or (ii) determining that the treatment is not effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In a related aspect, the invention provides a method for monitoring the effect of a treatment on development of a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;

g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the treatment is effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the treatment is not effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In another related aspect, the invention provides a method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and
h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In yet another related aspect, the invention provides a method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first...
miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and
h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

In a separate aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In a related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In another related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

In yet another related aspect, the invention provides a method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

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 following administration of the test compound;

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

h) (i) identifying that the test compound is useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).
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) 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, cerebrospinal fluid (CSF), 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]), 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), or various microfluidic technologies. 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. One of the preferred types of techniques are RT-PCR-based techniques as such techniques allow to achieve good sensitivity and specificity.

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 quality control (QC) and normalization methods can be used in the present invention:

a) Ubiquitous miRNAs can be used for QC by comparing their concentrations in subject’s plasma with pre-established normal values.

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.

**Kits of the Invention**

In conjunction with the above diagnostic, monitoring and screening methods, the present invention provides various kits comprising one or more primer and/or probe sets specific for the detection of the diagnostic miRNA pairs.

Non-limiting examples of the kits of the invention include:

1. A kit for detecting PD comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-323-3p, miR-107/miR-323-3p, miR-127/miR-323-3p, miR-181b/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-411, miR-107/miR41 1, miR-491-5p/miR-41 1; miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-146a, miR-155/miR-16, miR-155/miR-132, miR-155/miR-323-3p, miR-155/miR-41 1, miR-491-5p/miR-146b, miR-155/miR-146a, and miR-155/miR-146b.

2. A kit for differentiating PD from MCI comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-335, miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-134, miR-181b/miR-132, let-
7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-49l-5p/miR-134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-49l-5p/miR-132, miR-107/miR-323-3p, miR-127/miR-134, miR-49l-5p/miR-874, miR-49l-5p/miR-323-3p, miR-127/miR-432, let-7e/miR-134, let-7e/miR-411, miR-49l-5p/miR-411, miR-107/miR-874, miR-181a/miR-9*, miR-49l-5p/miR-210, miR-127/487b, miR-181a/miR-874, miR-9/miR-9*, miR-107/miR-487b, miR-107/miR-432, miR-9/miR-335, miR-181a/miR-132, miR-181b/miR-210, and miR-99b/miR-132; let-7e/miR-146a, miR-107/miR-146a, miR-49l-5p/miR-146a, miR-107/miR-146b, miR-49l-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, miR-155/miR-411, and miR-744/miR-146a.

3. A kit for differentiating PD from AD comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of: let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-411, miR-99b/miR-335, miR-49l-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-411, miR-181a/miR-335, let-7e/miR-9*, miR-49l-5p/miR-411, let-7e/miR-132, miR-181b/miR-132, miR-9/miR-9*, miR-9/miR-134, miR-107/miR-134, miR-181b/miR-874, let-7e/miR-134, miR-107/miR-9*, miR-127/miR-335, miR-9/miR-132, miR-181b/miR-9*, miR-49l-5p/miR-132, let-7e/miR-210, miR-49l-5p/miR-9*, miR-107/miR-323-3p, miR-49l-5p/miR-127, miR-49l-5p/miR-134, let-7e/miR-874, miR-181b/miR-210, miR-9/miR-874, miR-9/miR-485-3p, miR-744/miR-134, miR-181b/miR-323-3p; miR-107/miR-146a, miR-49l-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-49l-5p/miR-146b, miR-9/miR-146a, let-7e/miR-146b, miR-9/miR-146b, let-7e/miR-146a, miR-155/miR-874, miR-155/miR-9*, miR-155/miR-411, miR-744/miR-146a, miR-155/miR-210, miR-155/miR-146b, miR-744/miR-146b, miR-155/miR-146a, miR-155/miR-134, and miR-181b/miR-146b.

4. A kit comprising primers and/or probes specific for one or more combinations of pairs of miRNAs selected from the group consisting of:

(a) miR-181b/miR-323-3p and miR-99b/miR-9*,

(b) miR-49l-5p/miR-487b and miR-9/miR-146a, and

(c) miR-49l-5p/miR-210 and miR-181a/miR-146b.
Such kits can further include primer and/or probe sets specific for the detection of QC and additional normalizer miRNAs.

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 troubleshooting 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.

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: Selection of miRNAs for testing

The methods of the instant invention are based on the use of miRNAs enriched in different brain areas as numerators and denominators, which significantly improves test sensitivity and specificity. Table 1 below presents lists of brain-enriched miRNAs, miRNAs enriched in synapses, axons, dendrites and spines ("synapse and/or neurite miRNAs") and miRNAs enriched in different brain areas.

Table 1. miRNAs enriched in brain, different brain areas and neuronal compartments

<table>
<thead>
<tr>
<th>Brain areas and compartments</th>
<th>Enriched miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole brain</td>
<td>Let-7a,c,e, 7, 9, 96, 98, 99a,b, 103, 105, 106a, 107, 124a, 125a, 125b, 127, 128a, 129, 132, 134, 135a, 137, 138, 139, 149, 151, 153, 154, 181a, 181b, 181c, 182, 183, 184, 190, 204, 211, 212, 213, 218, 219-3p, 219-5p, 221,</td>
</tr>
<tr>
<td>Brain areas and compartments</td>
<td>Enriched miRNAs</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Cortex</td>
<td>9, 107, 124a, 125a, 125b, 128a, 132, 134, 154, 181b, 212, 213, 222, 323, 330-3p, 338-5p, 342, 381, 382, 425, 433, 491-5p, 885</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>7, 124a, 125a, 128a, 132, 136, 138, 212, 338, 451</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>9, 103, 124a, 125b, 128, 132, 134, 137, 138, 181a, 181b, 181c, 204, 212, 213, 218, 338, 381, 382, 425, 432, 489, 592, 874, 885</td>
</tr>
<tr>
<td>Amygdala</td>
<td>103, 134, 138, 182, 183, 222, 323-3p, 369, 381, 382</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>218, 219, 338, 451, 486</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>7, 96, 132, 99a,b, 154, 182, 212, 213, 328, 329, 335, 369, 381, 411, 432, 433, 487b</td>
</tr>
<tr>
<td>Midbrain, Substantia nigra</td>
<td>Let-7a,b,c,d,e, 9, 99a,b, 125a,b, 127-3p, 129-3p, 134, 149, 181a, 204, 329, 338, 340, 379, 383, 410, 425, 432, 433, 487a,b, 744, 9*, 99b*, 129*, 340*</td>
</tr>
</tbody>
</table>

Since neuroinflammatory process are involved in PD pathogenesis, several miRNAs associated with inflammatory process were also tested (miR-146a,b, miR-155 and miR-31). miR-210 associated with hypoxia in combination with brain-enriched miRNAs was also analyzed, and muscle-enriched miR-206 was tested to check if movement disorders characteristic of PD affect its concentration in plasma. Finally, ubiquitous miR-16 and miR-196a, which is not practically expressed in the brain, was tested as potential denominator.

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. 2006; 439: 283-289; Lugli et al. J. Neurochem. 2008; 106: 650-661; Bicker and Schratt.

Table 2. miRNAs used in the study

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Brain enrichment</th>
<th>Enriched in</th>
<th>Present in synapses</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7c</td>
<td>+</td>
<td>MB, PG, Cer</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-7</td>
<td>+</td>
<td>PG</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-9</td>
<td>+</td>
<td>FC, MB, Hip, Cer</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-9*</td>
<td>+</td>
<td>MB</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-16</td>
<td></td>
<td></td>
<td>Ubiquitous</td>
<td></td>
</tr>
<tr>
<td>miR-31</td>
<td></td>
<td></td>
<td>Inflammatory</td>
<td></td>
</tr>
<tr>
<td>miR-99b</td>
<td>+</td>
<td>MB</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-107</td>
<td>+</td>
<td>FC</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-127-3p</td>
<td>+</td>
<td>PG, MB, FC</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-132</td>
<td>+</td>
<td>PG, Hip</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-134</td>
<td>+</td>
<td>MB, Hip, PG</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-138</td>
<td>+</td>
<td>Hip, FC</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-146a</td>
<td></td>
<td></td>
<td>Inflammatory</td>
<td></td>
</tr>
<tr>
<td>miR-146b</td>
<td></td>
<td></td>
<td>Inflammatory</td>
<td></td>
</tr>
<tr>
<td>miR-155</td>
<td></td>
<td></td>
<td>Inflammatory</td>
<td></td>
</tr>
<tr>
<td>miR-181a</td>
<td>+</td>
<td>MB, FC, Hip</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-181b</td>
<td>+</td>
<td>FC, Hip</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>miR-196</td>
<td></td>
<td></td>
<td>Low in brain</td>
<td></td>
</tr>
<tr>
<td>miR-206</td>
<td></td>
<td></td>
<td>Muscle-enriched</td>
<td></td>
</tr>
<tr>
<td>miR-210</td>
<td></td>
<td></td>
<td>Hypoxia-activated</td>
<td></td>
</tr>
<tr>
<td>miR-323-3p</td>
<td>+</td>
<td>FC, MB</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>
Example 2: Quantitative Analysis

Mann-Whitney U-test was used to evaluate significance of differentiation of any two patient groups by various biomarker miRNA pairs. Bonferroni correction was applied for estimating significant P-values. In all experiments (differentiation of PD from age-matched control [AMC], MCI and AD) 32 miRNAs were tested, thus P-value<0.0001 (calculated as 0.05/496; 496 here indicates the total number of miRNA pairs examined) was considered significant. A standard formula for a case-control study (Eng J. Radiology. 2003; 227:309-313) was applied for estimating the sample size required to produce statistical power 0.90.

Example 3: Differentiation of PD patients from age-matched controls (AMC)

Plasma samples were obtained from 20 PD patients and 20 AMC (± 2.5 years). Concentrations of brain-enriched miRNAs, inflammation-associated miRNAs, muscle-enriched miR-206 and several ubiquitous miRNAs in plasma were analyzed using RT-qPCR with primers and probes for each individual miRNA (Life Technologies). The amount of RNA equivalent to 25 µL of plasma were taken in each RT reaction, and the amount of miRNA (cDNA) equivalent to 2 µL plasma was taken into final PCR. The results obtained for each miRNA were normalized per potential normalizer miRNA, converted into Relative Concentration (RC) of miRNA according to the ABI protocol ($2^{-ΔCt}$), and compared with miRNA profiles from age-matched controls (AMC). The biomarker pairs were selected as described above. Both approaches gave similar results. Correlation of plasma concentrations, P-values for differentiation of PD patients
from AMC and AUC (Area under ROC curve) for best miRNA pairs are presented in Table 3 and in Figure 1.

**Table 3. Biomarker miRNA pairs for differentiating PD patients from AMC**

<table>
<thead>
<tr>
<th>Marker</th>
<th>R</th>
<th>Marker</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7e / miR-335</td>
<td>0.95</td>
<td>let-7e / miR-9*</td>
<td>0.97</td>
</tr>
<tr>
<td>miR-107 / miR-335</td>
<td>0.91</td>
<td>miR-491-5p / miR-9*</td>
<td>0.95</td>
</tr>
<tr>
<td>miR-491-5p / miR-335</td>
<td>0.93</td>
<td>Let-7e / miR-323-3p</td>
<td>0.85</td>
</tr>
<tr>
<td>miR-744 / miR-335</td>
<td>0.98</td>
<td>miR-107 / miR-323-3p</td>
<td>0.84</td>
</tr>
<tr>
<td>miR-99b / miR-335</td>
<td>0.91</td>
<td>miR-127 / miR-323-3p</td>
<td>0.84</td>
</tr>
<tr>
<td>miR-155 / miR-335</td>
<td>0.93</td>
<td>miR-181b / miR-323-3p</td>
<td>0.89</td>
</tr>
<tr>
<td>let-7e / miR-411</td>
<td>0.89</td>
<td>miR-99b / miR-323-3p</td>
<td>0.90</td>
</tr>
<tr>
<td>miR-107 / miR-411</td>
<td>0.90</td>
<td>miR-155 / miR-323-3p</td>
<td>0.83</td>
</tr>
<tr>
<td>miR-491-5p / miR-411</td>
<td>0.89</td>
<td>miR-491-5p / miR-323-3p</td>
<td>0.84</td>
</tr>
<tr>
<td>miR-155 / miR-411</td>
<td>0.93</td>
<td>let-7e / miR-146a</td>
<td>0.97</td>
</tr>
<tr>
<td>let-7e / miR-132</td>
<td>0.94</td>
<td>let-7e / miR-146b</td>
<td>0.96</td>
</tr>
<tr>
<td>miR-107 / miR-132</td>
<td>0.95</td>
<td>miR-491-5p / miR-146a</td>
<td>0.97</td>
</tr>
<tr>
<td>miR-491-5p / miR-132</td>
<td>0.94</td>
<td>miR-491-5p / miR-146b</td>
<td>0.97</td>
</tr>
<tr>
<td>miR-155 / miR-132</td>
<td>0.92</td>
<td>miR-744 / miR-146a</td>
<td>0.98</td>
</tr>
<tr>
<td>Let-7e / miR-134</td>
<td>0.92</td>
<td>miR-155 / miR-146a</td>
<td>0.96</td>
</tr>
<tr>
<td>miR-107 / miR-134</td>
<td>0.94</td>
<td>miR-155 / miR-146b</td>
<td>0.97</td>
</tr>
<tr>
<td>miR-491-5p / miR-134</td>
<td>0.91</td>
<td>miR-155 / miR-16</td>
<td>0.95</td>
</tr>
</tbody>
</table>

R - Correlation coefficient for numerator and denominator miRNAs; Table shows miRNA pairs with PO.0001.

Conclusions:

1. miRNAs enriched in midbrain (e.g., let-7e) and frontal cortex (e.g., miR-107), the brain areas suffering in PD, and present in neurites and synapses are the best numerators in miRNA pairs, demonstrating the increase in miRNA ratio from PD patients compared to AMC. Although the inventors could not identify in the literature data on enrichment of miR-491-5p in particular brain areas but it behaves as a very good numerator in miRNA pairs, distinguishing PD and MCI subjects.

2. Brain-enriched miRNAs from brain areas not involved in PD or significantly less damaged are among the best denominators in miRNA pairs capable of differentiating PD and AMC (e.g., miR-132 and miR-335-5p, enriched in hippocampus).
3. Inflammation-associated miRNAs are divided into two groups. miR-155 and to a lesser degree miR-31 are good numerators and miR-146a and miR-146b are good denominators, which indicates that they play a different role in PD development or are located in different cell types.

4. Muscle-enriched miR-206 is not a good marker for PD detection.

**Example 4: Differentiation between PD and MCI patients**

The scheme of experiments was the same as described in the Example 2 above but PD patients were compared with MCI patients to find out biomarker miRNA pairs capable of differentiating PD MCI, which is a heterogeneous syndrome, characteristic of early stages of AD, irontotemporal dementia, vascular dementia, some cases of PD and other neurodegenerative diseases. Data on ability of various miRNA pairs to differentiate PD and MCI patients are presented in Figure 2 and Table 4. Presented results demonstrate that principally the same miRNA pairs that distinguish PD from AMC differentiate PD from MCI.

**Table 4. Biomarker miRNA pairs for differentiating PD patients and MCI patients**

<table>
<thead>
<tr>
<th>Marker</th>
<th>R</th>
<th>Marker</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7c / miR-146a</td>
<td>0.97</td>
<td>miR-491-5p / miR-9*</td>
<td>0.94</td>
</tr>
<tr>
<td>let-7c / miR-335</td>
<td>0.95</td>
<td>miR-107 / miR-323-</td>
<td>0.84</td>
</tr>
<tr>
<td>let-7c / miR-9*</td>
<td>0.97</td>
<td>miR-127 / miR-134</td>
<td>0.96</td>
</tr>
<tr>
<td>miR-107 / miR-</td>
<td>0.99</td>
<td>miR-155 / miR-335</td>
<td>0.93</td>
</tr>
<tr>
<td>miR-107 / miR-335</td>
<td>0.91</td>
<td>miR-491-5p / miR-323-</td>
<td>0.85</td>
</tr>
<tr>
<td>miR-127 / miR-</td>
<td>0.95</td>
<td>miR-491-5p / miR-9*</td>
<td>0.84</td>
</tr>
<tr>
<td>miR-491-5p / miR-</td>
<td>0.97</td>
<td>miR-127 / miR-432</td>
<td>0.99</td>
</tr>
<tr>
<td>miR-491-5p / miR-9*</td>
<td>0.97</td>
<td>let-7c / miR-134</td>
<td>0.92</td>
</tr>
<tr>
<td>miR-491-5p / miR-9*</td>
<td>0.97</td>
<td>miR-107 / miR-874</td>
<td>0.81</td>
</tr>
<tr>
<td>miR-107 / miR-</td>
<td>0.97</td>
<td>miR-181a / miR-9*</td>
<td>0.89</td>
</tr>
<tr>
<td>miR-107 / miR-9*</td>
<td>0.97</td>
<td>miR-491-5p / miR-874</td>
<td>0.95</td>
</tr>
<tr>
<td>miR-744 / miR-335</td>
<td>0.93</td>
<td>miR-181b / miR-335</td>
<td>0.89</td>
</tr>
<tr>
<td>miR-491-5p / miR-9*</td>
<td>0.97</td>
<td>miR-99b / miR-335</td>
<td>0.91</td>
</tr>
<tr>
<td>miR-155 / miR-874</td>
<td>0.88</td>
<td>miR-107 / miR-210</td>
<td>0.93</td>
</tr>
<tr>
<td>let-7c / miR-132</td>
<td>0.94</td>
<td>miR-127 / miR-487b</td>
<td>0.94</td>
</tr>
<tr>
<td>miR-107 / miR-134</td>
<td>0.94</td>
<td>miR-181a / miR-874</td>
<td>0.87</td>
</tr>
<tr>
<td>miR-181b / miR-</td>
<td>0.93</td>
<td>miR-744 / miR-146a</td>
<td>0.98</td>
</tr>
<tr>
<td>let-7c / miR-210</td>
<td>0.94</td>
<td>miR-9 / miR-9*</td>
<td>0.98</td>
</tr>
<tr>
<td>Let-7c / miR-411</td>
<td>0.89</td>
<td>miR-155 / miR-210</td>
<td>0.94</td>
</tr>
<tr>
<td>miR-107 /miR-411</td>
<td>0.90</td>
<td>miR-99b / miR-9*</td>
<td>0.97</td>
</tr>
<tr>
<td>miR-491-5p / miR-</td>
<td>0.89</td>
<td>miR-107 / miR-487b</td>
<td>0.92</td>
</tr>
</tbody>
</table>
R - Correlation coefficient for numerator and denominator miRNAs; Table shows miRNA pairs with PO.0001.

Example 5: Differentiation between PD and AD patients

Again the scheme of experiments was the same as described in the Example 2 above but PD patients were compared with AD patients to find out biomarker miRNA pairs capable of differentiating these two neurodegenerative diseases. Data on ability of different miRNA pairs to differentiate PD and AD patients are presented in Figure 3 and Table 5. Presented results again demonstrate that principally the same miRNA pairs that distinguish PD from AMC and MCI differentiate PD from AD. Interestingly, in the two latter cases (differentiation of PD from AD and MCI) miR-210 associated with hypoxia behaves as a good denominator.

Table 5. Biomarker miRNA pairs for differentiating PD patients and AD patients

<table>
<thead>
<tr>
<th>Marker</th>
<th>R</th>
<th>Marker</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7e / miR-874</td>
<td>0.85</td>
<td>miR-107 / miR-432</td>
<td>0.91</td>
</tr>
<tr>
<td>miR-181b / miPv-</td>
<td>0.87</td>
<td>miR-9 / miR-335</td>
<td>0.94</td>
</tr>
<tr>
<td>let-7e / miR-146b</td>
<td>0.97</td>
<td>miR-181a / miR-132</td>
<td>0.89</td>
</tr>
<tr>
<td>miR-107 / miR-132</td>
<td>0.95</td>
<td>miR-181b / miR-210</td>
<td>0.91</td>
</tr>
<tr>
<td>miR-155 / miR-41</td>
<td>0.93</td>
<td>miR-99b / miR-132</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 5. Biomarker miRNA pairs for differentiating PD patients and AD patients
<table>
<thead>
<tr>
<th>Marker</th>
<th>R</th>
<th>Marker</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-9 / miR-146b</td>
<td>0.97</td>
<td>let-7e / miR-874</td>
<td>0.85</td>
</tr>
<tr>
<td>let-7e / miR-146a</td>
<td>0.97</td>
<td>miR-181b /miR-210</td>
<td>0.91</td>
</tr>
<tr>
<td>miR-155 /miR-874</td>
<td>0.88</td>
<td>miR-9 / miR-874</td>
<td>0.85</td>
</tr>
<tr>
<td>miR-155 / miR-9*</td>
<td>0.97</td>
<td>miR-155 / miR-16</td>
<td>0.92</td>
</tr>
<tr>
<td>miR-744 /miR-146a</td>
<td>0.94</td>
<td>miR-155 / miR-134</td>
<td>0.88</td>
</tr>
<tr>
<td>let-7e / miR-9*</td>
<td>0.97</td>
<td>miR-9 /miR-485-3p</td>
<td>0.89</td>
</tr>
<tr>
<td>miR491-5p / miR-41</td>
<td>0.89</td>
<td>miR744 / miR-134</td>
<td>0.92</td>
</tr>
<tr>
<td>miR-155 /miR-210</td>
<td>0.94</td>
<td>miR-181b /miR-323-3p</td>
<td>0.82</td>
</tr>
<tr>
<td>let-7e / miR-132</td>
<td>0.86</td>
<td>miR-181b /miR-146b</td>
<td>0.94</td>
</tr>
<tr>
<td>miR-181b / miR-132</td>
<td>0.93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R - Correlation coefficient for numerator and denominator miRNAs; Table shows miRNA pairs with AU0.9 and P<0.0001.

**Summary**

Sensitivity and specificity of PD detection and differentiation from MCI and AD is very high, reaching for some pairs 100% accuracy:

**PD versus AMC**

let-7e/miR-335; let-7e/miR-41 1; miR-107/miR-146a; miR-107/miR-335; miR-107/miR-41 1; miR-155/miR-335; miR-181b/miR-335; miR-491-5p/miR-335; miR-491-5p/miR-41 1; miR-155/miR-146a

**PD versus MCI**

let-7e/miR-146a; let-7e/miR-335; let-7e/miR-miR-41 1; miR-107/miR-146a; miR-107/miR-335; miR-127/miR-323-3p; miR-491-5p/miR-146a; miR-491-5p/miR-335; miR-491-5p/miR-9*; miR-744/miR-335; miR-491-5p/miR-146b

**PD versus AD**

miR-107/miR-146a; miR-107/miR-335; miR-127/miR-323-3p; let-7e/miR-41 1; miR-99b/miR-335; miR-491-5p/miR-146a; miR-491-5p/miR-335; miR-155/miR-132; miR-155/miR-335; miR-744/miR-335.

It is also important that some biomarker miRNA pairs detecting PD or differentiating it from other neurodegenerative pathologies with lower accuracy being combined also demonstrate high sensitivity and specificity (Figure 4). Table 6 presents typical data demonstrating significance of correlation between numerator and denominator in biomarker miRNA pairs - as higher is correlation as lower is P-value for differentiating two subject cohorts.
Table 6. The importance of correlation between numerator and denominator miRNAs in biomarker miRNA pairs.

<table>
<thead>
<tr>
<th>Compared</th>
<th>Marker</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD – AMC</td>
<td>let-7c/miR-335</td>
<td>0.95</td>
<td>0.00E+00</td>
</tr>
<tr>
<td></td>
<td>let-7c/miR-370</td>
<td>0.74</td>
<td>1.08E-03</td>
</tr>
<tr>
<td></td>
<td>let-7c/miR-451</td>
<td>0.4</td>
<td>2.59E-01</td>
</tr>
<tr>
<td>PD – MCI</td>
<td>miR-155/miR-335</td>
<td>0.93</td>
<td>9.60E-06</td>
</tr>
<tr>
<td></td>
<td>miR-155/miR-370</td>
<td>0.72</td>
<td>1.31E-01</td>
</tr>
<tr>
<td></td>
<td>miR-155/miR-451</td>
<td>0.52</td>
<td>3.08E-01</td>
</tr>
<tr>
<td>PD – AD</td>
<td>miR-107/miR-335</td>
<td>0.91</td>
<td>0.00E+00</td>
</tr>
<tr>
<td></td>
<td>miR-107/miR-16</td>
<td>0.75</td>
<td>1.90E-04</td>
</tr>
<tr>
<td></td>
<td>miR-107/miR-370</td>
<td>0.71</td>
<td>1.30E-02</td>
</tr>
</tbody>
</table>

Compared - compared subject cohorts, R - Correlation coefficient for numerator and denominator miRNAs, P - P-value.

Table 7 presents lists of miRNAs used as numerators and denominators in the best biomarker miRNA pairs (capable of differentiating PD from AMC, MCI and AD with P-value < 2E-04).

Table 7. The list of most common numerator and denominator miRNAs in biomarker miRNA pairs capable of differentiating various subject cohorts

<table>
<thead>
<tr>
<th>Compared cohorts</th>
<th>Numerators</th>
<th>Denominators</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Name</td>
</tr>
<tr>
<td>PD-AMC</td>
<td>miR-107</td>
<td>miR-335</td>
</tr>
<tr>
<td></td>
<td>let-7c</td>
<td>miR-411</td>
</tr>
<tr>
<td></td>
<td>miR-491-5p</td>
<td>miR-132</td>
</tr>
<tr>
<td></td>
<td>miR-155</td>
<td>miR-134</td>
</tr>
<tr>
<td></td>
<td>miR-99b</td>
<td>miR-146a</td>
</tr>
<tr>
<td></td>
<td>miR-127</td>
<td>miR-146b</td>
</tr>
<tr>
<td></td>
<td>miR-744</td>
<td>miR-323-3p</td>
</tr>
<tr>
<td>PD-MCI</td>
<td>let-7c</td>
<td>miR-9*</td>
</tr>
<tr>
<td></td>
<td>miR-107</td>
<td>miR-132</td>
</tr>
<tr>
<td></td>
<td>miR-127</td>
<td>miR-146a</td>
</tr>
<tr>
<td></td>
<td>miR-155</td>
<td>miR-146b</td>
</tr>
<tr>
<td></td>
<td>miR-181a</td>
<td>miR-335</td>
</tr>
<tr>
<td></td>
<td>miR-181b</td>
<td>miR-411</td>
</tr>
<tr>
<td></td>
<td>miR-370</td>
<td>miR-432</td>
</tr>
</tbody>
</table>
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 detecting a first neurodegenerative disease in a subject, which method comprises:
   a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the first neurodegenerative disease;
   b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the first neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the first neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the first neurodegenerative disease;
   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 control ratio, and
   e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

2. A method for detecting a first neurodegenerative disease in a subject, which method comprises:
   a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
   b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first
miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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 control ratio, and

e) (i) identifying the subject as being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNAs calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with the first neurodegenerative disease when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

3. The method of claim 1 or claim 2, further comprising refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (d)-(e) of claim 1 or claim 2:

f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease, and

g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

4. The method of claim 1 or claim 2, further comprising refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and/or with the steps (a)-(e) of claim 1 or claim 2:

f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease;

g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is (i) enriched in a brain
area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;

h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);

i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;

j) (i) identifying the subject as being afflicted with the second neurodegenerative disease in addition to the first neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

5. The method of any one of claims 1-4, wherein said first neurodegenerative disease is Parkinson's disease (PD).

6. The method of claim 3 or claim 4, wherein said first neurodegenerative disease is Parkinson's disease (PD) and said second neurodegenerative disease is selected from the group consisting of Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBGD), Pick's disease, and olivopontocerebellar atrophy (OPCA).

7. A method for detecting Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject, wherein said first brain-enriched miRNA is neuronal miRNA enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA is (i) enriched in a brain area(s) which is not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;

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

d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and

e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.

8. A method for detecting Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) comparing the ratio of the levels of the miRNA calculated in step (c) with a corresponding control ratio, and

e) (i) identifying the subject as being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding control ratio or (ii) identifying the subject as not being afflicted with PD when the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding control ratio.
9. The method of claim 7 or claim 8, further comprising refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and with the steps (d)-(e) of claim 7 or claim 8:
f) comparing the ratio of the levels of the miRNAs calculated in step (c) with the standard range of ratios of said miRNAs characteristic of a second neurodegenerative disease different from PD;
g) (i) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (c) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) not excluding the diagnosis of the second neurodegenerative disease if the ratio of the levels of the miRNAs calculated in step (c) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

10. The method of claim 7 or claim 8, further comprising refining the diagnosis by the following steps, which steps can be performed simultaneously or sequentially with each other and with the steps (a)-(e) of claim 7 or claim 8:
f) measuring the level of a third brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said third brain-enriched miRNA is enriched in a brain area(s) affected by a second neurodegenerative disease and is a pre-identified numerator in biomarker miRNA pair for said second neurodegenerative disease;
g) measuring the level of a fourth brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said fourth brain-enriched miRNA is and is a pre-identified denominator in biomarker miRNA pair for said second neurodegenerative disease and is (i) enriched in a brain area(s) which is not affected by the second neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the second neurodegenerative disease and is different from the cell type where the third miRNA is enriched, or (iii) is enriched in the same brain area as the third miRNA, but its expression and/or secretion change differently than expression and/or secretion of the third miRNA during development of the second neurodegenerative disease;
h) calculating the ratio of the levels of the miRNAs measured in steps (f) and (g);
i) comparing the ratio of the levels of the miRNAs calculated in step (h) with the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease;

j) (i) identifying the subject as being afflicted with the second neurodegenerative disease in addition to PD if the ratio of the levels of the miRNAs calculated in step (h) falls within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease, or (ii) excluding the diagnosis of the second neurodegenerative disease in the subject if the ratio of the levels of the miRNAs calculated in step (h) does not fall within the standard range of ratios of said miRNAs characteristic of the second neurodegenerative disease.

11. The method of any one of claims 3, 4, 9, and 10, wherein the standard range of ratios of miRNAs characteristic of the second neurodegenerative disease is a statistically validated predetermined range of values established by determining the ratios of the same miRNAs in a large cohort of subjects diagnosed with the second neurodegenerative disease.

12. The method of claim 11, wherein the cohort of subjects diagnosed with the second neurodegenerative disease represents a full range of development stages of said second neurodegenerative disease.

13. The method of claim 11, wherein the cohort of subjects diagnosed with the second neurodegenerative disease represents one or more development stages of said second neurodegenerative disease.

14. The method of claim 9 or claim 10, wherein said second neurodegenerative disease is selected from the group consisting of Alzheimer's disease (AD), Mild Cognitive Impairment (MCI), Huntington's disease (HD), prion-caused diseases, frontotemporal dementia (FTD), Lewy body dementia, vascular dementias, Amyotrophic Later Sclerosis (ALS), chronic traumatic encephalopathy (CTE), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), corticobasal degeneration (CBDG), Pick's disease, and olivopontocerebellar atrophy (OPCA).

15. A method for monitoring changes in development of a neurodegenerative disease in a subject, which method comprises:
a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluids samples as in step (a), wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

16. A method for monitoring changes in development of a neurodegenerative disease in a subject, which method comprises:

a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluids samples as in step (a), wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that the neurodegenerative disease in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that the neurodegenerative disease in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

17. The method of claim 15 or claim 16, wherein the subject had been previously diagnosed with said neurodegenerative disease.

18. A method for monitoring changes in development of Parkinson's disease (PD) in a subject, which method comprises:

a) measuring the level of a first brain-enriched miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first brain-enriched miRNA is enriched in midbrain and/or frontal cortex;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid samples as in step (a), wherein said second brain-enriched miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first brain-enriched miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first brain-enriched miRNA during PD development;

c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;

d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and

e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).
19. A method for monitoring changes in development of Parkinson's disease (PD) in a subject, which method comprises:
a) measuring the level of a first miRNA in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid samples as in step (a), wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b) for each bodily fluid sample;
d) comparing the ratios of the levels of the miRNA calculated in step (c) between the earlier collected and later collected bodily fluid sample(s), and
e) (i) determining that PD in the subject has progressed if the ratio of the levels of the miRNA calculated in step (c) is increased in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s), or (ii) determining that PD in the subject has not progressed if the ratio of the levels of the miRNA calculated in step (c) is not changed in the later collected bodily fluid sample(s) as compared to the earlier collected sample(s).

20. The method of claim 18 or claim 19, wherein the subject had been previously diagnosed with PD.

21. A method for monitoring the effect of a treatment on development of a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell
type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain
area as the first miRNA, but its expression and/or secretion change differently than expression
and/or secretion of the first miRNA during development of the neurodegenerative disease;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid
sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid
sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each
bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and
optionally comparing the ratios of the levels of the miRNA calculated in step (f) between
different samples in step (d), and
h) (i) determining that the treatment is effective for said neurodegenerative disease if the
ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s)
calculated in step (f), or (ii) determining that the treatment is not effective for said
neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not
higher than the corresponding ratio(s) calculated in step (f).

22. A method for monitoring the effect of a treatment on development of a neurodegenerative
disease in a subject who had been previously diagnosed with said neurodegenerative disease,
which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject
prior to initiation of the treatment, wherein said first miRNA (i) is enriched in a brain area(s)
affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from
the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first
miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an
inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid
sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and
h) (i) determining that the treatment is effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the treatment is not effective for said neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

23. A method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;
g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).

24. A method for monitoring the effect of a treatment on development of Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample collected from the subject prior to initiation of the treatment, wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;

b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid sample(s) collected from the subject in the course of or following the treatment;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

f) calculating the ratio of the levels of the miRNA measured in steps (d) and (e) for each bodily fluid sample;

g) comparing the ratios of the levels of the miRNA calculated in steps (c) and (f), and optionally comparing the ratios of the levels of the miRNA calculated in step (f) between different samples in step (d), and

h) (i) determining that the PD treatment is effective if the ratio of the levels of the miRNA calculated in step (c) is higher than the corresponding ratio(s) calculated in step (f), or (ii) determining that the PD treatment is not effective if the ratio of the levels of the miRNA calculated in step (c) is not higher than the corresponding ratio(s) calculated in step (f).
25. A method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first brain-enriched miRNA is enriched in a brain area(s) affected by the neurodegenerative disease;

b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second brain-enriched miRNA (i) is enriched in a brain area(s) which is not affected by the neurodegenerative disease, or (ii) is enriched in a brain cell type which is not affected by the neurodegenerative disease, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during development of the neurodegenerative disease;

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

d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;

e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);

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 following administration of the test compound;

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

h) (i) identifying that the test compound is useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).
26. A method for identifying a compound useful for slowing down the progression or treating a neurodegenerative disease in a subject who had been previously diagnosed with said neurodegenerative disease, which method comprises:

a) measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA (i) is enriched in a brain area(s) affected by the first neurodegenerative disease or (ii) is an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA (i) is an inflammation-associated miRNA if the first miRNA is a brain-enriched miRNA or (ii) is a brain-enriched miRNA if the first miRNA is an inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating the neurodegenerative disease if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

27. A method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first brain-enriched miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is enriched in midbrain and/or frontal cortex;
b) measuring the level of a second brain-enriched miRNA in the same bodily fluid sample collected from the subject, wherein said second miRNA is (i) enriched in brain areas which are not affected by PD, or (ii) is enriched in a brain cell type which is not affected by PD, or (iii) is enriched in the same brain area as the first miRNA, but its expression and/or secretion change differently than expression and/or secretion of the first miRNA during PD development;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid sample(s) as in step (d);
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 following administration of the test compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

28. A method for identifying a compound useful for slowing down the progression or treating Parkinson's disease (PD) in a subject who had been previously diagnosed with PD, which method comprises:
a) measuring the level of a first miRNA in a bodily fluid sample, wherein said bodily fluid sample(s) is collected from the subject prior to test compound administration, and wherein said first miRNA is (i) a brain-enriched neuronal miRNA enriched in midbrain and/or frontal cortex or is (ii) an inflammation-associated miRNA;
b) measuring the level of a second miRNA in the same bodily fluid sample collected from
the subject, wherein said second miRNA is (i) an inflammation-associated miRNA if the first
miRNA is a brain-enriched miRNA or is (ii) a brain-enriched miRNA if the first miRNA is an
inflammation-associated miRNA;
c) calculating the ratio of the levels of the miRNA measured in steps (a) and (b);
d) measuring the level of the same first miRNA as in step (a) in one or more bodily fluid
samples collected from the subject following administration of a test compound;
e) measuring the level of the same second miRNA as in step (b) in the same bodily fluid
sample(s) as in step (d);
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 following administration of the test
compound;
g) comparing the ratio of the levels of the miRNA calculated in steps (c) and (f), and
h) (i) identifying that the test compound is useful for slowing down the progression or
treating PD if the ratio of the levels of the miRNA calculated in step (f) is lower than the ratio of
the levels of the miRNA calculated in step (c); (ii) identifying that the test compound is not
useful for slowing down the progression or treating PD if the ratio of the levels of the miRNA
calculated in step (f) is not lower than the ratio of the levels of the miRNAs calculated in step (c).

29. The method of any one of claims 15, 16, 21, 22, 25, and 26, wherein the neurodegenerative
disease is Parkinson's disease (PD).

30. The method of any one of claims 1-3, 7-9, 15, 16, 18, 19, and 21-28, wherein the first brain-
enriched miRNA is a neuronal miRNA.

31. The method of any one of claims 1, 3, 7, 9, 15, 18, 21, 23, 25, and 27, wherein the first brain-
enriched miRNA is enriched in neurons and the second brain-enriched miRNA is enriched in
glial cells.

32. The method of any one of claims 1-3, 7-9, 15, 16, 18, 19, and 21-28, wherein the first brain-
enriched miRNA is synapse and/or neurite miRNA.
33. The method of any one of claims 2, 16, 22, and 26, wherein the second miRNA is a brain-
enriched miRNA, which (1) is enriched in a brain area(s) which is not affected by the first
neurodegenerative disease or (2) is enriched in a brain cell type which is not affected by the first
neurodegenerative disease.

34. The method of any one of claims 1, 3, 7, 9, 15, 18, 21, 23, 25, and 27, wherein the pair of the
first miRNA and the second miRNA is selected from the group consisting of:
let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-
335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-
132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-
323-3p, miR-107/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, let-7e/miR-41 1,
miR-107/miR-4 1 1, and miR-49 1-5p/miR-4 1 1.

35. The method of any one of claims 2, 3, 8, 9, 16, 19, 22, 24, 26, and 28, wherein the pair of the
first miRNA and the second miRNA is selected from the group consisting of:
miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-
146a, miR-155/miR-16, miR-155/miR-132, miR-155/miR-323-3p, miR-155/miR-41 1, miR-491-
5p/miR-146b, miR-155/miR-146a, and miR-155/miR-146b.

36. The method of claim 3 or claim 9, wherein the pair of the first miRNA and the second
miRNA is selected from the group consisting of:
let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-33 5,
miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-134,
miR-181b/miR-132, let-7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-491-5p/miR-
134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-491-5p/miR-132, miR-
107/miR-323-3p, miR-127/miR-134, miR-491-5p/miR-874, miR-491-5p/miR-323-3p, miR-
127/miR-432, let-7e/miR-134, let-7e/miR-41 1, miR-107/miR-41 1, miR-491-5p/miR-41 1, miR-
107/miR-874, miR-181a/miR-9*, miR-491-5p/miR-2 10, miR-181b/miR-335, miR-99b/miR-335,
miR-107/miR-210, miR-127/487b, miR-181a/miR-874, miR-9/miR-9*, miR-107/miR-487b,
miR-107/miR-432, miR-9/miR-335, miR-181a/miR-132, miR-181b/miR-210, and miR-
99b/miR-132.
37. The method of claim 3 or claim 9, wherein the pair of the first miRNA and the second miRNA is selected from the group consisting of:
let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-411, miR-99b/miR-335, miR-491-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-411, miR-181a/miR-335, let-7e/miR-9*, miR-491-5p/miR-411, let-7e/miR-132, miR-181b/miR-132, miR-9/miR-9*, miR-9/miR-134, miR-107/miR-134, miR-181b/miR-874, let-7e/miR-134, miR-107/miR-132, miR-107/miR-9*, miR-127/miR-335, miR-9/miR-132, miR-181b/miR-9*, miR-491-5p/miR-132, let-7e/miR-210, miR-491-5p/miR-9*, miR-107/miR-323-3p, miR-491-5p/miR-323-3p, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-210, miR-9/miR-874, miR-9/miR-485-3p, miR-744/miR-134, and miR-181b/miR-323-3p.

38. The method of claim 3 or claim 9, wherein the pair of the first miRNA and the second miRNA is selected from the group consisting of:
let-7e/miR-146a, miR-107/miR-146a, miR-491-5p/miR-146a, miR-107/miR-146b, miR-491-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, and miR-744/miR-146a.

39. The method of claim 3 or claim 9, wherein the pair of the first miRNA and the second miRNA is selected from the group consisting of:
miR-107/miR-146a, miR-491-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, let-7e/miR-146b, miR-9/miR-146b, let-7e/miR-146a, miR-155/miR-874, miR-155/miR-9*, miR-744/miR-146a, miR-155/miR-210, miR-155/miR-146b, miR-155/miR-411, miR-744/miR-146b, miR-155/miR-146a, miR-155/miR-134, and miR-181b/miR-146b.

40. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, comprising measuring the level and calculating the ratios of the levels for two or more different pairs of miRNA.

41. The method of claim 40, comprising measuring the level and calculating the ratios of the levels for one or more pair combinations selected from the group consisting of:
(a) miR-181b/miR-323-3p and miR-99b/miR-9*,
(b) miR-491-5p/miR-487b plus miR-9/miR-146a, and
(c) miR-491-5p/miR-210 plus miR-181a/miR-146b.
42. The method of any one of claims 1-4 and 7-10, comprising measuring the levels of the miRNAs in two or more bodily fluid samples collected from the subject, wherein the samples have been collected at spaced apart time points.

43. The method of any one of claims 15, 16, 18, 19, and 46, wherein the bodily fluid samples are obtained several months apart.

44. The method of claim 43, wherein the bodily fluid samples are obtained 3-6 months apart.

45. The method of any one of claims 1, 3, 7, 9, 15, 18, 21, 23, 25, and 27, wherein the second miRNA is a brain-enriched miRNA selected from the group consisting of miRNAs which are mainly expressed in brain areas not involved in PD, miRNAs which are mainly expressed in glial cells, and brain-enriched miRNAs downregulated in PD.

46. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, further comprising normalizing the levels of the first and second miRNA to the level of a normalizer miRNA.

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

48. The method of any one of claims 1, 2, 7, and 8, wherein the control ratio is a predetermined value which represents a statistically validated threshold ratio of the levels of said first and second miRNAs (a single "cut-off" value) equal to the highest possible value within the range of corresponding values in age-matched healthy subjects.

49. The method of any one of claims 1, 2, 7, and 8, wherein the control ratio is the ratio of the levels of said first and second miRNAs in a similarly processed bodily fluid sample from the same subject collected in the past.

50. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, wherein the subject is human.

51. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, wherein the subject is an experimental animal.
52. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, wherein the bodily fluid is selected from the group consisting of blood plasma, serum, urine, cerebrospinal fluid (CSF), and saliva.

53. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, which method comprises the step of collecting the bodily fluid sample(s) from the subject.

54. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, wherein the level of the miRNAs is determined using a method selected from the group consisting of hybridization, RT-PCR, and sequencing.

55. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, wherein, prior to measuring miRNA level, the miRNA is purified from the bodily fluid sample.

56. The method of any one of claims 1-4, 7-10, 15, 16, 18, 19, and 21-28, further comprising the step of reducing or eliminating degradation of the miRNAs.

57. The method of any one of claims 1, 2, 15, and 16, further comprising administering a therapeutic or preventive treatment to the subject that has been diagnosed as having the neurodegenerative disease or as being at risk of progression to a more severe form of the neurodegenerative disease.

58. The method of any one of claims 7, 8, 18, and 19, further comprising administering a therapeutic or preventive treatment to the subject that has been diagnosed as having PD or as being at risk of progression to a more severe form of PD.

59. The method of claim 58, wherein the therapeutic treatment comprises administration of one or more compounds selected from the group consisting of dopamine-replenishing compounds, dopamine mimicking compounds, dopamine enhancers, catechol o-methyltransferase (COMT) inhibitors, dopamine receptor agonists, monoamine oxidase (MAOIs) inhibitors, amantadine, anti-cholinergic compounds, antiglutamatergic compounds, neurturin, anti-apoptotic compounds, promitochondrial compounds, calcium channel blockers, growth factors, and compounds targeting alpha-synuclein.

60. The method of claim 58, wherein the therapeutic treatment comprises a surgical procedure.
61. The method of claim 60, wherein the surgical procedure is selected from the group consisting of implantation of a battery-powered electrode in the brain, thalamotomy, pallidotomy, subthalamotomy, and dopamergic cell transplant.

62. The method of claim 58, wherein the preventive treatment is a change(s) in lifestyle or physical therapy.

63. The method of any one of claims 1-4, 7-10, 15, 16, 18, and 19, further comprising recruiting the subject in a clinical trial.

64. The method of any one of claims 8, 19, 24, and 28 wherein the second miRNA is a brain-enriched miRNA, which (1) is enriched in a brain area(s) which is not affected by PD or (2) is enriched in a brain cell type which is not affected by PD.

65. A kit for detecting Parkinson's disease (PD) comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of:
let-7e/miR-335, miR-107/miR-335, miR-491-5p/miR-335, miR-744/miR-335, miR-99b/miR-335, let-7e/miR-9*, miR-491-5p/miR-9*, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-134, miR-107/miR-134, miR-99b/miR-134, miR-491-5p/miR-134, let-7e/miR-323-3p, miR-107/miR-323-3p, miR-127/miR-323-3p, miR-181/miR-323-3p, miR-99b/miR-323-3p, miR-491-5p/miR-323-3p, miR-99b/miR-323-3p, miR-155/miR-335, let-7e/miR-146b, miR-491-5p/miR-146a, let-7e/miR-146a, miR-744/miR-146a, miR-155/miR-16, miR-155/miR-132, miR-155/miR-323-3p, miR-155/miR-41 1, miR-491-5p/miR-146b, miR-155/miR-146a, and miR-155/miR-146b.

66. A kit for differentiating Parkinson's disease (PD) from Mild Cognitive Impairment (MCI) comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of:
let-7e/miR-335, let-7e/miR-9*, miR-107/miR-335, miR-127/miR-323-3p, miR-491-5p/miR-335, miR-491-5p/miR-9*, miR-107/miR-9*, miR-744/miR-335, let-7e/miR-132, miR-107/miR-132, miR-491-5p/miR-132, let-7e/miR-210, miR-181b/miR-9*, miR-181a/miR-335, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-874, miR-107/miR-132, miR-491-5p/miR-132, miR-107/miR-323-3p, miR-127/miR-134, miR-491-5p/miR-874, miR-491-5p/miR-323-3p, miR-
127/miR-432, let-7e/miR-134, let-7e/miR-41, miR-107/miR-41, miR-491-5p/miR-41, miR-107/miR-874, miR-181a/miR-9*, miR-491-5p/miR-2 10, miR-181b/miR-335, miR-99b/miR-335, miR-107/miR-210, miR-127/miR-487b, miR-181a/miR-874, miR-9/miR-9*, miR-107/miR-487b, miR-107/miR-432, miR-9/miR-335, miR-181a/miR-132, miR-181b/miR-210, and miR-99b/miR-132; let-7e/miR-146a, miR-107/miR-146a, miR-491-5p/miR-146a, miR-107/miR-146b, miR-491-5p/miR-146b, miR-155/miR-874, let-7e/miR-146b, miR-155/miR-9*, miR-155/miR-335, miR-155/miR-41, and miR-744/miR-146a.

67. A kit for differentiating Parkinson's disease (PD) from Alzheimer's disease (AD) comprising primers and/or probes specific for one or more pairs of miRNAs selected from the group consisting of:
let-7e/miR-335, miR-107/miR-335, miR-127/miR-323-3p, let-7e/miR-41, miR-99b/miR-335, miR-491-5p/miR-335, miR-127/miR-134, miR-744/miR-335, miR-9/miR-335, miR-181b/miR-335, miR-107/miR-41, miR-181a/miR-335, let-7e/miR-9*, miR-491-5p/miR-4 11, let-7e/miR-132, miR-181b/miR-132, miR-9/miR-9*, miR-9/miR-134, miR-107/miR-134, miR-181b/miR-874, let-7e/miR-134, miR-107/miR-132, miR-107/miR-9*, miR-127/miR-335, miR-9/miR-132, miR-181b/miR-9*, miR-491-5p/miR-132, let-7e/miR-210, miR-491-5p/miR-9*, miR-107/miR-323-3p, miR-491-5p/miR-323-3p, miR-491-5p/miR-134, let-7e/miR-874, miR-181b/miR-210, miR-9/miR-874, miR-9/miR-485-3p, miR-744/miR-134, and miR-181b/miR-323-3p; miR-107/miR-146a, miR-491-5p/miR-146a, miR-155/miR-132, miR-155/miR-335, miR-107/miR-146b, miR-491-5p/miR-146b, miR-9/miR-146a, miR-155/miR-132, miR-155/miR-874, miR-9/miR-9*, miR-155/miR-41 1, miR-744/miR-146a, miR-155/miR-210, miR-155/miR-146b, miR-744/miR-146b, miR-155/miR-146a, miR-155/miR-134, and miR-181b/miR-146b.

68. A kit comprising primers and/or probes specific for one or more combinations of pairs of miRNAs selected from the group consisting of:
(a) miR-181b/miR-323-3p and miR-99b/miR-9*,
(b) miR-491-5p/miR-487b and miR-9/miR-146a, and
(c) miR-491-5p/miR-2 10 and miR-181a/miR-146b.
69. The kit of any one of claims 65-68, further comprising miRNA isolation and/or purification means.

70. The kit of any one of claims 65-68, further comprising instructions for use.

71. A method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) calculating the mean level of each miRNA measured in step (b);
(d) calculating the difference between the mean miRNA levels calculated in step (c);
(e) comparing the differences between the mean miRNA levels calculated in step (d) between all studied miRNAs and selecting as potential biomarker pairs those miRNA pairs for which the difference calculated in step (d) for one miRNA is at least 1.5 times the difference calculated for the other miRNA;
(f) calculating Spearman's rank correlation coefficient (r) for each potential biomarker miRNA pair selected in step (e), and
(g) identifying the miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if its (r) value calculated in step (f) is at least 0.8.

72. A method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) calculating Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNAs measured in step (b);
(d) selecting as potential biomarker pairs those miRNA pairs which have the (r) value calculated in step (c) of at least 0.8;
(e) calculating the mean level of each miRNA selected in step (d);
(f) calculating the difference between the mean miRNA levels calculated in step (e);
(g) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if the difference calculated in step (f) for one miRNA is at least 1.5 times the difference calculated for the other miRNA.

73. A method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting at least four miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) calculating Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNAs measured in step (b);
(d) selecting as potential biomarker pairs those miRNA pairs which have the (r) value calculated in step (c) of at least 0.8;
(e) calculating P-value of two subject cohorts separation for each miRNA pair selected in step (d), and
(f) identifying a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this pair differentiates two subject cohorts with a statistically significant P-value.

74. A computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the mean level of each miRNA measured in step (b);
(d) electronically calculating a difference between the mean miRNA levels calculated in step (c);
(e) selecting from the group of measured miRNAs a set of potential miRNA pairs each comprising a first miRNA and a second miRNA, wherein the calculated difference in the mean level in step (d) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA;

(f) electronically calculating the Spearman's rank correlation coefficient (r) for each potential miRNA pair selected in (e);

(g) selecting from the set of potential miRNA pairs those miRNA pairs, which are suitable for the diagnosis and/or monitoring of the pathology, wherein the (r) value calculated in step (f) is at least 0.8, and

(h) displaying all or part of the miRNA pairs selected in step (g).

75. A computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:

(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;

(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;

(c) electronically calculating the Spearman's rank correlation coefficient (r) for all possible pairs of individual miRNAs measured in step (b);

(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the (r) value calculated in step (c) is at least 0.8;

(e) electronically calculating the mean level of each miRNA selected in step (d);

(f) electronically calculating the difference between the mean miRNA levels calculated in step (e);

(g) selecting from the group of measured miRNAs a set of suitable miRNA biomarker pairs each comprising a first miRNA and a second miRNA, wherein for each suitable biomarker miRNA pair, the calculated difference in the mean level in step (f) of the first miRNA is at least 1.5 times the calculated difference in the mean level of the second miRNA, and

(h) displaying all or part of the suitable biomarker miRNA pairs selected in step (g).

76. A computer-implemented method for selecting a biomarker miRNA pair for diagnosis and/or monitoring of a pathology, said method comprising the following steps:
(a) selecting a group of miRNAs known to be enriched in an organ affected by the pathology;
(b) measuring the level of each miRNA selected in step (a) in bodily fluid samples from two subject cohorts;
(c) electronically calculating the Spearman's rank correlation coefficient (r) of the levels measured in step (b) for all possible pairs of individual miRNAs;
(d) selecting from the group of measured miRNAs a set of potential biomarker miRNA pairs, wherein the (r) value calculated in step (c) is at least 0.8;
(e) electronically calculating P-value of two subject cohorts separation for each miRNA pair selected in step (d);
(f) selecting a miRNA pair as a suitable biomarker pair for diagnosis and/or monitoring of said pathology if this miRNA pair differentiates two subject cohorts with a statistically significant P-value, and
(g) displaying all or part of the suitable biomarker miRNA pairs selected in step (f).

77. The method of any one of claims 71-76, wherein the miRNA level is measured by a method selected from the group consisting of RT-PCR-based methods, miRNA array-based methods, new generation sequencing, and hybridization.

78. The method of any one of claims 71-76, wherein the two subject cohorts are (1) subjects having the pathology and (2) age, gender and ethnicity-matched healthy subjects.

79. The method of any one of claims 71-76, wherein the two subject cohorts are subjects having two different pathologies of the organ.

80. The method of any one of claims 71-76, wherein the two subject cohorts are (1) younger subjects and (2) older subjects.

81. The method of any one of claims 71-76, wherein the two subject cohorts are age and ethnicity-matched (1) males and (2) females.

82. The method of any one of claims 71-76, wherein the bodily fluid is selected from the group consisting of plasma, serum, cerebrospinal fluid (CSF), urine, and saliva.
83. The method of any one of claims 71-76, wherein the subject is human.

84. The method of any one of claims 71-76, wherein the subject is an experimental animal.

85. The method of claim 73 or claim 76, wherein the P-value is calculated in step (e) using Mann-Whitney test.

86. The method of claim 73 or claim 76, wherein the statistically significant P-value is at least 0.05.

87. The method of any one of claims 71-76, wherein the pathology is a neurodegenerative disease.

88. The method of claim 87, wherein the neurodegenerative disease is selected from the group consisting of Parkinson's disease (PD), Alzheimer's disease (AD) and Mild Cognitive Impairment (MCI).

89. The method of any one of claims 71-76, wherein the organ affected by the pathology is brain.
Figure 1A

Figure 1B

<table>
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<tr>
<th>Name</th>
<th>miR-7e / miR-132</th>
<th>miR-7e / miR-146a</th>
<th>miR-7e / miR-335</th>
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Figure 2A

Figure 2B

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Figure 2C

Figure 2D

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Figure 2E

Figure 2F

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Figure 3A

Figure 3B

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Figure 3C

Figure 3D

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Figure 3E

Figure 3F

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Figure 4A

Figure 4B
Figure 4C

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**INTERNATIONAL SEARCH REPORT**

**International application No.**
PCT/US 14/65959

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**A. CLASSIFICATION OF SUBJECT MATTER**

**IPC(8)**  
C12Q 1/68, C10B 40/05, C40B 30/04 (2015.01)

**CPC**  
C12Q 1/68/03, C12Q 1/68/13, C12Q 1/68/51

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**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

CPC: C12Q 1/68/03, C12Q 1/68/13, C12Q 1/68/51

---

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>WO 2012/145636 A1 (SHEINERMAN, et al.) 26 October 2012 (26.10.2012) claims 1, 2, 12, 16, 20, 21; pg 25, ls 14-28; pg 27, ls 14-20; pg 51, ls 26 to pg 52, ln 15</td>
<td>1-4, 15-17, 21, 22, 25, 26, 33, (48,49)/(1,2), 57</td>
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<td>WO 2013/036936 A1 (KHO, et al.) 14 March 2013 (14.03.2013) claims 1, 19, 23; para [0007], [0008], [0118],[0128], [0143],[0148],[0157],[0160]</td>
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Additional documents are listed in the continuation of Box C.

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**H**  
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.

---

Date of the actual completion of the international search  
08 April 2015 (08.04.2015)

Date of mailing of the international search report  
28 APR 2015

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

Authorized officer: Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

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Form PCT/ISA/210 (second sheet) (January 2015)
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.: 5, 6, 11-14, 30-32, 34-47, 50-56, 63 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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 1-4, 7-10, 15-29, 33, 48, 49, 57-62, 64, drawn to a method comprising measuring relative level of two circulating microRNAs.

Group ii claims 65-70, drawn to a kit.

Group iii claims 71-89, drawn to a method comprising calculating the Spearman’s rank correlation coefficient (r) for pairs of individual miRNAs.

******************************************************************************* 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.: 1-4, 7-10, 15-29, 33, 48, 49, 57-62, 64

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

The inventions listed as Groups I-III 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:

Special Technical Features
The inventions of Groups I and II do not include the shared or common technical feature of a method comprising measuring relative level of two circulating microRNAs, as required by Group I.

The inventions of Group III do not include the shared or common technical feature of a method comprising measuring relative level of two circulating microRNAs, as required by Group I.

Common Technical Features
The inventions of Groups I and III share the technical feature of a method comprising measuring relative level of two circulating microRNAs for diagnosing a pathology. However, this shared technical feature does not represent a contribution over prior art as being anticipated by WO 2012/145363 A1 (26 October 2012) to Sheinerman, et al. (hereinafter “Sheinerman”) (pg 51, in 38 to pg 52, in 15, “Example 4; MCI detection by analysis of miRNAs in plasma...” 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... 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”. pg 27, in 14-19, “Figures 17A-F present analysis of associations between miR128 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 7 were calculated”). As said technical feature was known in the art at the time of the invention, this cannot be considered special technical feature that would otherwise unify the groups.

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