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(54) Title: MIRNA BIOMARKERS OF ALZHEIMER'S DISEASE

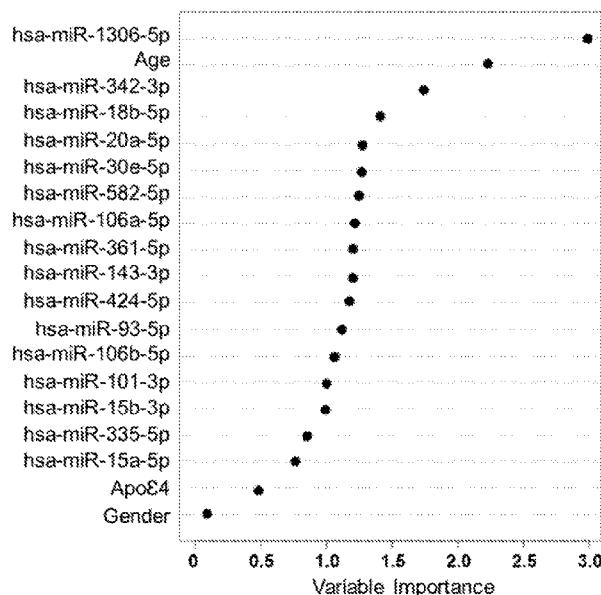


Figure 2D

(57) Abstract: The present disclosure relates to miRNA biomarkers, the level of expression of which, is instructive as to Alzheimer's disease (AD) including identifying onset and various stages of AD, its various clinical manifestations and amyloidosis-related conditions.



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MIRNA BIOMARKERS OF ALZHEIMER'S DISEASE

FILING DATA

[0001] This application is associated with and claims priority from Australian Provisional Patent Application No. 2014901970, filed on 26 May 2014, entitled "Biomarkers", the entire contents of which, are incorporated herein by reference.

BACKGROUND

FIELD

[0002] The present disclosure relates to nucleic acid biomarkers, the level of expression of which, is instructive as to Alzheimer's disease (AD) including identifying onset and various stages of AD, its various clinical manifestations and amyloidosis-related conditions.

DESCRIPTION OF RELATED ART

[0003] Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Alzheimer's disease (AD) is a progressive degenerative disorder that causes loss of memory, cognitive disturbances and behavioral changes. It is a debilitating condition leading to significant rates of morbidity and mortality. It is the fourth largest cause of death in the United States and affects five percent of people over age 65 and 20 percent of

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people over age 80. To date, there has been no established treatment which will prevent the onset or delay the progression of AD although there is substantial research ongoing worldwide towards identifying potential treatments, including a number of drug trials.

[0006] This form of neurodegeneration is characterized by the formation of intracellular neurofibrillary tangles, neuronal and synaptic loss and the accumulation of beta amyloid (A β) into amyloid plaque material (Breteler *et al.* (1992) *Epidemiol Rev* 14:59-82). A β is proteolytically cleaved from the amyloid precursor protein (APP) [Cole and Vassar (2008) *J Biol Chem* 283:29621-29625]. The most characteristic neuropathological feature of AD is the deposition of A β into plaques in the brain parenchyma and cerebral blood vessels leading to neuronal loss and cerebral atrophy (Terry *et al.* (1981) *Ann. Neurol* 10:184-192).

[0007] Positron emission tomography (PET) [Cole and Vassar (2008) *supra*] scanning using a carbon-11-labeled Pittsburgh compound B (¹¹C-PiB) to observe A β burden of the brain has shown that A β deposition is a slow pathological process which occurs during a period of 17-30 years (Villemagne *et al.* (2013) *The Lancet Neurology* 12:357-367). Thus, the asymptomatic or presymptomatic nature of pre-clinical dementia presents a challenge for early diagnosis and stratification of AD patients. Diagnosis of AD has made some progress in the recent decade by recruiting large flagship studies which has produced promising diagnostic methods such as PET imaging (Villemagne *et al.* (2013) *supra*) and CSF biomarkers (Hansson *et al.* (2006) *Lancet Neurology* 5:228-234) to detect A β burden. Although these methods have shown high diagnostic accuracy for AD, the high costs and invasive nature of PET imaging and CSF collection are not ideal for routine clinical testing.

[0008] MicroRNAs (miRNAs) are non-coding RNA species of approximately 22 nucleotides in length which are transcribed in all tissues and cells (Krol *et al.* (2010) *Nat Rev Genet* 11:597-610) and which bind to complementary sites at the 3' untranslated region (3'UTR) of their corresponding mRNA targets resulting in down-regulation of gene expression (Hannon (2004) *Nat Rev Genet* 5:522-531). miRNAs can be released into the extracellular environment by binding to RNA-binding proteins or through secretion in cell-

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derived plasma microvesicles such as exosomes (Valadi *et al.* (2007) *Nat Cell Biol* 9:654-659; Mitchell *et al.* (2008) *Proceedings of the National Academy of Sciences* 105:10513-10518; Bellingham *et al.* (2012) *Front Physiol* 3:124). The presence of miRNA can, therefore, reflect the physiological state of the biological system. Recent developments in high throughput next-generation sequencing (NGS) have allowed the ability to profile miRNA in biological fluids (Cheng *et al.* (2013) *Front Genet* 4:150). Profiles of deregulated miRNA isolated from plasma and serum (Mitchell *et al.* (2008) *supra*; Skog *et al.* (2008) *Nat Cell Biol* 10:1470-1476) suggest they have diagnostic potential for human disease.

[0009] A non-invasive and high throughput biological fluid-based test is required for improved population-based screening and patient care in order to refer patients for further examination. Furthermore, with numerous drug trials aiming to treat AD, a fluid-based test is needed to enrich cohorts of AD cases followed by monitoring the potential benefits and side-effects of therapeutic drugs.

SUMMARY

[0010] Nucleotide sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

[0011] The microRNAs (miRNAs) defined by miR nomenclature are listed in Table 1 with reference to a sequence identifier number.

[0012] Abbreviations used herein are defined in Table 2

[0013] Enabled herein is a method for the stratification of human subjects with respect to AD and its various clinical manifestations and stages including the early diagnosis of asymptomatic or presymptomatic individuals. The method disclosed herein is also applicable to other amyloidosis-related conditions. The stratification is based on an association between deregulated miRNA expression and AD. Deregulated miRNAs can be detected in a range of biological samples such as samples comprising or enriched for membranous microvesicles, blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion and interstitial fluid. An example of a membranous microvesicle is an exosome. Such structures may first require chemical or mechanical disruption to release the miRNAs or the miRNAs may be free circulating miRNA. The association between miRNA expression and AD is validated through unbiased next generation sequencing (NGS) followed by qRT-PCR. An miRNA signature is selected from an NGS training set in the form of a panel which is used to predict future or potential cognitive decline, early onset AD, the various stages of AD as well as other amyloidosis-related conditions.

[0014] The miRNA expression profile is the level of expression of one or more miRNAs or 2 or more miRNA comprising a nucleotide sequence selected from the listing consisting

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of SEQ ID NOs: 1 through 16 or a precursor form of each of SEQ ID NOs:1 through 16 compared to a first knowledge base generated from training data. The training data represent the correlation of expression levels of the miRNAs with subjects of known status with respect to AD. The first knowledge base, for example, may be miRNA expression levels in healthy individuals (healthy or normal controls). Alternatively, the first knowledge base may comprise levels of expression of the miRNAs in unhealthy controls. Hence, elevation or reduction in expression is dependent on the type of control. In an embodiment, the first knowledge base enables determination of ΔC_t levels of a particular miRNAs obtained using qPCR and then ΔC_t cut-off values established which correlate with the AD condition. Expression fold changes and levels of expression can also be measured of individual miRNAs and/or ratios determined of ΔC_t levels, fold levels of expression between any 2 or more of SEQ ID NOs:1 through 17. The assay may or may not require control sample to be run side-by-side.

[0015] Hence, enabled herein is a diagnostic rule based on the application of a comparison of levels of miRNA expression in a control sample or based on predetermined ΔC_t cut-off values. In another embodiment, the diagnostic rule is based on application of statistical and machine learning algorithms. Such an algorithm uses the relationships between miRNA expression and disease status observed in training data (with known disease status) to infer relationships which are then used to predict the status of patients with unknown status (test data). Practitioners skilled in the art of data analysis recognize that many different forms of inferring relationships in the training data may be used without materially changing the scope of the present invention.

[0016] Enabled herein is an assay to stratify a human subject with respect to Alzheimer's disease (AD) or a symptom associated with AD or other amyloidosis-related condition, the method comprising screening a biological sample from the subject for an miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NOs:1 through 17 or a precursor form of each of SEQ ID NOs:1 through 17 or their corresponding cDNA forms or their corresponding cDNA forms wherein the level of expression of a selected miRNA compared to a control is indicative of a stage in the

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development of AD. By "control" includes a ΔCt cut-off value based on known ΔCt levels of particular miRNAs obtained using qPCR. Ratios of expression or fold changes or ΔCt levels between 2 or more of SEQ ID NOs:1 through 17 may also be determined.

[0017] In an embodiment, SEQ ID NO:17 is excluded. Accordingly, the level of expression in the first knowledge base is based on levels in healthy individuals. In this aspect, the expression of one or more miRNAs comprising nucleotide sequences selected from SEQ ID NOs:1 through 13 is elevated relative to the healthy control and the expression of one or more miRNAs comprising nucleotide sequences selected from SEQ ID NOs:14 through 16 is reduced relative to a healthy control in a subject stratified as having a stage of AD or is at risk of developing AD. Reference to any of SEQ ID NOs:1 through 16 as well as SEQ ID NO:17 includes precursor forms thereof. In an embodiment, the biological sample comprises membranous microvesicles or a sample enriched for membranous microvesicles, blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion or interstitial fluid. Free circulating miRNAs may also be measured.

[0018] An example of a membranous microvesicle is an exosome. Microvesicles and exosomes may first require chemical or mechanical disruption to release the miRNAs. The miRNAs may also be captured on a solid support such as a bead or the side of a reaction vessel. Hence, the sample may comprise or be enriched for membrane microvesicles or miRNAs released from exosomes or membrane vesicles. A non-microvesicle sample of free miRNA is also contemplated herein.

[0019] The ability to recognise asymptomatic or presymptomatic AD individuals enables early medical and behavioral intervention thereby improving quality of life outcomes.

[0020] Hence, taught herein is a cost effective, low risk, minimally invasive test to detect onset of and to monitor stages of AD as well as other amyloidosis-related conditions. The assay can also be used to monitor treatment protocols. Importantly, the miRNA profile

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enables diagnosis of AD in subjects who present as healthy individuals yet have a high amyloid burden and/or other risk factors.

Table 1
Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Nucleotide sequence of hsa-miR-30e-5p
2	Nucleotide sequence of hsa-miR-101-3p
3	Nucleotide sequence of hsa-miR-15a-5p
4	Nucleotide sequence of hsa-miR-20a-5p
5	Nucleotide sequence of hsa-miR-93-5p
6	Nucleotide sequence of hsa-miR-106b-5p
7	Nucleotide sequence of hsa-miR-18b-5p
8	Nucleotide sequence of hsa-miR-106a-5p
9	Nucleotide sequence of hsa-miR-585-5p
10	Nucleotide sequence of hsa-miR-143-3p
11	Nucleotide sequence of hsa-miR-335-5p
12	Nucleotide sequence of hsa-miR-361-5p
13	Nucleotide sequence of hsa-miR-424-5p
14	Nucleotide sequence of hsa-miR-342-3p
15	Nucleotide sequence of hsa-miR-15b-3p
16	Nucleotide sequence of hsa-miR-1306-5p
17	Nucleotide sequence of hsa-miR-3065-5p

[0021] The present invention extends to the detection of precursor forms of the miRNAs which comprise the sequences of any one of SEQ ID NOs:1 through 17.

Table 2
Abbreviations

Abbreviation	Definition
Δ Ct	Delta Ct
AD	Alzheimer's disease
ApoE ϵ 4	Apolipoprotein ϵ 4
AUC	Area under receiver operating curve
CDR	Clinical dementia ratings
FDR	False discovery rate
HC	Healthy control
MCI	Mild cognitive impairment
miRNA	Micro-RNA
MMSE	Mini-mental state examination
NGS	Next generation sequences
PAM	Partitioning around meloids
PET	Positron emission tomography
ROC	Receiver operating characteristic
SUVr	Standard uptake value ratio

BRIEF DESCRIPTION OF THE FIGURES

[0022] Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0023] **Figure 1** is a diagrammatic representation showing hierarchical clustering of differentially expressed exosomal miRNA biomarkers obtained from healthy controls (HC) and patients with Alzheimer's disease (AD). Using the deep sequencing data obtained from the discovery set, hierarchical clustering is performed using Partek Genomics Suite on significantly differentially expressed miRNA using Euclidean average $\log_2$ (AD, MCI and HC) ≤ 0.05 and ± 1.2 fold change); There are two major nodes of the dendrogram. Node 1 contains 15 miRNA which are found to be up-regulated (hsa-miR-361-5p, hsa-miR-30e-5p, hsa-miR-93-5p, hsa-miR-15a-5p, hsa-miR-143-3p, hsa-miR-335-5p, hsa-miR-106b-5p, hsa-miR-101-3p, hsa-miR-424-5p, hsa-miR-106a-5p, hsa-miR-18b-5p, hsa-miR-3065-5p, hsa-miR-20a-5p, hsa-miR-3065-5p and hsa-miR-582-5p). Node 2 contains 3 miRNA which are found to be down-regulated (hsa-miR-1306-5p, hsa-miR-342-3p, and hsa-miR-15b-3p, Table 3). Patient samples are arranged by attributes. HC, healthy control; MCI, mild cognitive impairment; and AD, Alzheimer's disease. The miRNAs are defined in Table 1 with reference to the sequence listing.

[0024] **Figures 2A through D** are graphical representations of clustering and random forest testing of correlated miRNA identified in healthy controls and patients with Alzheimer's disease within the discovery set. A) PAM clustering of the 16 miRNA used for validation. Two main clusters are found. One cluster included miRNAs up-regulated in AD, and one for those down-regulated in AD. These 2 main clusters are broken into 2 further cluster search generating 4 clustered miRNA groups. B) miRNA in clusters 1 and 3 showed increased mean expression across ApoE ϵ 4 allele and clinical classification. Cluster 2 and 4 showing decrease mean expression across ApoE ϵ 4 allele and clinical classification. C) Box plots of miRNA clusters displaying associations with ApoE ϵ 4 allele status and clinical classification. D) Variable selection *via* Random Forest analyses ordered

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by importance of contribution towards clinical classification. The miRNAs found in each cluster are listed in Table 4.

[0025] Figure 3 is a graphical representation of box plots showing validated miRNA differentially expressed in healthy controls (HC) and patients with Alzheimer's disease (AD). Mean centred and scaled data are plotted between HC and AD patients. HC patients are represented by blue circular dots while AD patients are represented by red triangular dots.

DETAILED DESCRIPTION

[0026] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any element or integer or method step or group of elements or integers or method steps.

[0027] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a biomarker" includes a single biomarker, as well as two or more biomarkers; reference to "an miRNA" includes a single miRNA, as well as two or more miRNAs; reference to "the disclosure" includes single and multiple aspects taught by the disclosure; and so forth. Aspects taught and enabled herein are encompassed by the term "invention". All such aspects are enabled within the width of the present invention. All variants referred to herein are included by the term "forms" of the invention.

[0028] The use of numerical values in this specification, unless expressly indicated otherwise, are stated as approximations as though the values are preceded by the word "about". Furthermore, the manner of defining a control, and the selection of miRNA may result in different outcomes. Notwithstanding, the skilled person would compensate for these parameters without departing from the essence or scope of the present invention. Also, the disclosure of any ranges is intended as a continuous range including every value between minimum and maximum values. In addition, the subject protocol extends to ratios of expression levels of two or more miRNA biomarkers providing a numerical value associated with a level of likelihood of an individual having AD or is at risk of developing AD or its symptoms such as cognitive impairment.

[0029] The present disclosure teaches the identification of microRNAs (miRNAs) or precursor forms thereof, the expression of which, is statistically associated with AD, a stage of AD including asymptomatic or presymptomatic AD and/or a risk of developing

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AD. AD manifests itself as a spectrum of symptoms from asymptomatic features to mild cognitive impairment to severe cognitive decline and other symptoms or outcomes of major neurodegeneration. The ability to identify asymptomatic or presymptomatic or early onset AD subjects enables early clinical and behavioral intervention leading to significant improvement in quality of life for the individual and surrounding social and family networks. The method described herein can also be used to detect other amyloidosis-related conditions.

[0030] Hence, enabled herein is a diagnostic rule based on the application of a comparison of levels of miRNA expression in a control sample or based on a ΔCt cut-off value selected from a set of predetermined ΔCt levels obtained using qPCR and which are associated with a healthy individual with or at risk of developing AD or other amyloidosis-related condition. In another embodiment, the diagnostic rule is based on application of statistical and machine learning algorithms. Such an algorithm uses the relationships between miRNA expression and disease status observed in training data (with known disease status) to infer relationships which are then used to predict the status of patients with unknown status (test data). Practitioners skilled in the art of data analysis recognize that many different forms of inferring relationships in the training data may be used without materially changing the scope or essence of the present invention.

[0031] The instant disclosure teaches an assay to stratify a human subject with respect to AD or a symptom associated with AD or other amyloidosis-related condition, the method comprising screening a biological sample from the subject for one or more miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NO:1 through 16 or precursor forms thereof or their corresponding cDNA forms wherein the level of expression of the one or more miRNAs is indicative of a stage of AD or of the amyloidosis-related condition. For the sake of brevity, reference to AD also includes other amyloidosis-related conditions. Ratios of expression levels or expression folds or ΔCt levels between 2 or more of SEQ ID NOs:1 through 17 may also be determined.

[0032] The sample may comprise a sample comprising or enriched for membranous

microvesicles (e.g. exosomes), blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion or interstitial fluid. Free circulating miRNA is hence encompassed as a sample. If membranous microvesicles such as exosomes are sampled, they may first require chemical or mechanical disruption to release the miRNA.

[0033] In an embodiment, enabled herein is an assay to stratify a human subject with respect to AD or a symptom associated with AD, the method comprising obtaining a sample comprising or enriched for membranous microvesicles and subjecting the microvesicles to mechanical or chemical disruption to release miRNA and screening for one or more miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NO:1 through 16 or precursor forms thereof wherein the level of expression of the one or more miRNAs is indicative of a stage of AD.

[0034] Further taught herein is an assay to stratify a human subject with respect to AD or a symptom associated with AD, the method comprising subjecting a sample comprising or enriched for membranous microvesicles to mechanical or chemical disruption to release miRNA and treating one or more miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NO:1 through 16 or a precursor form thereof or a corresponding cDNA form to chemical processing and then determining the level of expression of the miRNAs wherein the level of expression of the one or more miRNAs is indicative of a stage of AD. By "chemical processing" in this context includes reverse transcription to generate cDNA, bisulfite treatment or chemical coupling to an immobilized support or to a solid phase. Free miRNA in a biological sample may also be assayed.

[0035] Reference to "membranous microvesicles" includes exosomes. The sample may or may not be a purified sample of microvesicles. A non-microvesicle sample may also be employed. A "stage of AD" means from presymptomatic to early onset to from mild to severe forms of AD. It also encompasses individuals with a likelihood of developing AD.

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[0036] Reference to "presymptomatic AD" includes a subject having no to mild cognitive impairment but having a risk factor for development of AD such as one or more ApoEε4 alleles and/or a high amyloid burden. This may also be determined by PET imaging.

[0037] The miRNA expression profile is the level of expression of one or more miRNAs comprising a nucleotide sequence selected from the listing consisting of SEQ ID NOs: 1 through 16 or precursor forms thereof or corresponding cDNA compared to a first knowledge base generated from training data. Ratios of levels of expression of 2 or more of SEQ ID NOs:1 through 17 can also form the training data. The training data represent the correlation of expression levels of the miRNAs with subjects of known status with respect to AD. The first knowledge base, for example, may be miRNA expression levels in healthy individuals (healthy or normal controls). Alternatively, the first knowledge base may comprise levels of expression of the miRNAs in unhealthy controls. Hence, elevation or reduction in expression is dependent on the type of control. Alternatively, Δ Ct levels in healthy or AD patients are predetermined using qPCR and a Δ Ct cut-off value selected which is associated with AD or with a healthy person.

[0038] In an embodiment, the level of expression in the first knowledge base is based on levels in healthy individuals. In this aspect, the expression of one or more miRNAs comprising nucleotide sequences selected from SEQ ID NOs:1 through 13 is elevated relative to the healthy control and the expression of one or more miRNAs comprising nucleotide sequences selected from SEQ ID NOs:14 through 16 is reduced relative to a healthy control in a subject stratified as having a stage of AD or is at risk of developing AD.

[0039] In an embodiment, the level of deregulation relative to a healthy control of individual miRNA expression correlates to a sensitivity of from about 40% to about 100% and a specificity of from about 30% to 100%. Collectively, however, determination of miRNAs defined by SEQ ID NOs:1 through 16 results in a sensitivity of about 87% or a specificity of about 77%. In this regard, another miRNA is hsa-miR-3065-5p (SEQ ID NO:17) which is not always detected by qRT-PCR. This miRNA is still regarded as part

of the present invention.

[0040] The term "deregulation" includes up-regulation and down-regulation. Hence, in an embodiment, the miRNA panel can be divided into two clusters based on up or down-regulation.

[0041] The up-regulation cluster includes SEQ ID NOs:1 through 13 and the down-regulation cluster includes SEQ ID NOs:14 through 16. Each of these clusters can be further divided based on APOE ϵ 4 allele genotyping and clinical classification. Hence, SEQ ID NOs:1 through 8 and 9 through 13 are in clusters 1 and 3, respectively, which exhibit a mean increase in expression relative to a healthy control and SEQ ID NO:14 and 15 and SEQ ID NO:16 are in clusters 2 and 4, respectively, which exhibit showing a mean decrease in expression relative to a healthy control. In a particular embodiment, SEQ ID NO:16 (hsa-miRNA 1306-5p) is assayed. In an alternative embodiment, the level of miRNA expression correlates with an unhealthy subject having AD or a certain likelihood of developing AD.

[0042] To determine whether a subject is considered a candidate for AD or to determine the stage of AD, the expression of one or more of SEQ ID NOs:1 through 16 may be assayed. This includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or all 16 miRNAs. SEQ ID NO:17 may also be assayed. Precursor forms of RNAs comprising a nucleotide sequence selected from SEQ ID NOs:1 through 17 may also be screened. Corresponding cDNA forms may also be assayed.

[0043] Enabled herein is an assay to stratify a human subject with respect to AD or a symptom associated with AD, the method comprising screening a biological sample from the subject for miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NOs:1 through 16 or precursor forms thereof or their corresponding cDNA forms wherein up-regulated levels of SEQ ID NOs:1 through 13 or precursor forms thereof and/or down-regulated levels of SEQ ID NOs:14 through 16 or precursor forms thereof or their corresponding cDNA forms relative to a healthy control are indicative of the subject

having AD or is at risk of developing symptoms of AD. The biological sample may comprise or be enriched for a membranous microvesicles such as exosomes or may be a non-membranous fluid sample. The latter may comprise free circulating miRNAs.

[0044] In an embodiment, the level of deregulation is measured relative to a control amount. The control may be a statistically validated level or range which defines up-regulation or down-regulation or normal regulation or the control may be determined on a subject of known AD status (e.g. an unhealthy or disease control). Alternatively, predetermined ΔC_t levels are obtained by qPCR associated with a healthy or not healthy subject and a ΔC_t cut-off value selected to diagnose AD. This avoids the need for a healthy control to be run each time in the assay.

[0045] Hence, enabled herein is a first knowledge base of levels of expression of miRNAs in subjects with known status with respect to AD. The first knowledge base is determined from training data following a trial of subjects. When the level of expression of one or more miRNAs is determined in a subject with unknown status, this creates a second knowledge base. By comparison between the first and second knowledge bases, the clinician can determine an index of probability that any one of tested subject has AD or is at risk of developing AD.

[0046] The control level may be determined using any suitable method, such as the analysis of test results relative to a standard result which reflects individual or collective results obtained from individuals with known AD status. This form of analysis enables the design of kits which require the collection and analysis of a single membranous microvesicles sample, being a test sample of interest, relative to the control. The control level may be determined from the subjects of a specific cohort and hence there may be different controls to test samples derived from different cohorts. Accordingly, there may be determined a number of control values or ranges which correspond to cohorts which differ in respect of characteristics such as age, gender, ethnicity or health status. The "control level" may be a discrete level or a range of levels.

[0047] Hence, the first knowledge base may comprise data from healthy controls or from patients with known levels of AD development. Hence, "deregulated" expression needs to be defined relative to whether the control (i.e. first knowledge base) is a healthy control or a disease control (or unhealthy control). A "disease control" is data from individuals with a known stage of AD. As indicated above, the first knowledge base may also comprise ΔC_t levels based on qPCR from which a ΔC_t cut-off value is selected based on correlation to AD. Expression levels or expression fold changes or ΔC_t levels of individual miRNAs or ratios thereof from 2 or more of SEQ ID NOs:1 through 17 can also be used.

[0048] To the extent that the miRNA product is present in a biological sample, the sample may be directly tested or else all or some of the nucleic acid material present in the sample may be isolated or enriched prior to testing. To this end, when screening for changes to the level of expression of the miRNA biomarkers, one may screen for the RNA transcripts themselves or cDNA which has been transcribed therefrom. The assay may be conducted on the sample directly or molecules derived therefrom pretreated prior to testing, for example, inactivation of live virus or being run on a gel. The sample may be freshly harvested or it may have been stored (for example by freezing) prior to testing or otherwise treated prior to testing. In an embodiment, the sample is a sample comprising or enriched for membranous microvesicles (e.g. exosomes), blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion or interstitial fluid.

[0049] As indicated above, whilst the subject assay is directed to AD, symptoms of AD can arise in other conditions associated with aberrant, unwanted or otherwise inappropriate amyloidosis. Hence, the miRNA expression profile may be used to screen for AD as well as Down's syndrome, hereditary cerebral hemorrhage with amyloidosis, amyloidosis associated with chronic inflammation, various forms of malignancy, familial Mediterranean fever, amyloidosis associated with multiple myeloma and other B-cell dyscrasias, amyloidosis associated with type II diabetes, prion disease, long term hemodialysis, carpal tunnel syndrome, amyloidosis associated with senile cardiac amyloid, familial amyloidotic polyneuropathy and amyloidosis associated with endocrine tumors.

[0050] In an embodiment, the condition is AD or is a stage of AD. As indicated above, a stage of AD includes asymptomatic or presymptomatic AD.

[0051] The subject being assayed may undergo additional testing after being stratified as having or being at risk of developing AD. The additional testing includes a range of behavioral or psychological evaluations such as level of cognitive ability as well as genetic testing for missense mutations in the chromosome 21 gene coding for β -amyloid precursor protein, a chromosome 14q gene coding for a 467 amino acid protein with 7 putative membrane-spanning domains and inheritance of the APoE ϵ 4 allele of the gene encoding apolipoprotein E. The presence of one or 2 alleles of APoE ϵ 4 together with the miRNA signature of the present invention enables the clinician to diagnose for the presence or absence of AD or to assess the likelihood of development of AD or to assess the stage of AD. Physiological testing such as PET imaging may also be conducted.

[0052] A miRNA is typically approximately 22 nucleotides in length although the present assay extends to miRNAs of from 15 to 30 nucleotides in length or to any fragment or part of an miRNA. A precursor form may also be screened. A precursor may comprise 70-100 nucleotides and comprise a nucleotide sequence selected from SEQ ID NOs:1 through 17. Corresponding cDNA forms of SEQ ID NOs:1 through 17 may also be assayed.

[0053] Typically, miRNAs are endogenously transcribed from DNA, but not translated into protein. The DNA sequence that codes for an miRNA generally includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base hybridize to form a double stranded RNA hairpin loop, this forming the primary miRNA structure (pri-miRNA). A nuclear enzyme cleaves the base of the hairpin to form pre-miRNA. The pre-miRNA molecule is then actively transported out of the nucleus into the cytoplasm where the Dicer enzyme cuts 20-25 nucleotides from the base of the hairpin to release the mature miRNA. The miRNA can be released into the extracellular environment when the cell membrane is compromised during apoptotic or

necrotic death, secreted when bound to lipoproteins or secreted in cell-derived plasma microvesicles such as exosomes.

[0054] The standard nomenclature system is used herein to identify miRNA. For example, a list of miRNAs (miRs) from a homosapien (hsa) having particular sequences is provided in Table 1.

[0055] In terms of screening for the "level of expression" or changes in fold of expression of miRNAs, this may be achieved in a variety of ways including screening directly for any of the forms of miRNA or cDNA generated therefrom. Accordingly, either RNA or DNA based screening may be employed. Changes to the absolute levels of any of these markers is indicative of changes to the expression of the subject miRNA. Furthermore, ratios of expression changes are also useful in determining a diagnosis. Still further, the miRNA which is identified and measured may be a whole molecule or a fragment thereof. For example, one may identify only fragments of miRNA from a membranous microvesicle sample, depending on how it has been processed. Provided that the fragment comprises sufficient sequence to indicate its origin with a particular miRNA, fragmented miRNAs are useful in the context of the method of the subject assay.

[0056] Reference to "nucleic acid molecule" should be understood as a reference to both ribonucleic acid molecules and deoxyribonucleic acid molecules and fragments thereof. The subject assay extends, therefore, to both directly screening for miRNA levels in a membranous microvesicle sample or screening for the complementary cDNA which has been reverse-transcribed from an miRNA population of interest. It is well within the skill of the person of skill in the art to design methodology directed to screening for either RNA or DNA.

[0057] The term "fragment" means a portion of the subject nucleic acid molecule. This is relevant with respect to screening for modulated miRNA levels in a fluid sample or membranous microvesicle samples which may have been chemically (e.g. enzymatically) or mechanically treated since the subject miRNA may have been degraded or otherwise

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fragmented. One may, therefore, actually be detecting fragments of the subject RNA molecule, which fragments are identified by virtue of the use of a suitably specific probe. Alternatively, one or more precursors of SEQ ID NOs:1 through 17 may be detected.

[0058] Reference to a "membranous microvesicle" means any particle which is comprised of a cellular plasma membrane component. Such membranous microvesicles may adopt a structure which takes the form of a lumen surrounded by plasma membrane. Examples of membranous microvesicles include, but are not limited to, microparticles, exosomes, apoptotic blebs, apoptotic bodies, cellular blebs, extracellular vesicles and the like. In an embodiment, the membranous microvesicle is an exosome. Free circulating miRNAs may also be measured.

[0059] Reference to an "exosome" includes vesicles which are secreted by a wide variety of cell types.

[0060] The exosomes tested in the subject assay are generally enriched from a biological sample. By "biological sample" is meant any biological material derived from an individual. The miRNA may be located in fluid medium or in a medium which comprises exosomes. Hence, samples include, but are not limited to, a sample comprising or enriched from membranous microvesicles (e.g. exosomes), blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion, interstitial fluid and fluid which has been introduced into the body of an individual and subsequently removed such as, for example, the saline solution extracted from the lung following lung lavage or the solution retrieved from an enema wash. The biological sample may be tested directly or undergo some form of pre-treatment prior to testing. For example, the sample may require the addition of a reagent, such as a buffer, to immobilise the miRNA in the sample. It should be further understood that the sample which is the subject of testing may be freshly isolated or it may have been isolated at an earlier point in time and subsequently stored or otherwise treated prior to testing. For example, the sample may have been collected at an earlier point in time and frozen or otherwise preserved in order to facilitate

its transportation to the site of testing. In yet another example, the sample may be treated to neutralize any possible pathogenic infection, thereby reducing the risk of transmission of the infection to the technician.

[0061] The choice of sample may be dependent on the nature of the subject including age and overall health status.

[0062] In an embodiment, the biological sample comprises or is enriched for membranous microvesicles or free miRNAs. In an embodiment, the sample is whole blood, plasma serum, cerebral fluid, spinal fluid, interstitial fluid or a fraction thereof.

[0063] To the extent that the subject biological sample is harvested from an individual, the term "individual" should be understood to include a human of any age or at an age regarded as being at risk. Such age selection includes subjects from 40 to 49, 50 to 59, 60 to 69, 70 to 79, 80 to 89 and 90 to 100 years old. Whilst the subject assay is directed to humans, the assay may be trialled in an animal model such as a non-human primate or a rodent (e.g. mouse) model.

[0064] The miRNA markers are defined by SEQ ID NOs:1 through 17 although the preferred panel is SEQ ID NOs:1 through 16. Such markers include large precursor forms which comprise a sequence selected from SEQ ID NOs:1 through 17 or their corresponding cDNA forms. In an embodiment, up-regulated levels of one or more of SEQ ID NOs:1 through 13 and/or down-regulated levels of one or more of SEQ ID NOs:14 through 16 relative to a healthy control is regarded as a signature of AD or a risk of developing AD. The miRNA display a fold expression difference of more than 1.2 and a significance of $p \leq 0.05$. As indicated above, if a disease or unhealthy control is employed, the up- or down-regulation may be reversed. Also contemplated herein is a measurement of ratios of ΔC_t values, fold changes and levels of expression between any 2 of SEQ ID NOs:1 through 17 including any 2 of SEQ ID NOs:1 through 16.

[0065] Whilst a signature profile of two or more miRNAs is likely to be more convenient,

measurement of a single miRNA is also contemplated.

[0066] Hence, in another embodiment, there is provided a method of screening for the onset, predisposition to the onset or monitoring the progress of AD in an individual, the method comprising measuring the level of expression of SEQ ID NO:16 (hsa-miRNA-1306-5p) in a biological sample from individual wherein a higher level of expression of the miRNA relative to a healthy control level is indicative of the onset or predisposition of the onset of AD. A "biological sample" in this context includes a sample comprising or enriched from membranous microvesicles (e.g. exosomes) as well as whole blood, serum, plasma and the like.

[0067] Reference to the "onset" of AD should be understood as a reference to the commencement of changes to the brain which are characteristic of AD, such as A β plaque deposition or formation of neurofibrillary tangles. In this regard, these changes may be well advanced in that cognitive impairment has become evident. Alternatively, the physical changes to the brain may be at a very early stage such that symptoms of cognitive impairment are not yet evident. Assessment of an individual's predisposition to the development of AD is contemplated herein. Changed levels of the subject miRNA markers are proposed to be indicative of that individual's predisposition to developing AD.

[0068] Detection of converse changes in the levels of the miRNA marker may be desired under circumstances other diagnosis, for example, to monitor the effectiveness of therapeutic or prophylactic treatment directed to modulating AD onset or progression. For example, where elevated levels of the markers indicates that an individual has developed AD screening for a decrease in the levels of this markers subsequently to the onset of a therapeutic regime may be utilized to indicate reversal or other form of improvement of the subject individual's condition.

[0069] Contemplated herein is a method of monitoring treatment of a subject with or having a risk of developing AD or as a monitor of the effectiveness of therapeutic or prophylactic treatment regimes directed to inhibiting or otherwise slowing AD

development. In these situations, mapping the modulation of miRNA marker expression levels in a biological sample is a valuable indicator of the status of an individual or the effectiveness of a therapeutic or prophylactic treatment regime which is currently in use. Accordingly, a method contemplated herein extends to monitoring for changes in miRNA marker expression levels in an individual relative to their levels prior to treatment or during treatment, or relative to one or more earlier marker expression levels determined from a biological sample of the individual. Furthermore, taught herein is a medical protocol to treat a subject, the protocol comprising assessing or stratifying the subject with respect to AD by the assay herein described and then providing therapeutic, psychological and/or behavioral intervention to ameliorate any symptoms of AD.

[0070] Insofar as the sample comprises or is enriched for membranous microvesicles (e.g. exosomes), the membranous microvesicles may be derived from any suitable biological sample and may be either isolated from that sample or enriched therein. Methods for performing isolation or enrichment are known and it is within the skill of the person in the art to select and apply a method appropriate to the particular circumstances. For example, the microvesicles may be enriched for by subjecting the biological sample of which they are part to mechanical or chemical disruption such that nucleic acid material is released. The nucleic acid material would include miRNA.

[0071] When using biological fluids, generally, cellular contamination is removed by low speed centrifugation.

[0072] To the extent that it is sought to isolate and analyse the miRNA within the membranous microvesicles, it is necessary to lyse the microvesicles in order to expose their nucleic acid content and to thereafter analyse the miRNA subpopulation of nucleic acid molecules. To this end, the analysis of exosome RNA is often based on isolation of total RNA followed by PCR amplification of specific transcripts of interest. Methods for isolating and analysing total RNA are well known. Once a cell-free biological fluid is obtained, a high speed centrifugation spin can be used to pellet the exosomes which are lysed. Thus, the nucleic acids are exposed and extracted. However, a kit can also be used

which extracts the exosomal RNA without use of high-speed centrifugation.

[0073] There is a wide variety of methods which can be and have been used to isolate total RNA from samples.

[0074] miRNA amplification or probing steps require use of primers. Reference to a "primer" or an "oligonucleotide primer" should be understood as a reference to any molecule comprising a sequence of nucleotides, or functional derivatives or analogues thereof, the function of which includes hybridization to a region of a nucleic acid molecule of interest. It should be understood that the primer may comprise non-nucleic acid components. For example, the primer may also comprise a non-nucleic acid tag such as a fluorescent or enzymatic tag or some other non-nucleic acid component which facilitates the use of the molecule as a probe or which otherwise facilitates its detection or immobilization. The primer may also comprise additional nucleic acid components, such as the oligonucleotide tag. In another example, the primer may be a protein nucleic acid which comprises a peptide backbone exhibiting nucleic acid side chains. The design and synthesis of primers suitable for use in the subject assay would be well known to those of skill in the art.

[0075] Various techniques can be used to analyse an amplification product in order to determine relative miRNA expression levels. Their operational characteristics, such as ease of use or sensitivity, vary so that different techniques may be useful for different purposes. They include but are not limited to sequencing, pyrosequencing, enzyme digestion, microarray analysis, denaturing gradient gel electrophoresis, agarose gel based separation, melt curve analysis on real-time PCR cyclers, quantitative real-time PCR, denaturing high performance liquid chromatography, mass spectrometry, primer extension, oligonucleotide-ligation, mutation specific polymerase chain reaction, denaturing gradient, electrophoresis (DGGE), temperature gradient denaturing electrophoresis, constant denaturing electrophoresis, single strand conformational electrophoresis and denaturing high performance liquid chromatography (DHPLC).

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[0076] It is well within the skill of the person of skill in the art to select and apply an appropriate method of screening for the miRNA marker expression levels hereinbefore discussed.

[0077] Further enabled herein is an algorithm-based screening assay to screen biological samples from subjects for levels of selected miRNAs including a panel of miRNA. Generally, input data in the form of expression levels are collected based on miRNAs defined by SEQ ID NOs:1 through 16 or precursor forms thereof or their corresponding cDNA forms and subjected to an algorithm to assess the statistical significance of any elevation or reduction in levels which information is then output data. Computer software and hardware for assessing input data are encompassed by the present invention. In an embodiment, the samples include a sample comprising or enriched for membranous microvesicles, blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion or interstitial fluid.

[0078] Another aspect of the present invention contemplates a method of stratifying a subject with respect to AD a female, the method comprising subjecting the subject to a diagnostic assay to determine the levels of one or more of miRNAs defined by SEQ ID NOs:1 through 16 or precursor forms thereof or their corresponding cDNA forms to generate an index of probability of the subject having a stage of AD or is at risk of developing AD.

[0079] The present invention further provides the use the levels of one or more miRNAs selected from SEQ ID NOs:1 through 16 or precursor forms thereof or their corresponding cDNA forms in the generation of an index of probability for use in a diagnostic assay to predict the presence of AD or a risk of developing same.

[0080] The assay of the present invention permits integration into existing or newly developed pathology architecture or platform systems. For example, the present invention contemplates a method of allowing a user to determine the status of a subject with respect

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to AD, the method including:

- (a) receiving data in the form of levels of expression of one or more miRNAs defined by SEQ ID NOs:1 through 16 and/or from the user *via* a communications network;
- (b) processing the subject data *via* an algorithm which provides a likelihood index value;
- (c) determining the status of the subject in accordance with the results of the likelihood index value in comparison with predetermined values; and
- (d) transferring an indication of the status of the subject to the user *via* the communications network.

[0081] Conveniently, the method generally further includes:

- (a) having the user determine the data using a remote end station; and
- (b) transferring the data from the end station to the base station *via* the communications network.

[0082] The base station can include first and second processing systems, in which case the method can include:

- (a) transferring the data to the first processing system;
- (b) transferring the data to the second processing system; and
- (c) causing the first processing system to perform the algorithmic function to generate the likelihood index value.

[0083] The method may also include:

- (a) transferring the results of the algorithmic function to the first processing system; and
- (b) causing the first processing system to determine the status of the subject.

[0084] Reference to an "algorithm" or "algorithmic functions" as outlined above includes the performance of a multivariate analysis function. A range of different architectures and platforms may be implemented in addition to those described above. It will be appreciated that any form of architecture suitable for implementing the present invention may be used.

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However, one beneficial technique is the use of distributed architectures. In particular, a number of end stations may be provided at respective geographical locations. This can increase the efficiency of the system by reducing data bandwidth costs and requirements, as well as ensuring that if one base station becomes congested or a fault occurs, other end stations could take over. This also allows load sharing or the like, to ensure access to the system is available at all times. This particular method is also amenable for high throughput screening of ΔC_t levels based on qPCR wherein a ΔC_t value is selected for cut-off in terms of a subject having or is at risk of developing AD or who is healthy.

[0085] In this case, it would be necessary to ensure that another base station contains the same information and signature such that different end stations can be used.

[0086] It will also be appreciated that in one example, the end stations can be hand-held devices, such as PDAs, mobile phones, or the like, which are capable of transferring the subject data to the base station *via* a communications network such as the Internet, and receiving the reports.

[0087] In the above aspects, the term "data" means the levels or concentrations of the biomarkers. The "communications network" includes the internet. When a server is used, it is generally a client server or more particularly a simple object application protocol (SOAP).

EXAMPLES

[0088] Aspects disclosed herein are further described by the following non-limiting Examples. The ensuing Examples employ the following Materials and Methods.

Participants

[0089] Patients are divided into three groups: healthy controls (HC), mild cognitive impairment (MCI) and Alzheimer's disease (AD) based on the establish criteria from the National Institute of Neurological and Communicative Diseases and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDA-ADRDA) [McKhann *et al.* (1984) *Neurology* 34:939-944], which has been described in detail elsewhere (Ellis *et al.* (2009) *Int Psychogeriatr* 21:672-687). Blood samples are collected from all patients (fasting) from whole blood venepuncture into Sarstedt s-monovette serum-gel 7.5 ml tubes 01-1602-001 (Sarstedt, Germany). Serum-gel tubes are processed within two hours of collection and serum is snap frozen in liquid nitrogen. In the discovery set, serum from 49 patients (HC, 23; MCI, 3; AD, 23) are collected for deep sequencing and serum from 60 patients (HC, 36; MCI, 8; AD, 16) are collected for validation. All individuals included are assessed for full blood pathology testing (Melbourne Health and PathWest Laboratory Medicine, Melbourne, Australia), apolipoprotein $\epsilon 4$ (*ApoE $\epsilon 4$*) genotyping and assessment of cognitive functions (mini-mental state examination, MMSE) at the time of collection (Ellis *et al.* (2009) *supra*). Of these, 83 (discovery set, 23 and validation set, 60) patients had Amyloid-PET neuroimaging data available for assessment of cerebral A β accumulation. A β burden is expressed as (Cheng *et al.* (2013) *supra*) C-PiB standardized uptake value ratio (SUVR) as previously described (Villenmagne *et al.* (2013) *supra*). Patient data from both baseline and 54-month time points are available for analyses.

Procedures

[0090] Serum exosomal RNA is isolated by using the Plasma/serum exosomal RNA isolation kit (Norgen Biotek, Canada) from 1 ml serum per participant whereby the manufactures' protocol is followed. The total exosomal RNA yield, composition and quality is analyzed by the Agilent 2100 Bioanalyser using the Small RNA kit (Agilent).

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Exosomal RNA is converted into cDNA libraries using the Ion Total RNA-Seq Kit V2 (Life Technologies, Australia) and prepared for sequencing as described previously (Cheng *et al.* (2013) *Kidney International*). Pooled libraries with unique barcodes are loaded on 318 sequencing chips and run on the Ion Torrent Personal Genome Machine (Life Technologies, Australia) [Cheng *et al.* (2013) *supra*]. The sequences are then assessed for quality and primer-adaptor sequences are trimmed by the Torrent Suite software (version 3.4.1), followed by alignment to the human reference genome (HG19). The trimmed and aligned data is transferred to Partek Genomics Suite (Partek, Singapore) and mapped to known miRNA using miRBase V.20. Bioinformatics analysis and differential expression is performed using Partek Genomics Suite. The panel of candidate miRNA found highly associated with AD (including positive and negative controls) are used for validation on a new set of serum samples collected from AIBL. Serum samples for the validation study are spiked with synthetic *C. elegans* miR-39 (Qiagen, Australia) during exosomal RNA extraction to monitor extraction efficiency and for normalization purposes. Upon processing of the serum samples as above, 1ng of exosomal miRNA is converted to cDNA (TaqMan MicroRNA Reverse Transcription Kit, Applied Biosystems, Australia) according to the manufacturers' protocol with a primer pool containing 23 miRNA assays (TaqMan microRNA assays, 5x, Applied Biosystems). cDNA samples are pre-amplified (TaqMan PreAmp Master Mix Kit, Applied Biosystems) and qRT-PCR (TaqMan Fast Advanced Master Mix, Applied Biosystems) is performed using individual miRNA assays (TaqMan microRNA assays, 20x, Applied Biosystems) and run on the ViiA (Trade Mark) 7 Real-Time PCR System (Life Technologies) across a 384-well format. Reverse transcription and pre-amplification no template controls using primer pools and individual assays are also prepared to ensure there is no background amplification of miRNA assays. Raw data are uploaded to DataAssist (Applied Biosystems) to calculate delta Ct (ΔCt) and normalization against controls further statistical analysis.

Statistical analysis

[0091] To analyze the deep sequencing data, the number of reads of each miRNA are normalized to reads per million (RPM) across all samples. Low abundant miRNAs with less than 50 read counts across all samples are removed thus high abundant miRNAs are

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analyzed. Initial statistical analysis of miRNA expression changes is performed using the Partek Genomics Suite. Selection of miRNAs is based upon ANOVA comparing healthy control and AD groups (clinical classification at the time of collection). Probability values are adjusted for multiple testing using the False Discovery Rate (FDR) method. Significant changes in miRNA expression are expressed in fold change [LOG_2] and defined as $p(\text{AD Vs HC}) \leq 0.05$, $p(\text{AD, MCI and HC}) \leq 0.05$ and ± 1.2 fold change. Generalized Linear Modelling is performed to compare each miRNA between clinical classification chosen from the initial analyses adjusted for age, gender and ApoE ϵ 4 allele status. Further classification (discovery data set) and prediction (validation data set) analyses are performed using Random Forest analyses. From these models, sensitivity and specificity and related statistics are calculated. All statistical analyses are performed using the R statistical environment, version 3.02.

EXAMPLE 1***Exosomal miRNA discover set analyses***

[0092] The characteristics of the participants included in this study are shown in Table 3. The prevalence of ApoE ϵ 4 is significantly higher in the AD group compared with health controls ($p = 0.006$). AD patients performed significantly poorer in the MMSE ($p = 0.006$) and obtained low clinical dementia ratings [CDR] ($p < 0.0001$) and composite scores (composite score 1: $p < 0.0001$, composite score 2: $p < 0.0001$). Deep sequencing of the exosomal RNA extracted from serum samples is performed and sequences are mapped to miRBase V.20. Approximately half of the reads obtained (43%) from small RNA sequencing are miRNA. Overall, the sample cohort mapped to 1419 known miRNA. Upon performing normalization of reads and performing ANOVA analyses, 17 miRNA are found to be significantly deregulated (p_{ANOVA} (AD Vs HC) ≤ 0.05 , p_{ANOVA} (AD, MCI and HC) ≤ 0.05 and ± 1.2 fold change, Figure 1); 14 miRNA are found to be up-regulated (hsa-miR-361-5p, hsa-miR-30e-5p, hsa-miR-93-5p, hsa-miR-15a-5p, hsa-miR-143-3p, hsa-miR-335-5p, hsa-miR-106b-5p, hsa-miR-101-3p, hsa-miR-424-5p, hsa-miR-106a-5p, hsa-miR-18b-5p, hsa-miR-3065-5p, hsa-miR-20a-5p, and hsa-miR-582-5p) and 3 miRNA are found to be down-regulated (hsa-miR-1306-5p, hsa-miR-342-3p, and 15b-3p, Table 3). Upon post adjustment of age, ApoE ϵ 4 allele status and sex (Generalized Linear Modelling), the majority of miRNA remained statistically significant upon contrasting within all attribute groups (AD, MCI and HC) and between AD and HC (Table 4). The miRNAs listed above are defined by sequence identifier number (Table 1).

[0093] Upon performing Partitioning Around Medoids (PAM) clustering of the 17 miRNA, two main clusters are found, one for those miRNAs up-regulated in AD, and one for those down-regulated in AD. Further investigation of these revealed four clusters (Figure 2A), with stepwise increases in miRNA expression across ApoE ϵ 4 allele carriers and clinical classification (Figure 2B). Delineation into miRNA cluster averages, showed clear associations with ApoE ϵ 4 allele status (Figure 2C), confirming the stepwise increases shown by assessing miRNA by cluster. Variable selection via Random Forest analyses

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(Figure 2D) defined hsa-miR-1306-5p as contributing more towards clinical classification than age, indicating this miRNA is important in class separation.

[0094] Receiver Operating Characteristic (ROC) analyses statistics for each miRNA marker are shown in Table 5. The ROC analyses is performed using the raw sequence count data to estimate the performance of each miRNA to predict AD. Criterion cut points are chosen by taking the closest point on the curve to the top left corner of the ROC curve; approximating the most optimum performance for each marker. Independently, the miRNAs displayed between 35 and 100% sensitivity and specificity. Similar to the Random Forest model, hsa-miR-1306-5p showed the greatest combined, sensitivity and specificity (87.5% and 70%, $p = 0.0008$).

EXAMPLE 2***qRT-PCR validation set analyses***

[0095] All patients in the validation set (n = 60, Table 3) went through full assessment including neuroimaging performed at baseline (prior to this study) and 54 months (time of collection). Samples are spiked with synthetic cel-miR-39 as an external control and three highly abundant miRNA are run as potential endogenous controls (hsa-miR-451, hsa-miR-223-3p, and hsa-miR-339-5p.). For quality control assurance and normalization controls, cel-miR-39 is used as an external control and hsa-miR-451 is chosen as the endogenous control owing to its stable expression across all samples. Upon completion of the qRT-PCR assay, two patient samples did not display appropriate threshold levels of cel-miR-39 and hsa-miR-451, thus did not fulfil quality control measures and are removed. In addition, hsa-miR-3065-5p is found to be undetected (Ct >35) in 40 samples. As qRT-PCR could not detect hsa-miR-3065-5p in all patient samples by qRT-PCR, this miRNA is removed from the list of biomarker candidates.

[0096] Model validation (Random Forest, Figure 2D) using the 16 miRNA markers, age, gender and ApoEε4 allele status with 54 month clinical classification correctly diagnosed 13 out of 15 AD patients (sensitivity 87%, Table 6) and confirmed 27 out of 35 healthy control patients (specificity 77%, Table 6). Five HC patients (samples 7, 9, 16, 34 and 51) who are incorrectly classified as having AD are found to have high Aβ burden with an SUVR of greater than 1.5 across four time points of the AIBL longitudinal study, suggestive of future risk of clinical manifestation of AD. Three HC patients (sample 8, 17 and 39) had low SUVR, but who are APOE ε4 carriers are also classified as having AD. Case by case information is presented in Table 6. Box plots of qRT-PCR data for each miRNA marker stratified by clinical classification are presented in Figure 3.

[0097] As the cohort size for MCI patients is small, the model could not be trained to predict progression from HC to MCI or MCI to AD. Instead HC and AD thresholds are applied to predict the MCI patients in the validation set. Of the eight MCI patients at 54 months, two are classified as having AD (samples 27 and 53), while the remaining six are

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classified as HC (Table 6). Further investigation using levels of neocortical Beta Amyloid burden show those MCI patients classified as having AD had high levels of SUVR (greater than 1.5), suggesting that these patients may be on an AD type dementia pathway. Furthermore, three MCI patients who are classified as HC all have low SUVR and are negative for APOE ϵ 4, suggesting that they are not on an AD type dementia pathway. The remaining patients (samples 26, 28, 37, 49 and 29) incorrectly classified do not carry APOE ϵ 4 and are classified as HC. Interestingly a clear outlier (sample 49), which is APOE ϵ 4 negative, displayed the highest SUVR of 3.03 and a MMSE score of 10 at 54 months, was classified as healthy using miRNA biomarkers.

EXAMPLE 3***Development of assay***

[0098] The use of exosomes compared to cell-free or whole blood has a number of disease specific advantages for diagnostic purposes. First, isolating the enriched miRNA in exosomes from biological samples of AD patients removes the saturation of insignificant miRNA expressed in both AD and HC patients thus, providing a better performing predictive AD diagnosis. Secondly, miRNA originating from the brain have been demonstrated to cross the endothelial cellular layers of the blood brain barrier by transcytosis of exosomes across the endothelial layer in order to communicate between the brain and distant organs *via* biological fluids (Haqqani *et al.* (2013) *Fluids barriers CNS* 10:4). Thirdly, evidence has revealed that exosomes serve as a RNase-protective vesicle which shield miRNA from RNase-rich environments such as the circulatory system (Huang *et al.* (2013) *Bmc Genomics* 14:319). Furthermore, specific packaging of miRNA into exosomes (Gibbings *et al.* (2009) *Nat Cell Biol* 11:1143-1149) and increased secretion of microvesicles into peripheral blood of cancer patients compared to healthy patients (Mitchell *et al.* (2008) *supra*) suggests the importance of exosomes in the role of extracellular communication during disease. These factors allow the possibility of profiling disease specific miRNA that are found enriched from exosomes.

[0099] This study produced a large coverage of miRNA (1419 known miRNA) extracted from exosomal serum samples isolated for AD biomarker discovery by next-generation sequencing (NGS). The advantage of NGS is its ability to detect absolute counts of all miRNA present in samples. However, in order to successfully validate miRNA assays by qRT-PCR technology for diagnostic assays, miRNAs need to lie within the detectable range of qRT-PCR. Using the workflow carried out in this study, it is estimated that approximately 50 RPM must be obtained across the majority of biological samples for successful biomarker validation by qRT-PCR. Biomarkers studies attempting to validate a large panel consisting of low abundant miRNAs will result in unsuccessful validation of miRNAs and/or changes in direction of expression (Leidinger *et al.* (2013) *Genome Biol* 14:R78; Kumar *et al.* (2013) *PLoS ONE* 8:e69807). In this study, from the 1419 mapped

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miRNA in this cohort, 220 highly abundant miRNA are present in all samples across the study. Seventeen significantly differentially expressed exosomal miRNA are found in AD patients and 16 miRNA are successfully validated displaying consistent expression changes with AD and HC across both NGS and qRT-PCR data sets. The commonly used comparative delta Ct approach in qRT-PCR may not be applicable for diagnostic use as a healthy control group is required for every run. Thus, normalized delta Ct is applied into a Random Forest model to predict clinical classification. Such a model is able to predict clinical classification (at 54 months) with high accuracy (87% sensitivity and 77% specificity).

[0100] A possible limitation in the assay is an ability to diagnose those with late AD where the rate of pathogenesis, in particular A β deposition, slows in the later stages of AD when patients experience cerebral atrophy and severe cognitive impairment (Villenmagne *et al.* (2013) *supra*). However, in that case diagnosis is less critical and the assay may be used with other markers. Patients of the AIBL study are diagnosed according to NINCDS-ADRDA criteria, which is heavily reliant on cognitive and neuropsychological testing for a clinical diagnosis of probable AD. Thus, in this study, the diagnosis obtained by miRNA analysis is compared with the participant's classification by NINCDS-ADRDA criteria. The additional strength of this study comes from the extensive database of metadata collected at baseline through to 54 months. Each participant underwent a battery of assessment including APOE ϵ 4 genotyping, cognitive examination and Amyloid-PET neuroimaging. Amyloid-PET and ApoE ϵ 4 genotyping information for those HC patients incorrectly classified with AD suggested progression towards AD. Taking into consideration that these HC patients could actually be prodromal AD, the predicted specificity would increase to 91.4%. Ultimately, the objective and purpose of the exosomal miRNA biomarker test is to predict future cognitive decline in asymptomatic or presymptomatic individuals and during the progression of patients with early dementia. This study represents a significant step towards developing a cost-effective, non-invasive and low risk diagnostic test to detect the onset and monitor various stages of AD in order for physicians to provide optimal care for patients.

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[0101] Here, serum samples are analyzed for exosomal miRNA associated with AD together with metadata which included cognitive and amyloid-PET imaging. A16-miRNA expression profile is generated for the diagnosis of AD with sensitivity and specificity of 87% and 77%, respectively. Importantly, the exosomal miRNA AD signature is able to predict cognitively healthy patients presenting with high amyloid burden as probable AD.

[0102] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure contemplates all such variations and modifications. The disclosure also enables all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features or compositions or compounds.

Table 3
Demographics and clinical make up of discovery and validation set

Discovery Set	HC	MCI	AD	p value*
N	23	3	23	
Age (mean $\pm$ SD)	73.07 (7.57)	76.91 (4.4)	78.89 (7.33)	0.034
Gender (F/M)	10/13	1/2	13/9	0.484
ApoE4 (-ve/+ve)	18/5	1/2	7/15	0.006
MMSE (mean $\pm$ SD)	29 (1.25)	23 (5.66)	15 (8.66)	<0.0001
Composite score 1 (mean $\pm$ SD)	0.26 (0.73)	-2.04 (0.80)	-2.41 (0.25)	<0.0001
Composite score 2 (mean $\pm$ SD)	0.10 (1.10)	-1.79 (1.18)	-2.10 (0.90)	<0.0001
Validation Set	HC	MCI	AD	p value*
N	36	8	16	
Age (mean $\pm$ SD)	78.55 (6.52)	78.91 (6.3)	78.29 (7.55)	0.963
Gender (F/M)	21/14	4/4	10/5	0.737
ApoE4 (-ve/+ve)	27/8	6/2	2/13	0.0001
MMSE (mean $\pm$ SD)	30 (1.08)	29 (2.03)	21 (8.14)	<0.0001
Composite score 1 (mean $\pm$ SD)	0 (0.59)	-1.16 (0.65)	-2.02 (0.65)	<0.0001
Composite score 2 (mean $\pm$ SD)	0 (0.91)	-0.63 (0.7)	-1.64 (0.4)	0.046

*MMSE = mini-mental state examination. APOE = apolipoprotein E. *Compared with healthy controls*

Table 4
Number of reads obtained from deep sequencing data per miRNA across each clinical classification

Cluster	miRNA	HC	MCI	AD	Fold change	P Value	p value (GLM)* All groups (Unadjusted)	All groups (Adjusted)	HC vs AD (Unadjusted)	HC vs AD (Adjusted)
1	hsa-miR-30e-5p	Mean (SD) 85.38 (18.81)	Mean RPM (SD) 160.39 (34.93)	Mean RPM (SD) 111.26 (35.59)	1.30	1.10E-04	0.02	0.03	0.01	0.01
1	hsa-miR-101-3p	75.14 (20.29)	164.98 (118.45)	107.38 (63.26)	1.43	0.01	0.10	0.44	0.07	0.01
1	hsa-miR-15a-5p	2472.22 (769.66)	4168.34 (2182.56)	3254.91 (1407.36)	1.32	0.02	0.05	0.13	0.04	0.01
1	hsa-miR-20a-5p	1774.54 (484.33)	3191.99 (1824.21)	2818.21 (1568.42)	1.59	9.53E-03	0.02	0.10	0.02	0.02
1	hsa-miR-93-5p	2577 (601.67)	3941.08 (1307.43)	3389.06 (1236.83)	1.32	9.26E-03	0.03	0.18	0.03	0.02
1	hsa-miR-106b-5p	658.56 (209.64)	1244.95 (693.21)	900.7 (462.92)	1.37	0.02	0.09	0.40	0.07	0.01
1	hsa-miR-18b-5p	64.7 (20.93)	105.09 (53.34)	96.48 (42.46)	1.49	6.85E-03	0.01	0.05	0.01	0.03
1	hsa-miR-106a-5p	318.95 (84.52)	396.55 (130.17)	450.94 (218.61)	1.41	0.03	0.04	0.23	0.05	0.01
2	hsa-miR-1306-5p	46.72 (22.26)	49.45 (14.6)	30.72 (15.19)	0.66	0.01	0.01	0.05	0.01	0.04
3	hsa-miR-3065-5p [#]	34.45 (23.2)	54.11 (7.36)	52.86 (27.71)	1.53	0.04	0.01	0.10	0.01	0.02
3	hsa-miR-582-5p	15.84 (10.82)	29.42 (16.55)	25.41 (12.43)	1.60	0.02	0.01	0.06	0.01	0.02
3	hsa-miR-143-3p	111.69 (53.6)	181.94 (51.29)	149.21 (52.8)	1.34	0.02	0.02	0.07	0.02	0.03

The average number of total reads obtained per sample was 780, 371. Reads were mapped to 1419 known mature miRNA using miRBase V-20. miRNA with lower than 50 reads across all samples were removed leaving 225 abundant miRNA for analysis. Raw reads were normalized to read per million (RPM). RPM values for each sample. ANOVA analysis was performed by comparing cohort groups with healthy controls. *P values are the result of Generalized Linear Modeling pre and post adjustment with age, sex and APOE ε4 allele. miRBase v.20 accession numbers. [#]Hsa-miR-3065-5p was not used in the validation set as it was found to be undetectable using qRT-PCR.

Table 5*Receiver operating characteristics of miRNA exosomal Alzheimer's disease biomarkers*

miRNA	AUC (95% CI)	p value	Sensitivity	Specificity	Threshold
hsa-miR-30e-5p	75.31 (57.92 - 92.71)	0.01	56.25	90.00	108.07
hsa-miR-101-3p	60.31 (39.15 - 81.48)	0.31	43.75	100.00	117.01
hsa-miR-15a-5p	70.00 (52.25 - 87.75)	0.04	37.50	100.00	3741.71
hsa-miR-20a-5p	64.69 (45.04 - 84.34)	0.14	43.75	100.00	2803.99
hsa-miR-93-5p	67.50 (48.33 - 86.67)	0.08	37.50	100.00	3869.47
hsa-miR-106b-5p	57.50 (36.66 - 78.34)	0.46	43.75	90.00	866.54
hsa-miR-18b-5p	71.25 (53.33 - 89.17)	0.03	50.00	100.00	101.53
hsa-miR-106a-5p	62.81 (41.53 - 84.09)	0.20	50.00	90.00	391.25
hsa-miR-1306-5p	81.88 (67.37 - 96.39)	0.0008	87.50	70.00	34.99
hsa-miR-582-5p	70.94 (53.59 - 88.29)	0.03	43.75	95.00	26.88
hsa-miR-143-3p	71.25 (53.66 - 88.84)	0.03	87.50	55.00	111.39
hsa-miR-335-5p	66.88 (48.86 - 84.89)	0.09	93.75	35.00	119.78
hsa-miR-361-5p	68.13 (50.34 - 85.91)	0.07	93.75	50.00	93.82
hsa-miR-424-5p	74.06 (57.62 - 90.50)	0.01	50.00	90.00	208.91
hsa-miR-342-3p	69.06 (51.46 - 86.66)	0.05	81.25	55.00	1146.51
hsa-miR-15b-3p	66.25 (48.15 - 84.35)	0.10	100.00	30.00	184.99

AUC=Area under receiver operating curve. CI = Confidence interval.

Table 6
Demographics, clinical make up and classification of patients of the validation set at baseline and 54 months of the AIBL flagship study

Sample #	Age	Gender	ApoEε4	MMSE Baseline	MMSE 54 m	PiB SUVR Baseline	PiB SUVR 54 m	Class. Baseline	Class. 54 m	Validation Class.
<i>Patients diagnosed correctly according to clinical classification</i>										
2	67	F	+	28	24	2.57	2.97	AD	AD	AD
46	73	F	+	25	21	2.04	NI	AD	AD	AD
50	59	F	+	23	25	1.59	NI	AD	AD	AD
40	69	F	+	30	26	2.6	NI	HC	AD	AD
48	78	M	+	30	16	2.25	2.39	HC	AD	AD
43	68	M	+	30	17	2.27	2.55	HC	AD	AD
5	75	F	+	27	21	2.64	2.82	MCI	AD	AD
4	84	M	+	26	23	2.44	2.48	MCI	AD	AD
58	77	F	+	27	26	2.06	NI	MCI	AD	AD
59	80	F	+	26	26	2.2	NI	MCI	AD	AD
55	76	F	+	27	27	2.1	2.31	MCI	AD	AD
47	84	F	+	22	2	2.55	NI	MCI	AD	AD
1	69	M	+	26	10	2.16	NI	MCI	AD	AD
3	81	F	-	29	29	1.06	1.13	HC	HC	HC
23	81	F	-	28	30	1.16	1.07	HC	HC	HC
12	81	F	-	30	30	1.26	1.24	HC	HC	HC
13	88	F	-	30	30	2.18	2.3	HC	HC	HC
19	80	M	-	30	29	2.03	2.41	HC	HC	HC
42	86	F	-	30	30	1.36	NI	HC	HC	HC
10	74	F	-	30	30	1.14	1.04	HC	HC	HC
11	63	M	-	29	28	1.36	1.31	HC	HC	HC
36	65	F	-	30	30	1.18	1.23	HC	HC	HC
32	72	F	-	26	29	1.35	1.61	HC	HC	HC
25	78	M	-	28	29	2.01	2.27	HC	HC	HC
18	78	F	-	29	28	1.87	NI	HC	HC	HC
15	76	F	-	28	26	1.79	2.03	HC	HC	HC
14	74	F	-	28	28	1.07	1.12	HC	HC	HC
22	76	F	-	28	30	1.13	NI	HC	HC	HC
20	76	M	-	26	29	1.27	1.25	HC	HC	HC
21	74	F	-	27	28	1.02	1.02	HC	HC	HC

Sample #	Age	Gender	ApoEε4	MMSE Baseline	MMSE 54 m	PiB SUVR Baseline	PiB SUVR 54 m	Class. Baseline	Class. 54 m	Validation Class.
35	67	F	-	29	30	1.08	1.04	HC	HC	HC
33	63	M	-	30	29	1.06	1.05	HC	HC	HC
45	61	F	-	29	30	1.11	1.22	HC	HC	HC
38	72	M	-	30	30	1.21	1.21	HC	HC	HC
44	71	M	-	30	30	1.22	1.3	HC	HC	HC
52	74	M	-	30	27	1.15	1.09	HC	HC	HC
54	77	M	-	30	30	1.18	1.3	HC	HC	HC
60	61	M	-	29	30	1.07	1.03	HC	HC	HC
56	76	F	-	29	28	1.19	1.39	HC	HC	HC
24	72	M	-	29	30	1.17	1.2	HC	HC	HC
<i>Patients misdiagnosed from clinical classification</i>										
9	73	F	+	29	29	1.69	1.75	HC	HC	AD
51	82	M	+	30	29	2.33	2.37	HC	HC	AD
7	79	F	+	28	27	2.07	NI	HC	HC	AD
16	74	M	+	29	30	1.35	1.52	HC	HC	AD
34	72	M	+	29	30	1.57	1.78	HC	HC	AD
17	76	F	+	30	30	1.11	1.08	HC	HC	AD
8	69	F	+	30	30	1.21	NI	HC	HC	AD
39	68	F	+	30	30	1.06	1.15	HC	HC	AD
27	70	M	+	26	28	2.05	2.56	MCI	MCI	AD
53	76	M	+	26	30	1.65	2.06	MCI	MCI	AD
57	82	F	-	29	29	1.1	1.22	MCI	MCI	HC
41	78	F	-	27	30	1.05	1.2	HC	MCI	HC
6	79	M	-	27	29	1	1.03	MCI	MCI	HC
26	68	F	-	30	29	1.4	NI	MCI	MCI	HC
28	78	M	-	28	25	2.16	2.38	MCI	MCI	HC
37	64	F	-	30	25	1.62	1.82	MCI	MCI	HC
49	82	M	-	20	10	3.04	3.03	AD	AD	HC
29	65	F	-	24	6	1.92	2.12	MCI	AD	HC

Positive APOE ε4 is indicated by "+". Negative for APOE ε4 is indicated as "-". MMSE = mini-mental state examination. Classification obtained using miRNA exosomal biomarkers are indicated under validation classification. PiB = Carbon-11-labelled Pittsburgh compound B. SUVR = standard uptake value ratio. Patients who presented with a low Aβ burden in the brain have a SUVR <1.5. Patients who presented with high Aβ burden in the

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brain have a $SUVR > 1.5$.²Class. = Classification. Clinical classification at baseline and 54 months is diagnosed by NINCDS-ADRDA criteria for AD. Classification obtained using miRNA exosomal biomarkers are indicated under validation classification.

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CLAIMS:

1. An assay to stratify a human subject with respect to Alzheimer's disease (AD) or a symptom associated with AD or other amyloidosis-related condition, said method comprising screening a biological sample from the subject for an miRNA comprising a nucleotide sequence selected from the list consisting of SEQ ID NOs:1 through 17 or precursor forms thereof or corresponding cDNA forms wherein the level of expression of a selected miRNA or ratio of levels of expression from 2 or more of SEQ ID NOs:1 through 17 compared to a control is indicative of a stage in the development of AD or the amyloidosis-related condition.
2. The assay of Claim 1 wherein the sample is as sample comprising or enriched for membranous microvesicles, blood, serum, plasma, urine, lymph, cerebrospinal fluid, ascites, saliva, mucus, stool, biopsy specimens, breast milk, gastric juice, pleural fluid, semen, sweat, tears, hair, vaginal secretion or interstitial fluid.
3. The assay of Claim 2 wherein the sample comprises or is enriched for membranous microvesicles or free circulating miRNA released from membranous microvesicles.
4. The assay of Claim 3 wherein the membranous microvesicles are serum derived exosomes.
5. The assay of Claim 1 wherein the miRNA comprises a nucleotide sequence selected from the listing consisting of SEQ ID NOs:1 through 17 or precursor forms thereof.
6. The assay of Claim 5 wherein the level of expression of miRNAs defined by SEQ ID NOs:1 through 13 or a ratio of expression level of 2 or more of SEQ ID NOs:1 through 13 compared to a control is indicative of a stage in the development of AD.

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7. The assay of Claim 5 wherein the level of expression of miRNA defined by SEQ ID NOs:14 through 16 or ratio of expression levels of 2 or more of SEQ ID NOs:14 through 17 compared to a control is indicative of a stage in the development of AD.
8. The assay of Claim 6 wherein the level of expression of miRNA defined by SEQ ID NOs:1 through 13 is up-regulated relative to a healthy control.
9. The assay of Claim 7 wherein the level of expression of miRNAs defined by SEQ ID NOs:14 through 16 is down-regulated relative to a healthy control.
10. The assay of Claim 1 wherein the control is a ΔCt cut-off value based on a correlation of ΔCt levels determined by qPCR and AD or a correlation of ratios of ΔCt levels.
11. The assay of Claim 5 wherein the level of expression of 2 to 16 of the miRNAs is determined.
12. The assay of Claim 11 wherein the level of expression of the miRNA defined by SEQ ID NO:16 is determined.
13. The assay of any one of Claims 1 to 12 further comprising a psychological, behavioral, physiological and/or genetic assessment.
14. The assay of Claim 13 wherein the psychological assessment determines the presence and/or level of cognitive impairment.
15. The assay of Claim 13 wherein the genetic assessment comprises a determination of the APOE ϵ 4 genotype, wherein the presence of one or more alleles of APOE ϵ 4 in combination with altered expression of the miRNA relative to a control is an indicator of AD or a risk of developing same.

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16. The assay of Claim 13 wherein the physiological assessment is amyloid-PET neuroimaging.
17. The assay of any one of Claims 1 to 16 wherein the stage of AD is asymptomatic or presymptomatic.
18. The assay of any one of Claims 1 to 16 wherein the stage of AD is early onset.
19. The assay of any one of Claims 1 to 16 wherein the stage of AD results in mild cognitive impairment.
20. The assay of any one of Claims 1 to 19 wherein the miRNAs are amplified with primers specific to one or more of the miRNAs.
21. The assay of Claim 20 wherein the miRNAs are immobilized to a solid support prior to amplification or miRNA-specific amplicons are immobilized to a solid support.
22. The assay of Claim 1 used to monitor a therapeutic protocol for the treatment of AD in a subject.
23. A kit comprising components and reagents when used in the assay of any one of Claims 1 to 21.
24. A medical protocol to treat a subject, said protocol comprising assessing or stratifying the subject with respect to AD by the assay of Claim 1 and then providing therapeutic, psychological and/or behavioral intervention to ameliorate any symptoms of AD.

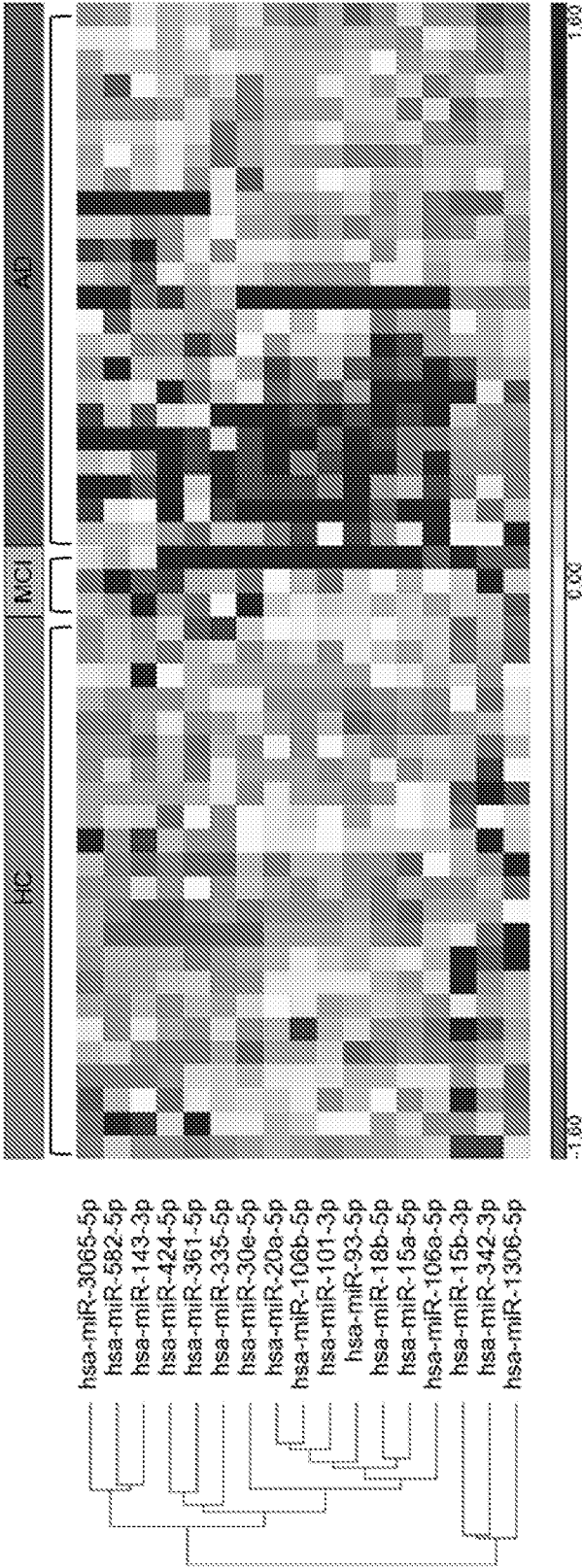
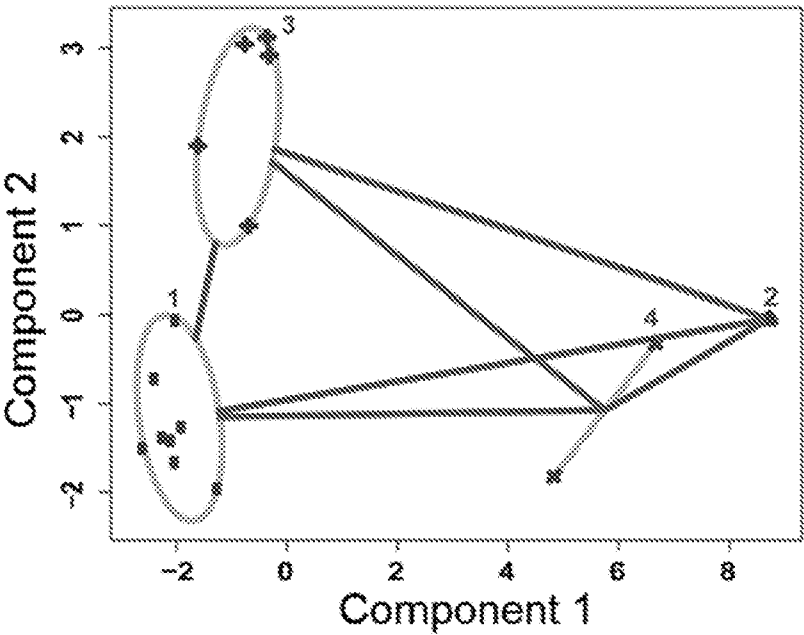


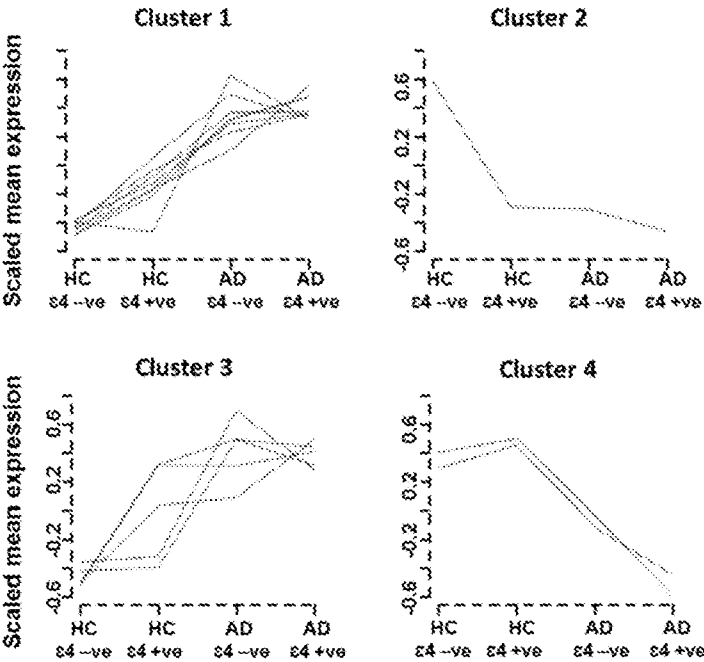
Figure 1

2/5

A



B



Figures 2A and B

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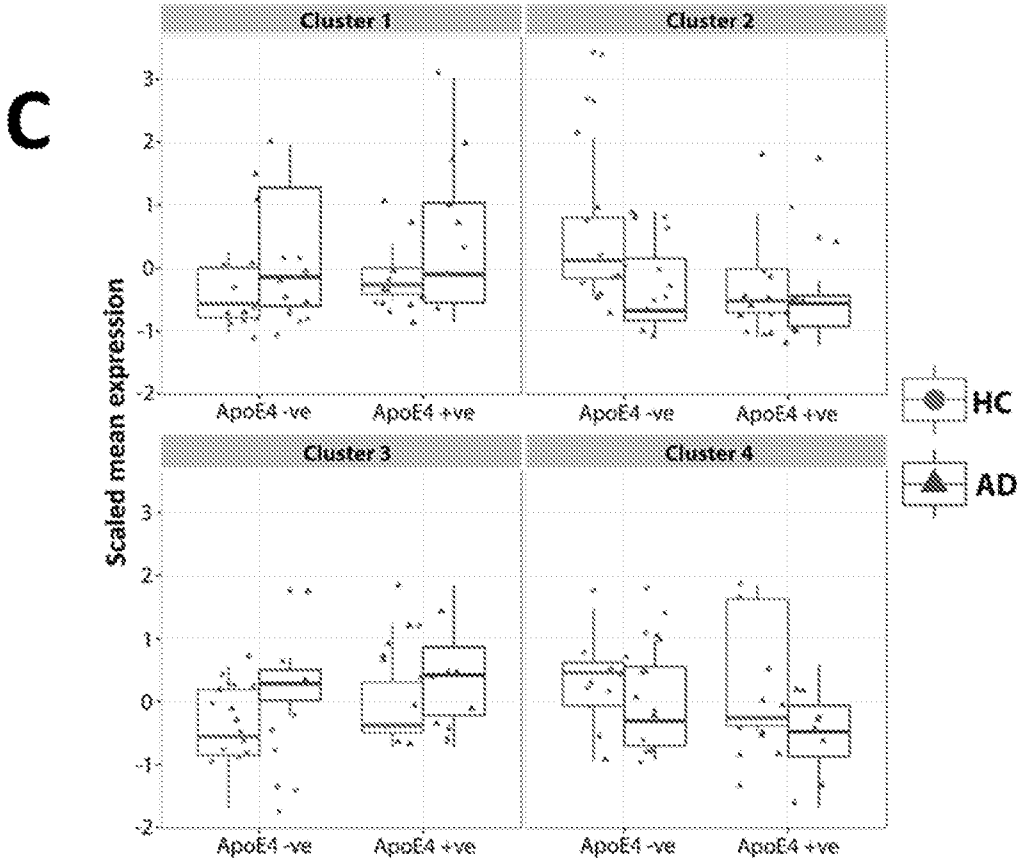


Figure 2C

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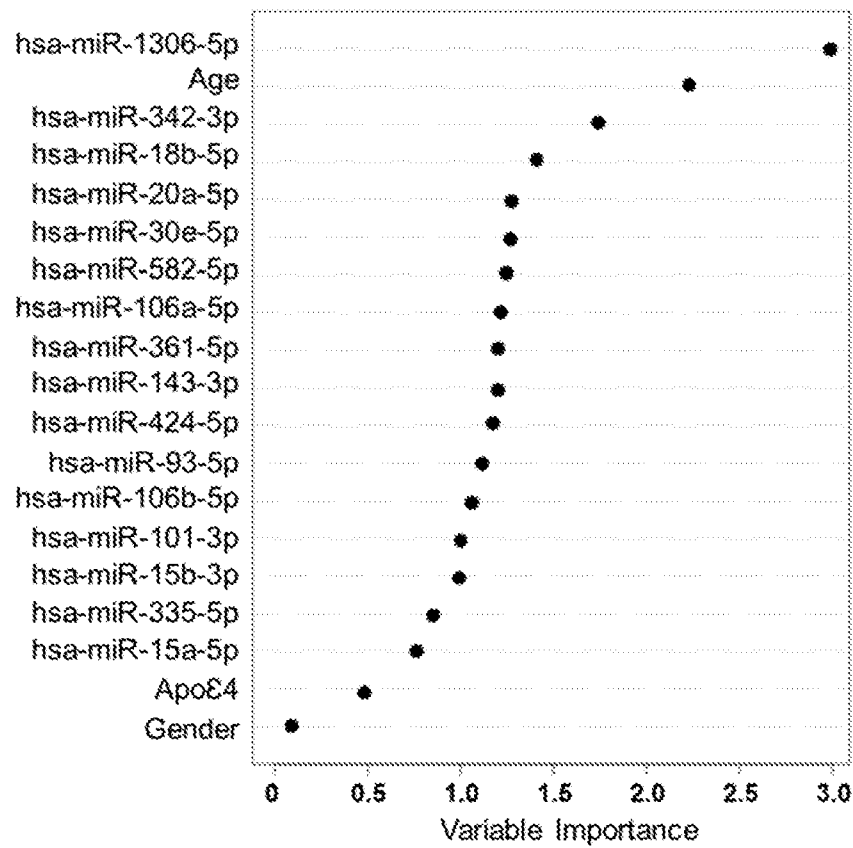


Figure 2D

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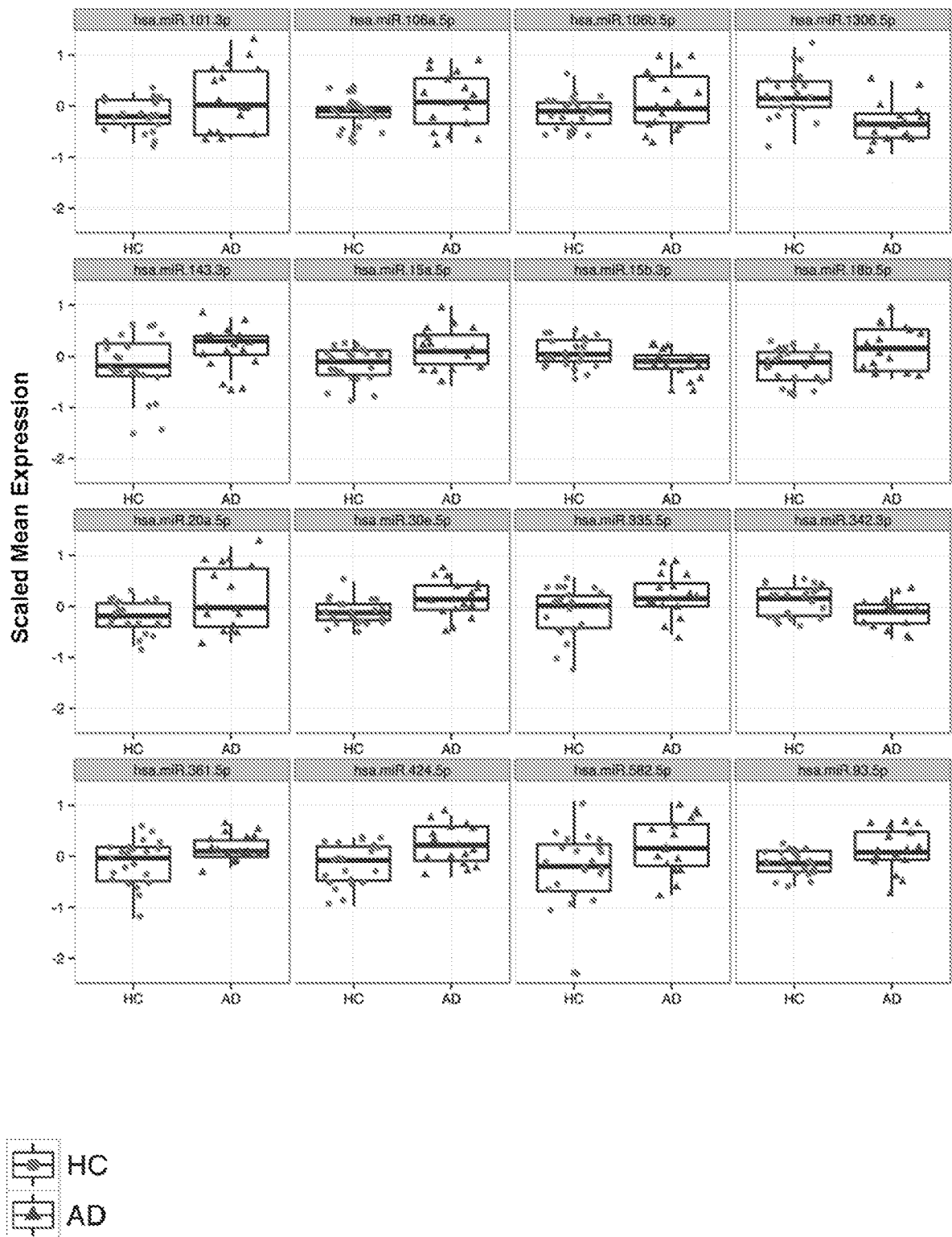


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/050264

A. CLASSIFICATION OF SUBJECT MATTER C12Q 1/68 (2006.01)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) GenomeQuest: SEQ ID 1, 7, 9, 14, 16; CAPLUS, MEDLINE, BIOSIS, EMBASE via STN; EPODOC, WPIAP, \$PATEN via EPOQUE; Google: mir-30e, mir-18b, mir-582, mir-342, mir-1306, alzheimers, amyloid, hill, cheng, doecke		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 22 June 2015	Date of mailing of the international search report 22 June 2015	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Felix White AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832565	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2015/050264
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Lau, P, et al "Alteration of the microRNA network during the progression of Alzheimer's disease", EMBO Molecular Medicine, 2013, vol. 5, pps 1613-1634	
A	WO 2014/075911 A1 (SIEMENS AKTIENGESELLSCHAFT et al.) 22 May 2014	
P,X	Cheng, L. et al "Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment", Molecular Psychiatry, 2014, doi:10.1038/mp.2014.127, advance online publication 28 October 2014 Whole document	1-24
X Y	Cogswell, J.P. et al "Identification of miRNA Changes in Alzheimer's Disease Brain and CSF Yields Putative Biomarkers and Insights into Disease Pathways" Journal of Alzheimer's Disease, 2008, vol 14, pps 27-41 Fig 1 Fig 1	1-2, 5-6, 8, 10, 11, 13-24 3-4
X Y	US 2012/0108650 A1 (JIN et al) 03 May 2012 [0022] [0022]	1-2, 5-6, 10, 11, 13-24 3-4
X Y	Meng, F. et al "Constructing and characterizing a bioactive small molecule and microRNA association network for Alzheimer's disease" Journal of the Royal Society Interface, 2014, vol. 11, 20131057 (March 6 2014) Paragraph 3.1 and Table S5 Paragraph 3.1 and Table S5	1-2, 5-6, 10, 11, 13-24 3-4
X Y	WO 2012/105826 A1 (ERASMUS UNIVESITY MEDICAL CENTER ROTTERDAM) 09 August 2012 Examples, Claims Examples, Claims	1-2, 5-6, 10, 11, 13-24 3-4
X Y	EP 2617433 A2 (SNU R&DB FOUNDATION) 24 July 2013 Claim 9 Claim 9	1-2, 5-6, 10, 11, 13-24 3-4
X Y	WO 2009/009457 A1 (UNIVERSITY OF LOUISVILLE RESEARCH FOUNDATION, INC.) 15 January 2009 Table 1 Table 1	1-2, 5-6, 8, 10, 11, 13-24 3-4
X Y	WO 2013/024469 A1 (ROSETTA GENOMICS LTD.) 21 February 2013 Table 2b Table 2b	1-2, 5, 7, 9-11, 13-24 3-4
X Y	Montag, J. et al "Upregulation of miRNA hsa-miR-342-3p in experimental and idiopathic prion disease" Molecular Neurodegeneration, 2009, vol. 4: 36 Abstract Abstract	1-2, 5, 7, 10, 11, 13-24 3-4
Form PCT/ISA/210 (fifth sheet) (July 2009)		

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2015/050264
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Augustin, R. et al "Computational identification and experimental validation of microRNAs binding to the Alzheimer-related gene ADAM10" BMC Medical Genetics, 2012, 13:35 Table 1, Fig 3	1-24
Y	Cheng, L. et al "Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood" Journal of Extracellular Vesicles, 2014, 3:23743 (March 26 2014) Page 13 l.h. column	3-4
Y	WO 2010/056337 A2 (CARIS MPI, INC.) 20 May 2010 Abstract, Fig 42, [0527]	3-4
P,X P,Y	WO 2015/006705 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 15 January 2015 Table 12, p. 62 Table 12, p. 62	1-2, 5-6, 8, 10-11, 13-24 3-4

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

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

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.:

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.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- The use of each miRNA of SEQ ID 1-17 in an assay to stratify a subject with Alzheimer's disease is considered to represent a separate invention.
- Therefore the claims comprise 17 separate inventive concepts.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is the use of a miRNA to stratify a subject with Alzheimer's disease.

However this feature does not make a contribution over the prior art because it is disclosed in: D1 (Lau et al, EMBO Mol Medicine 2013, vol 5, pps 1613-1634) and D2 (WO2014/075911 A1) which each disclose the relationship of many miRNAs with Alzheimer's disease.

Therefore in the light of this document this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

For the fee already paid, the ISA considered that it was possible to search and report on five miRNAs. As requested in a letter from the applicant dated 9 June 2015, the International Search Report and Opinion has been drawn up for SEQ ID NOs 1, 7, 9, 14 and 16)

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2015/050264	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2014/075911 A1	22 May 2014	EP 2733219 A1	21 May 2014
		EP 2733220 A1	21 May 2014
		WO 2014075822 A1	22 May 2014
		WO 2014075939 A1	22 May 2014
US 2012/0108650 A1	03 May 2012		
WO 2012/105826 A1	09 August 2012		
EP 2617433 A2	24 July 2013	CN 103189069 A	03 Jul 2013
		JP 2013542921 A	28 Nov 2013
		JP 5710001 B2	30 Apr 2015
		KR 20120088009 A	08 Aug 2012
		KR 101235256 B1	21 Feb 2013
		US 2013184331 A1	18 Jul 2013
		WO 2012036433 A2	22 Mar 2012
WO 2009/009457 A1	15 January 2009		
WO 2013/024469 A1	21 February 2013	US 2014206777 A1	24 Jul 2014
WO 2010/056337 A2	20 May 2010	AU 2011223789 A1	20 Sep 2012
		AU 2011237669 A1	01 Nov 2012
		AU 2011291599 A1	07 Mar 2013
		AU 2012220872 A1	12 Sep 2013
		AU 2012267884 A1	23 Jan 2014
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			

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Form PCT/ISA/210 (Family Annex)(July 2009)

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2015/050264	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		AU 2012271516 A1	23 Jan 2014
		AU 2012294458 A1	27 Feb 2014
		CA 2742324 A1	03 Jun 2010
		CA 2743211 A1	20 May 2010
		CA 2791905 A1	09 Sep 2011
		CA 2795776 A1	13 Oct 2011
		CA 2808417 A1	23 Feb 2012
		CA 2827894 A1	30 Aug 2012
		CA 2838728 A1	13 Dec 2012
		CA 2839530 A1	20 Dec 2012
		CA 2844671 A1	14 Feb 2013
		CN 102301002 A	28 Dec 2011
		CN 102308004 A	04 Jan 2012
		CN 103025890 A	03 Apr 2013
		CN 103237901 A	07 Aug 2013
		CN 103492590 A	01 Jan 2014
		CN 103782174 A	07 May 2014
		CN 103797131 A	14 May 2014
		CN 103874770 A	18 Jun 2014
		EP 2350320 A2	03 Aug 2011
		EP 2358912 A2	24 Aug 2011
		EP 2542696 A1	09 Jan 2013
		EP 2556172 A1	13 Feb 2013
		EP 2606353 A1	26 Jun 2013
		EP 2678448 A1	01 Jan 2014
		EP 2718721 A1	16 Apr 2014
		EP 2721179 A2	23 Apr 2014
		EP 2730662 A1	14 May 2014
		EP 2742154 A2	18 Jun 2014
		GB 2465088 A	12 May 2010
		GB 2465088 B	18 Jul 2012
		GB 2463401 A	17 Mar 2010
		GB 2463401 B	29 Jan 2014
		HK 1142371 A1	18 Jul 2014
		IL 212591 A	31 Jul 2014
		IL 212768 A	31 Jul 2014

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		JP 2012507300 A	29 Mar 2012
		JP 2012508577 A	12 Apr 2012
		JP 2013521502 A	10 Jun 2013
		JP 2013526852 A	27 Jun 2013
		JP 2013540995 A	07 Nov 2013
		JP 2014507160 A	27 Mar 2014
		JP 2014519340 A	14 Aug 2014
		JP 2014522993 A	08 Sep 2014
		JP 2014526032 A	02 Oct 2014
		KR 20130043104 A	29 Apr 2013
		KR 20130056855 A	30 May 2013
		KR 20140067001 A	03 Jun 2014
		KR 20140072014 A	12 Jun 2014
		KR 20140076543 A	20 Jun 2014
		US 2010113290 A1	06 May 2010
		US 7888035 B2	15 Feb 2011
		US 2010184046 A1	22 Jul 2010
		US 7897356 B2	01 Mar 2011
		US 2011159506 A1	30 Jun 2011
		US 8192954 B2	05 Jun 2012
		US 2011151460 A1	23 Jun 2011
		US 8211653 B2	03 Jul 2012
		US 2011237450 A1	29 Sep 2011
		US 8278059 B2	02 Oct 2012
		US 2010203529 A1	12 Aug 2010
		US 2013005599 A1	03 Jan 2013
		US 2013045882 A1	21 Feb 2013
		US 2013178383 A1	11 Jul 2013
		US 2013184169 A1	18 Jul 2013
		US 2013287772 A1	31 Oct 2013
		US 2014141986 A1	22 May 2014
		US 2014148350 A1	29 May 2014
		US 2014162888 A1	12 Jun 2014
		US 2014220580 A1	07 Aug 2014
		US 2014228233 A1	14 Aug 2014
		US 2015024961 A1	22 Jan 2015

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
		WO 2010062706 A2	03 Jun 2010
		WO 2011109440 A1	09 Sep 2011
		WO 2011127219 A1	13 Oct 2011
		WO 2012024543 A1	23 Feb 2012
		WO 2012115885 A1	30 Aug 2012
		WO 2012170711 A1	13 Dec 2012
		WO 2012174282 A2	20 Dec 2012
		WO 2013022995 A2	14 Feb 2013
WO 2015/006705 A2	15 January 2015		
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			