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APPLICATION NUMBER:  61/522,328
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| **Application Number:** | 61522328 |
| **International Application Number:** |  |
| **Confirmation Number:** | 6307 |
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| **First Named Inventor/Applicant Name:** | Ugo Pastorino |
| **Customer Number:** | 30623 |
| **Filer:** | Matthew Pavao/Victoria Hughes |
| **Filer Authorized By:** | Matthew Pavao |
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INVENTOR(S)

<table>
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<th>Given Name (first and middle [if any])</th>
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<tr>
<td>Ugo</td>
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Additional inventors are being named on the separately numbered sheets attached hereto.

TITLE OF THE INVENTION (500 characters max): Micro-RNA Biomarkers and Methods of Using Same

Direct all correspondence to: CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (check all that apply)

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SIGNATURE  / Matthew Pavao /  Date  August 11, 2011

TYPED or PRINTED NAME  Matthew Pavao, J.D., Ph.D.  REGISTRATION NO.  50,572

TELEPHONE  (617) 348-4707  Docket Number:  42823-501P01US
MICRO-RNA BIOMARKERS AND METHODS OF USING SAME

FIELD OF THE INVENTION

[0001] The present invention concerns methods for identifying and using, in prediagnostic and/or diagnostic stages, special molecular bio-markers identifiable in biological samples, such as for example whole blood, serum, plasma, saliva or bronchia condensate collected from an individual.

[0002] In more detail, the invention relates to methods for identifying individuals at risk of tumour, in particular pulmonary tumour. The invention also concerns methods for determining a presence and/or level of aggressiveness of a tumour, for example a pulmonary tumour, in an individual.

[0003] The invention also relates to diagnostic kits and apparatus usable for setting up one or more stages of the methods.

[0004] Further, the invention concerns methods and pharmaceutical compounds for treating an individual in whom presence of a tumour has been diagnosed, for example a pulmonary tumour.

[0005] The invention also concerns methods and pharmaceutical compounds for treating an individual in whom a risk of developing a tumour has been identified, for example a pulmonary tumour, for reducing and/or eliminating the risk of developing a tumour.

BACKGROUND OF THE INVENTION

[0006] As is known, tumours are one of the main causes of death in the world. In particular, pulmonary tumours are the highest in terms of incidence, as they represent about 12% of all the new cases of cancer, and constitute the main cause of death by cancer in the world, in both men and women.

[0007] In Europe about 400,000 new cases are diagnosed per year (80% men, 20% women). In Italy too the epidemiology of pulmonary cancer is similar, with an incidence of 34,000 cases per year of which 7,000 are women and 27,000 men.

[0008] Sadly the incidence and the mortality are very similar due to the highly lethal nature of pulmonary tumour: world-wide mortality 27,500, of which 22,000 mend and 5,500 women. This epidemiological data and the scarce level of treatability of the illness underline the importance of identifying methods which are able to identify as soon as possible any subjects who might be at risk of developing pulmonary cancer. Further, it is of great interest.
to develop procedures which can help in the correct diagnosis of tumours, in particular pulmonary tumours present in an individual subject under examination.

[0009] Notwithstanding these needs, tumour markers available today are for diagnostic use, i.e. they identify the patients when the disease has already developed such as to be identifiable with imaging methods (spiral CT scan). These markers are however few and not specific and essentially comprise biochemical markers such as the evaluation of the protein CEA (Carcinoembryonic Antigen) and some cytokeratins such as TPA, TPS and Cyfra 21.1.

[0010] Also known is a proteomic test (5-protein profile) on the serum, at present proposed by Vermillion Inc. and used to indicate a probability (score from 1 to 10) that ovarian masses might be of a malignant nature. This test is used for women who already present ovarian masses of a non-defined nature.

[0011] With specific reference to pulmonary tumours, although in recent years important improvements have been made in the treatment of oncological patients, there is however a need to develop more effective methods which can lead to a faster therapeutic intervention in clinical management of many types of tumours.

[0012] At present the majority of pulmonary tumours are diagnosed at a late stage, when the symptoms are clinically evident and, for example with reference to Non-small-cell lung carcinoma (NSCLC), only a third of patients with NSCLC exhibits a surgically-resectable disease, an approach which remains the most effective treatment for this type of tumour.

[0013] Notwithstanding recent progress in treatment of pulmonary cancer after resection and the use of specific treatments for determined molecular targets, the rate of healing of non-small-cell lung carcinoma (NSCLC) remains low due to the reappearance thereof in patients that are resistant to drugs or who present metastasis.

[0014] The effectiveness of the spiral CT scan in identification of pulmonary cancer in heavy smokers is under evaluation in various randomised clinical studies in Europe and the United States. Owing to the its high level of sensitivity there remain various critical points for its use in modern clinical practice, such as over-diagnosis of indolent nodules, with a consequently high frequency of non-necessary treatments and the verification of the effective impact on mortality.

[0015] In this context, in recent years microRNAs have been identified (herein below also MiRNA) as a new class of circulating bio-markers which by their nature seem to be very stable and highly specific tissue (Chen X, Cell Res, 2008). miRNAs are small non-coding
RNA molecules (length 19-25 nucleotides) having a regulatory function which are able to modulate the expression of several target genes involved in various molecular mechanisms, among which those involved in transformation processes.


[00018] Tests have also been carried out to determine whether miRNAs are stable, detectable and quantifiable not only in the tissues (both deep-frozen and fixed in formalin or paraffin) but also in the bodily fluids. The results of this research have demonstrated that miRNAs are also present in the blood circulation (whole blood, serum and plasma), where they are found in stable form protected by endogenous RNAsi. Circulating miRNAs are detectable and quantifiable and the studies which have taken their levels in oncological patients' biological fluids under examination have reported that some of them present deregulated levels with respect to healthy individuals (Heneghan HM et al., Ann Surg, 2010; Mitchell PS et al., Proc Natl Acad Sci USA, 2008; Chen X, Cell Res, 2008).


[00020] Notwithstanding the presence of diagnostic imaging systems and the studies relating to microRNAs, there is still the need to identify procedures which are able to identify, with a certain degree of anticipation, individuals at risk of developing pulmonary cancer and possibly able to predict the development of the forms of cancer, in particular pulmonary tumour, that are more aggressive and lethal. There is also a need to improve the degree of reliability of diagnostic techniques at present available.

SUMMARY OF THE INVENTION

[00021] In this situation, the aim of the present invention is to obviate one or more of the limitations in the known procedures and products.
Thus it is an aim of the invention to provide procedures for early determination of individuals who present a risk of developing a tumour, in particular a pulmonary tumour.

A further aim of the invention is to make available procedures which assist in the diagnosis of tumour, in particular pulmonary tumours, in human subjects.

A further aim of the invention is to make available procedures which can be easily set up in laboratories, by analysing biological samples collected from an individual.

A further aim of the invention is to provide procedures which enable satisfactory results to be obtained using samples of blood, serum or plasma.

A further aim of the invention is to define diagnostic kits and/or apparatus usable in the above-cited procedures in order to identify human subjects who are at risk of contracting a tumour and/or for assisting in the diagnosis of tumours present in human subjects.

A further aim of the invention is to provide pharmaceutical compounds and/or treatments which can be used to treat an individual in whom the presence of a pulmonary tumour has been diagnosed.

A final aim of the invention is to provide pharmaceutical compounds and/or treatments for reducing and/or eliminating the risk of developing a pulmonary tumour.

One or more of the set aims are substantially attained by a method and/or a kit and/or a compound and/or an apparatus in accordance with one or more of the accompanying claims.

Aspects of the invention are described herein below.

A first aspect concerns a procedure for identifying individuals at risk of a pulmonary tumour, the procedure comprising steps of:

- measuring, in at least a sample of biological fluid previously collected from a subject, a value of the level of expression of a plurality of microRNA molecules;
- determining when the measured values of the level of expression deviate with respect to a predetermined and respective control criterion.

The microRNA or miRNA molecules are thus used for identifying individuals at risk or in a stage in which the tumour has not yet manifested.

In a second aspect in accordance with the first aspect the step of determining comprises determining the level of expression of at least six miRNA from the miRNA listed in Tables I, II in a biological sample from a subject, and comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA.
from a control biological sample. For example determining may comprise determining - in a biological sample from a subject - the level of expression of the six miRNA listed in Table IIb or the level of expression of the six miRNA listed in Table IIb, and comparing the level of expression of said six miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample, wherein a change or deviation in the level of expression of said at least six miRNA in said biological sample from said control biological sample identifies a subject at risk of manifesting a tumor (Table Iib miRNA are used) or and aggressive tumor (Table Iib miRNA are used).

[00036] In a third aspect in accordance with the first aspect the step of determining comprises substeps of calculating a plurality of ratios or real differences determined by performing the ratio or respectively the difference between the measured values of the levels of expression of a predetermined number of pairs of the microRNA molecules, comparing each of the real ratios or differences with a respective control value, determining the real ratios or differences which deviate from the respective ratio value or control difference.

[00037] In a fourth aspect in accordance with the third aspect, a step is also included of determining a number or percentage of real ratios or differences which deviate from the respective control value and defining as an individual at risk an individual for whom at least a predetermined number or a predetermined percentage of the real ratios or differences deviates with respect to the respective ratio value or control difference.

[00038] In a fifth aspect, in accordance with any one of the preceding aspects, for each of the calculated ratios, a respective control ratio is associated represented by the ratio of the expression values for the microRNAs in a control sample relating to a biological fluid of the same type. The control ratio is in reality either a known value, or a determined value as a mean of measured values in a sufficiently large population of individuals, or a value relating to a fluid sample collected from a healthy individual.

[00039] In a sixth aspect, in accordance with any one of the preceding aspects, the procedure comprises the step of correlating the deviation of a predetermined number or a predetermined percentage of expression levels (i.e. real ratios or differences) with respect to the corresponding control criteria in the presence or absence of risk that the individual clinically presents a pulmonary tumour in a predetermined time.

[00040] In a seventh aspect according to the preceding aspect the predetermined time is comprised between one and three years, more optionally is comprised between 12 and 28 months. In other words the method of the invention is able to significantly anticipate the
determination of the risk of contracting a tumour with respect to traditional techniques (such as spiral CT) which have to wait for the disease to manifest at the level of lacerations or nodules of various mm.

[00041] In an eighth aspect in accordance with any one of the preceding aspects the procedure comprises a step of correlating the deviation of a predetermined number or a predetermined percentage of expression level with respect to the corresponding control criteria to the presence or absence of risk which the individual manifests clinically an aggressive pulmonary tumour in a predetermined time.

[00042] In a ninth aspect, according to the preceding aspect, the predetermined time is comprised between one and three years, more optionally between 12 and 28 months. In other words the method of the invention is able to significantly anticipate the determination of the risk of contracting an aggressive tumour with respect to the traditional techniques (such as spiral CT) which have to wait for the disease to manifest at the level of lacerations or nodules or various mm.

[00043] In a tenth aspect, in accordance with any one of the preceding aspects, calculating the plurality of real ratios or differences comprises using the expression values of a predetermined number or a predetermined percentage of the miRNAs of Table I and/or of Table II, optionally using the expression values of the miRNAs of Table Ib and/or of Table III.

[00044] In an eleventh aspect in accordance with any one of aspects 6th, 7th or 10th, calculating the plurality of real ratios or differences comprises using the expression values of a predetermined number or a predetermined percentage of the miRNAs belonging to the following list:

[00045] hsa-mirR-140-5p
[00046] hsa-mirR-660
[00047] hsa-mirR-320
[00048] hsa-mirR-28-3p
[00049] hsa-mirR-451
[00050] hsa-mirR-30c
[00051] hsa-mirR-92a
[00052] hsa-mirR-197
[00053] hsa-mirR-221
[00054] hsa-mirR-19b
[00055] hsa-mirR-15b
In a twelfth aspect, according to any one of aspects 8th, 9th or 10th, calculating the plurality of real ratios or differences comprises using the expression values of a predetermined number or a predetermined percentage of the miRNA of Table II or of the miRNA belonging to the following list:

| [00061] | hsa-miR-221 |
| [00062] | hsa-miR-451 |
| [00063] | hsa-miR-197 |
| [00064] | hsa-miR-486-5p |
| [00065] | hsa-miR-28-3p |
| [00066] | hsa-miR-660 |
| [00067] | hsa-miR-140-5p |
| [00068] | has-miR-148a |
| [00069] | has-miR-21 |
| [00070] | has-miR-101 |
| [00071] | has-miR-140-3p |
| [00072] | has-miR-142-3p |
| [00073] | has-miR-15b |
| [00074] | has-miR-19b |
| [00075] | has-miR-30b |
| [00076] | has-miR-30c |

In a thirteenth aspect according to any one of the preceding aspects, calculating the plurality of real ratios comprises determining a predetermined number or a predetermined percentage of ratios among the values of the expression levels, the ratios being selected from the group comprising ratios between values of expression levels of pairs of microRNA as in Table III, optionally in which at least 20% are determined, more optionally at least 50% and still more optionally all the ratios of Table III.

In a fourteenth aspect in accordance with the preceding claim, determining a predetermined number or a predetermined percentage of ratios comprises calculating at least 20% of the real ratios of Table III and in which it comprises a step of defining as an individual at risk of pulmonary tumour, optionally in a period comprised between one and
three years from a collection of the sample of biological fluid, an individual for whom at least 30%, optionally at least 50% of the real ratios calculated deviates with respect to the respective control ratio value.

[00079] In a fifteenth aspect in accordance with any one of aspects 13 or 14, in which the ratios are those of Table IIIb or are selected from among those of the following list:

[00080] Q₁ = hsa-mirR-106a / hsa-mirR-451
[00081] Q₂ = hsa-mirR-140-5p / hsa-mirR-320
[00082] Q₃ = hsa-mirR-140-5p / hsa-mirR-451
[00083] Q₄ = hsa-mirR-140-5p / hsa-mirR-660
[00084] Q₅ = hsa-mirR-140-5p / hsa-mirR-92a
[00085] Q₆ = hsa-mirR-15b / hsa-mirR-92a
[00086] Q₇ = hsa-mirR-17 / hsa-mirR-451
[00087] Q₈ = hsa-mirR-197 / hsa-mirR-451
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[00091] Q₁₂ = hsa-mirR-30b / hsa-mirR-92a
[00092] Q₁₃ = hsa-mirR-30c / hsa-mirR-451
[00093] Q₁₄ = hsa-mirR-30c / hsa-mirR-660
[00094] In a sixteenth aspect in accordance with any one of the preceding aspects, calculating the plurality of real ratios comprises determining a predetermined number or a predetermined percentage of ratios among the values of the expression levels, the ratios being selected from the group comprising ratios between values of expression levels of pairs of microRNAs as in Table IV, optionally in which at least 30% are determined, more optionally at least 50%, and still more optionally all the ratios of Table IV.

[00095] In a seventeenth aspect according to the preceding aspect, determining a predetermined number or a predetermined percentage of ratios comprises calculating at least 30% of the real ratios as in Table IV, and wherein the procedure comprises a step of defining as an individual at risk of aggressive pulmonary tumour, optionally in a period comprised between one and three years from collecting a sample of biological fluid, an individual for whom at least 50%, optionally at least 75% of the real ratios calculated deviates with respect to the respective control ratio value.

[00096] In an eighteenth aspect according to any one of aspects 16 or 17, the ratios are those of Table IVb or are selected from those of the following list:
[00097] $Q_1 =$ hsa-mirR-148a/ hsa-mirR-660
[00098] $Q_2 =$ hsa-mirR-140-5p/ hsa-mirR-486-5p
[00099] $Q_3 =$ hsa-mirR-140-5p/ hsa-mirR-660
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[00104] $Q_8 =$ hsa-mirR-28-3p/ hsa-mirR-451
[00106] $Q_{10} =$ hsa-mirR-28-3p/ hsa-mirR-660
[00107] In a nineteenth aspect of any one of the preceding aspects, the steps of the procedure are conducted in vitro.
[00108] In a twentieth aspect in accordance with any one of the preceding aspects, the biological fluid is one selected from a group comprising: whole blood, a fraction of blood, plasma, serum.
[00109] In a twenty-first aspect in accordance with any one of the preceding aspects the pulmonary tumour is one selected from the group comprising: small-cell lung cancer (SCLC), non small-cell lung cancer (NSCLC), pulmonary adenocarcinoma (ADC), bronchiolo-alveolar carcinoma (BAC), squamous-cell lung carcinoma (SCC), large-cell carcinoma (LC).
[00110] In a twenty-second aspect according to any one of the preceding aspects, the sample of biological fluid originates from a smoker individual who, at the moment of the collection of the sample, does not present a pulmonary tumour if subjected to imaging diagnostic methods, in particular the smoker individual not presenting nodules of dimensions of greater than 5mm if subjected to a spiral CT scan.
[00111] A twenty-third aspect concerns a medical kit for determining biomolecular markers present in a sample of human biological fluid, the kit comprising a platform having a plurality of sites, each of which is destined to receive a respective discrete quantity of the sample of biological fluid, each of the sites comprising a reagent capable of bonding with at least a respective microRNA of Table I and/or Table II, optionally wherein each of the sites comprises a reagent capable of bonding with at least a respective microRNA of Table Ib and/or Table IIb.
[00112] In a twenty-fourth aspect in accordance with the preceding aspect, the reagent includes at least a reagent selected from among the group comprising:
[00113] a polynucleotide comprising a nucleotide sequence of at least one of the
microRNAs as in Table I and/or Table II, optionally as in Table Ib and/or Table IIb;

[000114] a polynucleotide comprising a nucleotide sequence which is complementary to a sequence of at least one of the microRNAs as in Table I and/or Table II, optionally as in Table Ib and/or Table IIb;

[000115] a molecular probe configured such as to recognise a sequence of at least one of the microRNAs as in Table I and/or Table II, optionally as in Table Ib and/or Table IIb.

[000116] A twenty-fifth aspect concerns a medical apparatus comprising:

[000117] a unit defining a seating for receiving one or more of the kits of aspects 23rd or 24th;

[000118] means for determining a value of the level of expression of the microRNAs as in Table I and/or Table II;

[000119] means for calculating the values of the real ratios from among the values of levels of expression of pairs of microRNAs, the ratios being selected from those in Table III and/or Table IV, optionally those ratios of Table IIIb and/or those of Table IVb;

[000120] In a twenty-sixth aspect according to the preceding aspect, the means for determining the value of the expression level comprise one of the techniques selected from the group:

[000121] Quantitative Real-time PCR,
[000122] Microfluidic cards,
[000123] Microarrays,
[000124] RT – PCR, quantitative or semi-quantitative,
[000125] Northern blot,
[000126] Solution Hybridization,
[000127] Sequencing.

[000128] A twenty-eighth aspect comprises an in vitro procedure for identifying individuals at risk of tumour and/or for determining a presence of and/or an aggressiveness of a tumour in an individual, the process comprising steps of:

[000129] measuring, in at least a sample of biological fluid previously collected from a subject, a value of a level of expression of a plurality of microRNA molecules;

[000130] calculating a plurality of real ratios determined by calculating a ratio between the measured values of the levels of expression of a predetermined number of pairs of the microRNA molecules;

[000131] comparing each of the real ratios with a respective control value.

[000132] In a twenty-ninth aspect in accordance with the twenty-eighth aspect, the
process comprises determining a number or percentage of real ratios which deviate from the respective control value, defining, as an individual presenting a form of tumour, an individual for whom at least a predetermined number or a predetermined percentage of the real ratios deviates with respect to the respective control ratio value.

[000133] In a thirtieth aspect in accordance with the twenty-ninth, a respective control ratio is associated to each of the calculated ratios, represented by a ratio of the values of expression for the microRNAs in a control sample relative to a biological fluid of a same type.

[000134] In a thirty-first aspect, in accordance with the thirtieth or the twenty-ninth, calculating the plurality of real ratios comprises using the values of expression of a predetermined number of the miRNAs as in Table I and/or Table II and/or Table V and/or Table VI.

[000135] In a thirty-second aspect in accordance with any one of aspects from the 29th to 31st, calculating the plurality of real ratios comprises determining a predetermined number or a predetermined percentage of ratios from among the values of the levels of expression, the ratios being selected from among the group comprising ratios as in Table III, and/or Table IV and/or in Table VII and/or in Table VIII.

[000136] In a thirty-third aspect in accordance with the thirty-second, determining a predetermined number or a predetermined percentage of ratios comprises calculating at least 20% of the real ratios of Table VII and comprises a step of defining as an individual presenting a pulmonary tumour an individual for whom at least 30% of the predetermined number of real ratios as in Table VII which have been calculated deviate with respect to the control value.

[000137] In a thirty-fourth aspect in accordance with the thirty-third or the thirty-second,

[000138] determining a predetermined number or a predetermined percentage of ratios comprises calculating at least 20% of the real ratios of Table VIII and comprises a step of defining as an individual presenting an aggressive pulmonary tumour an individual in whom 50%, optionally at least 60%, of the real ratios which have been calculated deviate with respect to the respective control value.

[000139] In a thirty-fifth aspect, in accordance with the thirty-fourth or the thirty-third or the thirty-second, determining the predetermined number or a predetermined percentage of ratios comprises calculating at least 20% of the real ratios of Table III and comprises a step of defining as an individual at risk of a pulmonary tumour, optionally in a period comprised
between one and three years from a collection of the sample of biological fluid, an individual for whom at least 30%, optionally at least 50%, of the real ratios calculated deviate with respect to the respective control ratio value.

[000140] In a thirty-sixth aspect in accordance with the thirty-fifth, the thirty-fourth or the thirty-third or the thirty-second, determining the predetermined number or a predetermined percentage of ratios comprises calculating at least 30% of the real ratios of Table IV and comprises a step of defining as an individual at risk of an aggressive pulmonary tumour, optionally in a period comprised between one and three years from a collection of the sample of biological fluid, an individual for whom at least 50%, optionally at least 75%, of the real ratios calculated deviate with respect to the respective control ratio value.

[000141] In a thirty-seventh aspect in accordance with the any one of the aspects from the 28th to the 32nd, determining a predetermined number or a predetermined percentage of ratios comprises calculating the real ratios of Table VIIIb and wherein the procedure comprises a step of defining as an individual presenting a pulmonary tumor an individual for whom at least 80% of the real ratios as in Table VIIb which have been calculated deviate with respect to the control value.

[000142] In a thirty-eighth aspect in accordance with the any one of the aspects from the 28th to the 32nd, determining a predetermined number or a predetermined percentage of ratios comprises calculating the real ratios of Table VIIIb and wherein the procedure comprises a step of defining as an individual presenting an aggressive pulmonary tumor an individual for whom at least 80% of the real ratios as in Table VIIIb which have been calculated deviate with respect to the control value.

[000143] In a thirty-ninth aspect in accordance with the any one of the aspects from the 28th to the 32nd, determining a predetermined number or a predetermined percentage of ratios comprises calculating the real ratios of Table IIIb and wherein the procedure comprises a step of defining as individual at risk of a pulmonary tumour, optionally in a period comprised between one and three years from a collection of the sample of biological fluid, an individual for whom at least 80% of the real ratios as in Table IIIb which have been calculated deviate with respect to the control value.

[000144] In a fortieth aspect in accordance with the any one of the aspects from the 28th to the 32nd, determining a predetermined number or a predetermined percentage of ratios comprises calculating the real ratios of Table IVb and wherein the procedure comprises a step of defining as individual at risk of an aggressive pulmonary tumour, optionally in a period comprised between one and three years from a collection of the sample of biological fluid,
an individual for whom at least 80% of the real ratios as in Table IVb which have been calculated deviate with respect to the control value.

[000145] In a forty-first aspect in accordance with any one of the preceding aspects from the 28th to 40th, the biological fluid is one selected from among a group comprising: whole blood, a fraction of blood, plasma, serum; saliva or bronchial condensate.

[000146] In a forty-second aspect in accordance with any one of the preceding aspects from the 28th to 41st, the tumour is a pulmonary tumour selected from among a group comprising: small-cell lung cancer (SCLC), non small-cell lung cancer (NSCLC), pulmonary adenocarcinoma (ADC), bronchio-alveolar carcinoma (BAC), squamous-cell lung carcinoma (SCC), large-cell carcinoma (LC).

[000147] In a forty-third aspect, in accordance with any one of the preceding aspects from the 28th to 42nd, the sample of biological fluid originates from a smoker individual who, at the moment of the collection of the sample, presents a pulmonary tumour if subjected to imaging diagnostic methods, in particular the smoker individual presenting nodules of dimensions of greater than 5mm if subjected to a spiral CT scan.

[000148] In a forty-fourth aspect, a medical kit is provided for determining biomolecular markers present in a sample of human biological fluid, the kit comprising:

[000149] a platform, for example a support for receiving fluid samples, having a plurality of sites, each of which is destined to receive a respective discrete quantity of the sample of biological fluid,

[000150] each of the sites comprising a reagent capable of bonding with at least a respective microRNA of Table I and/or Table II, and/or Table V and/or Table VI, optionally a reagent capable of bonding with at least a respective microRNA microRNA as in Table Ib and/or Table IIb, and/or Table Vb and/or Table VIb.

[000151] In a forty-fifth aspect in accordance with the preceding aspect, the reagent includes at least a reagent selected from among a group comprising:

[000152] a polynucleotide comprising a nucleotide sequence of at least one of the microRNAs as in Table I and/or Table II, and/or Table V and/or Table VI, or a nucleotide sequence of at least one of the microRNAs as in Table Ib and/or Table IIb, and/or Table Vb and/or Table VIb;

[000153] a polynucleotide comprising a nucleotide sequence which is complementary to a sequence of at least one of the microRNAs as in Table I and/or Table II, and/or Table V and/or Table VI, optionally comprising a nucleotide sequence which is complementary to a sequence of at least one of the microRNAs as in Table Ib and/or Table IIb, and/or Table Vb
and/or Table VIb;

[000154] a molecular probe configured such as to recognise a sequence of at least one of the microRNAs as in Table I and/or Table II and/or Table V and/or Table VI, optionally a sequence of at least one of the microRNAs as in Table Ib and/or Table IIb, and/or Table Vb and/or Table VIb.

[000155] In a forty-sixth aspect a medical apparatus is provided, comprising:

[000156] a unit defining a seating for receiving one or more of the kits of the preceding claim,

[000157] means for determining a value of the level of expression of the microRNAs as in Table I and/or Table II, and/or Table V and/or Table VI, optionally the value of the level of expression of the microRNA as in Table Ib and/or Table IIb, and/or Table Vb and/or Table VIb;

[000158] means for calculating the values of the real ratios from among the values of levels of expression of pairs of microRNAs, the ratios being selected from those in Table III and/or Table IV and/or Table VII and/or Table VIII, optionally from those in Table IIIb and/or Table IVb and/or Table VIIb and/or Table VIIIb.

[000159] In a forty-third aspect in accordance with the preceding aspect, the means for determining the value of the level of expression comprise one from among the techniques selected from a group as follows:

[000160] Quantitative Real-time PCR,

[000161] Microfluidic cards,

[000162] Microarrays,

[000163] RT – PCR, quantitative or semi-quantitative,

[000164] Northern blot,

[000165] Solution Hybridization,

[000166] Sequencing.

[000167] In a forty-eighth aspect a method is comprised for treating an individual in whom a presence of a pulmonary tumour has been diagnosed or in whom a risk of developing a pulmonary tumour has been identified, respectively for treatment of the pulmonary tumour or for reducing and/or eliminating the risk of developing a pulmonary tumour, the method comprising following steps:

[000168] measuring a level of expression of at least an miRNA listed in Table I or Table II or Table V or Table VI present in a sample of biological fluid previously taken from the individual,
determining the miRNAs having measured values of a level of expression which deviate with respect to a predetermined and respective control criterion;

altering the level of expression of the miRNAs for which the levels of expression deviate with respect to the respective control criterion.

In a forty-nineth aspect the step of measuring comprises measuring a level of expression of at least a miRNA listed in Table Ib or Table IIb or Table Vb or Table VIb present in a sample of biological fluid previously taken from the individual.

In a fiftieth aspect in accordance with the preceding aspect the step of altering the level of expression of the miRNAs comprises:

administering to the individual an effective quantity of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI, or of one of more of the miRNA listed in Table Ib or Table IIb or Table Vb or Table VIb, if the level of expression measured of the miRNA or the miRNAs is lower than a respective control level of expression.

In a fifty-first aspect according to the 49th or 50th aspect, the step of altering the level of expression of the miRNAs comprises administering to the individual an effective quantity of at least a compound for inhibiting the expression of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI, or listed in Table Ib or Table IIb or Table Vb or Table VIb, if the measured level of expression of one or more of the miRNA or miRNAs is higher than the control level of expression.

In a fifty-second aspect, in accordance with the 50th aspect, the method comprises restoring the values of levels of expression to a control level of expression for the miRNAs which are under-expressed with respect to the respective control level of expression.

In a fifty-third aspect in accordance with any one of aspects from the 48th to the 52nd, the method comprises administering a therapeutically effective quantity of a compound comprising at least one of the miRNAs of Table I or Table II or Table V or Table VI, optionally at least one of the miRNA listed in Table Ib or Table IIb or Table Vb or Table VIb, chemically synthesised (miRNA mimetics) or recombinant.

In a fifty-fourth aspect according to any one of aspects from 51st to 53rd, the method comprises reducing the values of the level of expression to the control level of expression for miRNAs which are over-expressed with respect to the respective control level of expression.

In a fifty-fifth aspect in accordance with any one of the aspects from 48th to 54th, the method comprises administering a therapeutically effective quantity of a compound comprising at least an inhibitor of a microRNA of Table I or Table II or Table V or Table VI
or Table Ib or Table IIb or Table Vb or Table VIb.

[000179] In fifty-sixth aspect in accordance with the preceding aspect, the inhibitor comprises double-filament RNA.

[000180] In a fifty-seventh aspect according to the preceding aspect the method comprises short interfering RNA (siRNA), antisense nucleic acids (anti-miRNA oligonucleotides (AMOs), molecules of enzymatic RNA (ribozymes).

[000181] In a fifty-eighth aspect according to any one of aspects from the 55th to the 57th, the inhibitor is directed to a specific product of microRNA and interferes with the expression, for example by means of inhibition of a translation or induction of degradation, of a target gene of the microRNA.

[000182] In a fifty-nineth aspect in accordance with any one of the preceding aspects, the step of determining the miRNAs having measured values of the levels of expression which deviate with respect to the respective control criterion comprises:

[000183] calculating a plurality of real ratios determined by performing a ratio between the measured values of the levels of expression of a predetermined number of pairs of the microRNA molecules, the ratios being selected from a group comprising the ratios as in Table III and/or Table IV and/or Table VII and/or Table VIII, optionally the ratios being selected from a group comprising the ratios as in and/or Table IIIb and/or Table IVb and/or Table VIIb and/or Table VIIIb,

[000184] determining the real ratios which deviate from the respective control values,

[000185] identifying the miRNAs involved in the real ratios which deviate from the respective control value.

[000186] A sixtyth aspect concerns a pharmaceutical compound for treating an individual in whom has been diagnosed a pulmonary tumour or in whom a risk of developing a pulmonary tumour has been identified, respectively for treatment of the pulmonary tumour or for reducing and/or eliminating the risk of developing a pulmonary tumour, the compound comprising:

[000187] at least one, optionally at least six, of the miRNAs listed in Table I or Table II or Table V or Table VI, and/or

[000188] at least an inhibitor of the expression of at least one, optionally at least six, of the miRNAs listed in Table I or Table II or Table V or Table VI.

[000189] In a sixty-first aspect, according to the preceding aspect, the compound comprises a therapeutically effective quantity of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI.
[000190] A sixty-second aspect concerns a pharmaceutical compound for treating an individual in whom has been diagnosed a pulmonary tumour or in whom a risk of developing a pulmonary tumour has been identified, respectively for treatment of the pulmonary tumour or for reducing and/or eliminating the risk of developing a pulmonary tumour, the compound comprising:

[000191] at least one, optionally at least six, of the miRNAs listed in Table Ib or Table IIb or Table Vb or Table VIIb, and/or

[000192] at least an inhibitor of the expression of at least one, optionally of at least six, of the miRNAs listed in Table Ib or Table IIb or Table Vb or Table VIIb.

[000193] In a sixty-third aspect, according to the preceding aspect, the compound comprises a therapeutically effective quantity of at least one, optionally of at least six, of the miRNAs listed in Table Ib or Table IIb or Table Vb or Table VIIb.

[000194] In a sixty-fourth aspect in accordance with any one of aspects from the 60th to the 63rd, the quantity is able, for the miRNAs that are under-expressed with respect to the respective control level of expression, to restore the values of the level of expression to the respective control level of expression.

[000195] In a sixty-fifth aspect in accordance with any one of aspects from the 60th to the 64th, the therapeutically effective quantity comprises miRNA of Table I or Table II or Table V or Table VI, optionally the therapeutically effective quantity comprises the miRNA of Table Ib or Table IIb or Table Vb or Table VIIb, chemically synthesised or recombinant.

[000196] In a sixty-sixth aspect in accordance with any one of aspects from the 60th to the 64th, the compound comprises a therapeutically effective quantity of the inhibitor of the expression of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI, optionally all those listed in Table Ib or Table IIb or Table Vb or Table VIIb, the quantity being able, for the over-expressed miRNAs with respect to the respective control level of expression, to reduce the values of the level of expression to the respective control level of expression.

[000197] In a sixty-seventh aspect in accordance with the preceding aspect, the inhibitor comprises double-filament RNA, optionally short interfering RNA (siRNA), and/or antisense nucleic acids, and/or enzymatic RNA molecules (ribozymes).

[000198] In a sixty-eighth aspect in accordance with one of the preceding two aspects, the inhibitor is directed to a specific product of microRNA and interferes with the expression (by means of inhibition of translation or induction of degradation) of a target gene of the microRNA.
In a sixty-ninth aspect, a pharmaceutical compound is provided according to any one of claims from the 60th to the 68th, for preparation of a medicament usable in one of the therapeutic methods of any one of aspects from the 48th to 59th.

In a seventieth aspect in accordance with the preceding aspect the therapeutic method is a method for treating an individual in whom a presence of a pulmonary tumour has been diagnosed.

In a seventy-first aspect in accordance with the sixty-ninth, the therapeutic method is a method for treating an individual in whom a risk of developing a pulmonary tumour has been identified, in order to reduce and/or eliminate the risk of developing the pulmonary tumour.

In a seventy-second aspect, in accordance with any one of the preceding aspects, as a variant of the invention and alternatively to the real ratios (in the method, the medical kit and the apparatus) real differences are determined by performing the difference between the measured values of the expression levels of a predetermined number of pairs of the molecules of microRNA. In this case each of the differences is compared with a respective control value in order to determine the differences which deviate from the respective control value.

All the preceding aspects are equally applied by replacing the real ratios with real differences between the pairs of miRNA as in the appended tables.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In the specification, the singular forms also include the plural unless the context clearly dictates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. The references cited herein are not admitted to be prior art to the claimed invention. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.
Detailed Description of the Invention

[000206] There now follow some specific and non-limiting aspects of the invention. In particular, the steps of the research made by the inventors are described, and the results obtained are reported, with various aspects of the invention by way of example.

[000207] Definitions

[000208] The following terminology, when used in the description, claims and drawings of the present patent application, is intended to have the following meanings.


[000210] MicroRNA or miRNA: small RNA molecules (length 19-25 nucleotides), non-coding. In particular, reference is made to miRNA present in biological samples of human tissue, for example whole blood, plasma, serum, saliva or bronchial condensate.

[000211] miRNA ratios: real ratios determined by performing a ratio among the measured values of the expression levels of predetermined pairs of molecules of microRNA.

[000212] Aggressive tumour: a tumour, for example a pulmonary tumour, with a lethal prognosis or capable of causing death in 90% of patients within five years from diagnosis of the disease.

[000213] Individual at risk of tumour (aggressive or not according to the case studies): an individual who in the time of reference (1-3 years) following the collection of the biological sample has a risk of over 80% of developing a tumour, for example a pulmonary tumour, detectable using techniques such as spiral CT.

[000214] Research

[000215] The inventors investigated the expression profile of miRNA in the plasma of individuals enrolled in screening protocols using spiral CT. This investigation was done with the aim of verifying the capability of miRNAs as a new class of biomolecular markers for:

[000216] prediction of the risk of developing a tumour, in particular a pulmonary tumour, and

[000217] diagnosis of the tumour, in particular pulmonary tumour, and thus as a prognostic aid for discriminating patients with indolent or aggressive pulmonary lesions.

[000218] Plasma samples taken from smoker individuals were used, where the individuals were over 50 years old, in a time parameter of between one and two years before detection with CT spiral of the presence of a pulmonary tumour in the same individuals. Also used were samples of plasma collected at the moment of the appearance of the disease (detected using spiral CT). The plasma samples were obtained from patients who had developed a pulmonary tumour with various characteristics in terms of clinical
aggressiveness (indolent nodules or advanced and metastatic tumours) as well as from individuals who remained free of disease for the whole duration of the screening.

[000219] In a first stage of the research, identification was made of the microRNAs that were present in the plasma using microfluidic cards, model: TaqMan® of Applied Biosystems. Out of 378 microRNA analysed, 100 were present stably in the plasma of healthy smoker individuals used as the control group. Thus, with a large amount of starting data, there is a general agreement on the possibility of normalising the expression levels of the single microRNAs on the mean of the expression levels of the 100 microRNAs for each individual (Mestdagh P et al. Genome Biol, 2009). The data obtained using this type of normalisation were compared to those obtained by normalising on potential microRNA housekeeping (for example mir-16, mammU6, RNU44 or RNU48).

[000220] The inventors then thought of no longer using the values of the expression levels of the single microRNAs, but instead the ratios among pairs thereof. The value of the cycle threshold (Ct) obtained by qReal-Time PCR with the SDS 2.2.2® software (Applied Biosystems) was transformed into the corresponding expression value ($2^{-Ct}$). Then the ratio between the value of the expression level of each pair of microRNAs possible was calculated, obtaining 4950 total ratios: the 4950 total ratios were given by the formula $100 \times 99 / 2$ as the ratio between two miRNAs and the reciprocal contain the same data. Finally the variation of these ratios (called “miRNA ratios”) in the plasma of the various classes of patients was analysed in order to identify plasma biomarkers.

[000221] The results showed that the microRNAs present in the greatest amount in the ratios discriminating among the classes of patients are the same as those which emerge from the analyses performed by normalising on the mean value of the expression levels of the 100 microRNAs for each individual, thus validating the method based on the miRNA ratios for quantifying the involved microRNAs.

[000222] In greater detail, with the aim of identifying biomarkers in the plasma which are able to predict the appearance of the pulmonary tumour, the inventors studied the expression profile of microRNAs circulating in collected samples up to two years preceding the diagnosis of the disease and at the moment of surgery in patients of two independent clinical trials, as mentioned above, for early diagnosis of pulmonary tumour in high-risk individuals (age >50 years and smokers) using spiral CT. In the first training set, made up of 40 samples of plasma from 19 patients and 27 samples of plasma from healthy control individuals in 5 different pools, the miRNA expression levels were analysed using TaqMan MicroRNA Assays (Applied Biosystems) with the aim of identifying the significantly-
different miRNA ratios (p<0.05) between samples of plasma collected pre-disease, at the moment of surgery and from healthy individuals.

The specificity and sensitivity of the signatures of microRNAs thus obtained were compared with the validation set composed, as described above, of 32 plasma samples of 22 patients and 54 plasma samples of healthy control individuals, grouped in 10 different pools.

For the generalisation of the signatures used for predicting the aggressiveness of the disease, the inventors grouped the two cohorts (training set and validation set) with the aim of obtaining a sufficient number for the statistical analysis. The cases with unfavourable prognosis were first compared to the respective controls and the signatures thus obtained were tested to evaluate their effective capacity to discriminate the patients having poor prognosis from those having good prognosis.

**Completed tests – materials and equipment used**

As already mentioned, the signatures of the microRNAs identified in the various analyses were validated on two independent sets constituted by high-risk individuals (smokers of more than 50 years of age) enrolled in two different clinical trials for early identification of pulmonary tumour using low-dose spiral CT: a first set, or training set, made up of 40 samples of plasma from 19 patients and 27 samples of plasma from healthy control individuals grouped in 5 different pools and a second set or validation set (i.e. in a second set of individuals) made up of 32 samples of plasma from 22 patients and 54 samples of plasma from healthy control individuals, grouped in 10 different pools.

Figure 1 summarises the pathological clinical characteristics of the training set and the validation set selected for the analysis of the expression levels of the miRNAs in the plasma samples.

To determine the microRNA profile in the plasma samples the total RNA was extracted from 200μl of plasma using the mirVana™ PARIS™ Kit (Ambion), eluting in 50μl of elution buffer.

The expression levels were determined using q-Real Time PCR starting from 3μl of elute first using the Megaplex™ Pools Protocol on a microfluidic card, type A (Applied Biosystems), then the Multiplex™ Pools Protocol (Applied Biosystems).

All the data was extrapolated using the Sequence Detection System software (SDS 2.2.2®, Applied Biosystems), setting the threshold manually at 0.2 and the baseline between 3 and 18 cycles (on a total of 40).
Apart from the standard equipment for molecular biology, use was made of Real-time quantitative PCR 7900-HT (Applied Biosystems) and GeneAmp® 9700 Sequence Detection System (Applied Biosystems).

**Results**

The inventors identified the miRNAs, in particular those of appended Table I, as molecular biomarkers for the evaluation of the risk of manifesting pulmonary tumours within 1-3 years from the sample collection of biological fluid. The inventors also identified that the ratios among the miRNA expression values are ideal molecular biomarkers for investigation into the evaluation of the risk of contracting a pulmonary tumour within 1-3 years from the sample collection of biological fluid, such as whole blood, serum, plasma, saliva or bronchial condensate.

Using the expression levels of the miRNAs listed in Table I, the inventors specifically identified the ratios among the values measured of the expression levels relative to the pairs of microRNA listed in Table III. These ratios can be used for the evaluation of the risk of contracting pulmonary tumour within 1-3 years from the collection of the sample of biological fluid, giving extremely reliable prediction results.

In more detail, by calculating a sufficient number of real ratios selected from among those in Table III, for example at least 20% of them, and optionally at least 50%, it is possible to observe the progress with respect to control ratios. An individual is defined at high risk of pulmonary tumour, which might be detectable by spiral CT, in a period comprised between one and three years from the collection of the sample of biological fluid, for whom at least 30% of real ratios calculated deviates with respect to the respective value of the control ratio.

The inventors also identified the miRNAs, in particular those of appended Table II, as biomarkers for evaluation of the risk of contracting aggressive pulmonary tumour within 1-3 years from the sample collection of biological fluid. Further, in this case too, the ratios between the miRNA expression values were specifically identified as ideal molecular biomarkers to be investigated for the evaluation of the risk of contracting an aggressive pulmonary tumour within 1-3 years from the sample biological fluid collection, which might be whole blood, serum, plasma, saliva or bronchial condensate.

Further, by using the miRNA expression levels of Table II, the inventors identified the ratios between measure values of expression levels relative to pairs of microRNAs of Table IV for evaluation of the risk of contracting an aggressive pulmonary tumour within 1-3 years from the sample collection of biological fluid. In more detail, by
calculating a sufficient number of real ratios selected from among the ratios of Table IV, for example at least 30%, and optionally at least 50%, the progression of the ratios with respect to control ratios can be studied.

[000238] An individual is defined as at risk of contracting an aggressive pulmonary tumour in a period comprised between one and three years from the collection of the sample of biological fluid, if in that individual at least 50%, optionally at least 75%, of the real ratios calculated deviate with respect to the respective control ratio value.

[000239] The use of miRNA ratios also enables reliably predicting the development of pulmonary tumour, in particular of the more aggressive form, in high-risk individuals (more than 50 years of age and heavy smokers) up to two years before the disease is at a visible stage with the better imaging techniques at present available (spiral CT). Note also that the method using the calculation of the ratios, or miRNA ratios, described above can be actuated with a simple collection of a blood sample and is therefore entirely non-invasive, and allows the analysis to be performed rapidly and economically.

[000240] In addition to the above, the inventors found that the miRNAs, and in particular those listed in Table V, have a role as biomolecular markers for determining the actual presence of a pulmonary tumour in an individual, for diagnostic purposes.

[000241] The inventors also found that in this case the ratios between expression level values of miRNA pairs are valid biomarkers with a diagnostic and prognostic function. In detail, the miRNAs of Table V were used for determining the ratios of Table VII which represent ratios between measured values of expression levels of relative microRNA pairs and which are used to determine the actual presence (diagnosis) of a pulmonary tumour in an individual. In more detail, by calculating at least 20% of the real ratios of Table VII it is possible to define the individual presents a pulmonary tumour if at least 30% of the real ratios (as in Table VII) calculated deviate from the respective control value.

[000242] Lastly, the inventors have found that the miRNAs, and in particular those listed in Table VI, can be used as biomolecular markers for determining the actual presence of an aggressive pulmonary tumour in an individual (prognosis). The inventors have also found that in particular the ratios between values of expression levels of miRNA pairs are valid biomarkers with a diagnostic and prognostic function even in the case of an aggressive tumour.

[000243] In detail, the miRNAs of Table VI were used to determine the ratios of Table VIII where there is a list of ratios between the measured values of the expression levels relative to microRNA pairs of Table VI used for determining the actual presence of an
aggressive pulmonary tumour in an individual. In detail, by detecting at least 20% of the real ratios of Table VIII, it is possible to define an individual having an aggressive pulmonary tumour as one in whom at least 60% of the real ratios that have been calculated deviate with respect to the respective control value.

Thus the use of the described procedure can help also to resolve the problem of overdiagnosis and overtreatment in patients who are not at risk.

In greater detail, in a context of surveillance of the disease using spiral CT, the use of a test based on this method might enable selection of only a sub-group of patients at high risk of developing the disease to be subsequently kept under a more strict control. Further, the ability of the test to predict the patients who will develop a more aggressive disease, frequently not diagnosed by the CT scan, enables directing these individuals directly to specific pharmacological programmes (including giving up smoking) and/or the use of more specific diagnostic examinations based on the metabolic-biological characteristics such as PET with various tracers or body MRI, or a different local treatment such as stereotaxic radiotherapy, or other treatments besides.

Lastly, the use of miRNA ratios is an easily-applicable method with a potential current clinical use and which avoids the use of more profound and complex analysis.

Examples

The following Examples may relate to variants compared Tables I to VIII.

1. Identification of a signature based on miRNAs able to identify individuals at risk of developing pulmonary tumour (Figure 4A)

The samples of plasma collected 1-2 years before from patients in whom a tumour was later diagnosed using spiral CT were analysed and compared with the control pool, constituted by healthy individuals.

A signature was therefore identified in the training comprising 14 miRNA ratios made up of 14 microRNAs capable of correctly discriminating 18 out of 20 pre-disease samples from individuals who then will develop the disease (90% sensitivity), while only one control pool was positive for this signature (80% specificity). In the validation set the sensitivity was 80%, while the specificity was 90% (AUC-ROC=0.85, p<0.001).

The miRNA ratios of the first example were then listed and are reported also in figure 4A.

Q₁ = hsa-miR-106a / hsa-miR-451
The predictive capacity of this signature was validated in samples collected up to 28 months before the diagnosis of disease with spiral CT and the microRNAs most frequently deregulated were: mir-660, mir 140-5p, mir-451, mir-28-3p, mir-30c and mir-92.

2. Identification of the signature based on the miRNAs able to have diagnostic value (figure 4B).

Plasma samples collected at the moment of surgery or on identification of the disease by spiral CT were compared with the control pools. In the training set a panel of 16 miRNA ratios, made up of 13 microRNAs, correctly classify 16 out of 19 patients with a sensitivity of 84% and a specificity of 80%. In the validation set sensitivity is 75% and the specificity is 100% (AUC-ROC=0.88, p<0.0001).

A lower sensitivity in the validation set can be correlated to the presence of a greater number of small indolent nodules, of which two patients are part, whose blood samples were mis-matched both by the risk signature in the pre-disease samples, and by the signature in the samples taken in the presence of disease.

The miRNA ratios of the second example are listed herein below and are also reported in figure 4B.

Q₁= hsa-miR-106a/ hsa-miR-140-5p
Q₂= hsa-miR-106a/ hsa-miR-30c
Q₃= hsa-miR-106a/ hsa-miR-486-5p
Q₄= hsa-miR-140-5p/ hsa-miR-17
Q₅= hsa-miR-140-5p/ hsa-miR-660
Q₆= hsa-miR-15b/ hsa-miR-660
Q₇= hsa-miR-15b/ hsa-miR-92a
Q₈= hsa-miR-17/ hsa-miR-30c
Q₉= hsa-miR-17/ hsa-miR-451
Q₁₀= hsa-miR-17/ hsa-miR-486-5p
Q₁₁= hsa-miR-19b/ hsa-miR-451
Q₁₂= hsa-miR-19b/ hsa-miR-660
Q₁₃= hsa-miR-19b/ hsa-miR-92a
Q₁₄= hsa-miR-21/ hsa-miR-92a
Q₁₅= hsa-miR-28-3p/ hsa-miR-660
Q₁₆= hsa-miR-28-3p/ hsa-miR-92a

This diagnostic signature was then used to verify the presence of disease in the plasma samples collected before identification of the disease by spiral CT. In the training set, 11 out of 20 (55%) of the cases were classified as being in presence of disease and, very interestingly, of these 11, 10 are either pessimistic diagnosis cases or belonging to patients in whom the tumour was identified in the later years of the screening, or where more aggressive tumours with worse prognoses were identified.

Very similar results were obtained in the validation set, since in 10 out of 15 (66.6%) pre-disease samples the signature of the presence of disease was presented. There are only 4 miRNA ratios in common between the risk signatures and the diagnosis signatures; also partially different are the microRNAs involved: mir-17, mir-660, mir-92a, mir-106a, mir-19b are the most deregulated microRNAs at the moment of the diagnosis of pulmonary tumour.

3. Identification of a signature based on the miRNAs for risk of development of aggressive pulmonary tumour

The microRNA profiles of the pre-disease samples with unfavourable prognosis were identified and 10 miRNA ratios identified that were able to recognise 5 out of 5 patients in the first set, 4 out of 5 in the validation set and with a specificity in both of 100%. Note that mir-221, mir-660, mir-486-5p, mir-28-3p, mir-197, mir-106a, mir-451, mir-140-5p and mir-16 are the deregulated microRNAs.

The miRNA ratios of this third example are listed below and are also reported in figure 4C.

Q₁= hsa-miR-106a/ hsa-miR-660
Q₂= hsa-miR-140-5p/ hsa-miR-486-5p
Q₃ = hsa-miR-16/ hsa-miR-197
Q₄ = hsa-miR-197/ hsa-miR-486-5p
Q₅ = hsa-miR-197/ hsa-miR-660
Q₆ = hsa-miR-221/ hsa-miR-451
Q₇ = hsa-miR-221/ hsa-miR-660
Q₈ = hsa-miR-28-3p/ hsa-miR-451
Q₉ = hsa-miR-28-3p/ hsa-miR-486-5p
Q₁₀ = hsa-miR-28-3p/ hsa-miR-660

This signature was then tested on the pre-disease samples of the patients having a good prognosis in the training set and in the validation set. The signature classifies, respectively in the two sets, 33.3% and 45% of the samples; figure 2 illustrates a Kaplan-Meier survival curve of patients with or without the signature of risk of aggressive disease; the curve with the aggressive signature is represented in a continuous line and identified by RAD+ (risk of aggressive disease +) while the curve without the signature of risk of aggressive disease is represented by a discontinuous line and identified by RAD- (risk of aggressive disease -) in plasma samples collected 1-2 years before identification of the disease by spiral CT.

Of interest is the fact that the majority of the identified samples belong to individuals who developed the tumour between the III and the V year of screening, independently of the degree of the tumour. This supports the previous observation on the tumoral and normal samples of lung tissue, where a different profile of mircoRNA was present respectively in the tumoral and normal tissue of the same patients. It is worthy of note that among the patients having a tumour diagnosed in the second year of screening (all tumours at stage Ia and Ib), only one case with stage Ib exhibited the signature of aggressive risk.

4. Identification of a signature based on miRNAs for prognosis of patients identified by spiral CT (figure 4D)

The samples from patients having a pessimistic prognosis, collected at the moment of the diagnosis of the disease, were analysed, revealing a signature of 10 miRNA ratios, all containing mir-486-5p, which identifies 7 out of 8 patients with a pessimistic prognosis in the training set, 2 out of 3 of the validation set and no control pool in either data set.

The miRNA ratios of this fourth example are listed below and are also reported in figure 4D.
Further, only 2 out of 11 and 2 out of 13 patients having a good prognosis, respectively in the first and second set, are positive for this signature; figure 3 reports a Kaplan-Meier survival curve of patients with or without the signatures for presence of aggressive disease (respectively identified with the continuous line of PAD+, which stands for the presence of aggressive disease +, and with the broken line of PAD-, standing for the presence of aggressive disease - ) in plasma samples collected at the moment of identification of the disease by spiral CT.

Further, this signature was used to classify the pre-disease samples in both data sets. Half of the patients with pessimistic prognosis also present this aggressiveness signature, while for those with good prognosis of the 6 positives for this signature, 5 are tumours identified after the third year of screening.

Note that mir-486-5p, compared with mir-21, mir-126, mir-15b, mir-148a, mir-142-3p, mir-17, mir-197, mir-221, mir-28-3p and mir-106a, is always under-expressed in the plasma of patients with a pessimistic prognosis.

From the above-reported results, the inventors deduced that the microRNAs present in the plasma are useful for identifying the presence of the pulmonary tumour even 1-2 years before detection by spiral CT and further for predicting the development of types of more aggressive pulmonary cancer, indicating the possibility of selecting individuals at high risk on the basis of profiles of circulating microRNA.

Kits and apparatus for determining the biomolecular markers present in a sample of human biological fluid usable for determination of risk.

The inventors have also developed medical kits useful for effectively and simply applying the methods described above, for determining the risk of contracting a tumour, for example by using a sample of blood removed from an individual.
[000324] In its general form the kit comprises a platform having a plurality of sites, each of which is destined to receive a respective discrete quantity of the sample of biological fluid (for example whole blood, serum, plasma, saliva or bronchial condensate). Each site comprises a reagent capable of bonding with at least a respective of the microRNAs of Table I and/or Table II, in such a way as to enable detectability with the apparatus described herein below.

[000325] In the structural profile the platform can be a support of the microfluidic card with the miRNA of interest and with channellings for the distribution to the respective sites of a predetermined number of samples of biological fluid.

[000326] For example it can include at least one selected from a group comprising:

[000327] a polynucleotide comprising a nucleotide sequence of at least one of the microRNAs as in Table I and/or Table II,

[000328] a polynucleotide comprising a nucleotide sequence which is complementary to a sequence of at least one of the microRNAs as in Table I and/or Table II,

[000329] a molecular probe configured such as to recognise a sequence of at least one of the microRNAs as in Table I and/or Table II.

[000330] The described medical kit can also be used with a medical apparatus comprising a unit defining a seating for receiving one or more kits and means for determining the value of the expression of the microRNAs of Table I and/or Table II. The means for determining the value of the expression level comprise one from among the techniques selected from a group as follows:

[000331] Quantitative Real-time PCR
[000332] Microfluidic cards
[000333] Microarrays
[000334] Quantitative or semi-quantitative RT-PCR
[000335] Northern blot
[000336] Solution Hybridization
[000337] Sequencing

[000338] The apparatus can also exhibit means for calculating the values of the real ratios among values of expression levels of pairs of microRNAs as in Table III and/or as in Table IV. These means can comprise a programme and a processing unit in which the programme contains instructions which when carried out by the processor enable a calculation of the ratios. Alternatively an analog circuit can be provided which is able to perform the calculations.
Kits and apparatus for determining the biomolecular markers present in a sample of human biological fluid usable for diagnosis of tumours.

The inventors have also developed medical kits that are useful for effectively and simply setting up the above-described methods for tumour diagnosis, for example by using a blood sample collected from an individual.

In its general form the kit comprises a platform having a plurality of sites, each of which is destined to receive a respective discrete quantity of the sample of biological fluid (for example whole blood, serum, plasma, saliva or bronchial condensate). In the structural sense the platform can be a support for a micro-fluidic card with the miRNA of interest with channellings for the distribution to the respective sites of a predetermined number of samples of biological fluid. Each site comprises a reagent capable of bonding with at least a respective one of the microRNAs of Table V and/or Table VI.

For example the reagent includes at least one selected from the group comprising:

- a polynucleotide comprising a nucleotide sequence of at least one of the microRNAs as in Table V and/or Table VI;
- a polynucleotide comprising a nucleotide sequence which is complementary to a sequence of at least one of the microRNAs as in Table V and/or Table VI;
- a molecular probe configured such as to recognise a sequence of at least one of the microRNAs as in Table V and/or Table VI.

The described medical kit can also be used with a medical apparatus defining a seating for receiving one or more of the kits of the preceding claim, and means for determining a value of the level of expression of the microRNAs as in Table V and/or Table VI. The means for determining the value of the expression level comprise one selected from among the following group:

- Quantitative Real-time PCR,
- Microfluidic cards,
- Microarrays,
- RT – PCR, quantitative or semi-quantitative,
- Northern blot,
- Solution Hybridization,
- Sequencing.

The apparatus further comprises means for calculating the values of the real ratios among the values of expression levels of pairs of microRNA, the ratios being selected...
from among those of Table VII and/or Table VIII. The means can comprise a program and a processing unit in which the program contains instructions that when performed by the processor enable a calculation of the ratios. Alternatively an analog circuit can be provided which is capable of performing the same calculations.

[000355]

[000356] **Compounds and treatment**

[000357] The inventors have developed a method for treating an individual in whom the presence of a pulmonary tumour has been diagnosed or in whom a risk of developing a pulmonary tumour has been diagnosed, respectively for the treatment of the pulmonary tumour or in order to reduce and/or eliminate the risk of developing a pulmonary tumour.

[000358] The method comprises the following steps of measuring an expression level of at least an miRNA listed in Table I or Table II or Table V or Table VI, present in a sample of biological fluid previously collected from an individual, and then determining the miRNAs having values measured for the expression level which deviate with respect to a predetermined and respective control criterion. The evaluation of the deviation with respect to a control criterion can use the procedures of the miRNA ratios described above for the various cases.

[000359] Once the overexpressed or underexpressed miRNAs have been determined, the method comprises altering the expression level of the miRNAs whose levels of expression deviate with respect to the respective control criterion.

[000360] For example, in order to alter the expression level of the miRNAs the individual can be administered with a pharmaceutical compound having an effective quantity of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI if the expression level measured of the miRNA, or miRNAs, is lower than a respective control expression level.

[000361] Alternatively, or in addition to the above, it is also possible to administer the individual with a pharmaceutical compound having an effective quantity of at least a compound for inhibiting the expression of at least one of the miRNAs listed in Table I or Table II or Table V or Table VI if and for those miRNAs whose measured expression level is above the control expression level.

[000362] In this way the values of the expression level can be reset to the control expression level for the underexpressed miRNAs with respect to the respective control level of expression and/or it is possible to reduce the expression level for the overexpressed miRNAs.
With the aim of resetting the level of the underexpressed miRNAs a therapeutically effective quantity of a compound can be administered which comprises at least one of the miRNAs of Table I or Table II or Table V or Table VI, chemically synthesised (miRNA mimetics) or recombinant.

With the aim of reducing the expression level values to the control expression level for the overexpressed miRNAs with respect to the respective control expression level, a therapeutically effective quantity of a compound can be administered which comprises at least an inhibitor of a microRNA of Table I or Table II or Table V or Table VI. The inhibitor comprises, for example, one or more of the following: double-filament RNA, optionally short interfering RNA (siRNA), antisense nucleic acids (anti-miRNA oligonucleotides (AMOs), molecules of enzymatic RNA (ribozymes). The inhibitor is directed to a specific product of microRNA and interferes with the expression (by inhibition of the translation or induction of the degradation) of a target gene of the microRNA.

The administering of the above compounds (synthetic microRNAs or mimetic miRNAs and inhibitors of microRNA) can for example be done by means of viral systems or nanoparticles containing microRNA or microRNA inhibitor) linked covalently with lipids or encapsulated liposomes.

The administration methods can be the following, possibly combined with one another:

- intranasal instillation
- inhalation (aerosol)
- systemic (injection or infusion)
- direct inoculation in the tumour (where present and visible)
- intrapleural administration
- endopneumonic administration

In terms of dosage, continuous and prolonged dosage can be performed over time. As miRNA molecules are "naturally" present in the organism, no relevant toxicity will obtain.

Table I: microRNAs used for evaluation of the risk of manifesting a pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-451</td>
</tr>
</tbody>
</table>
Comparing the miRNAs listed in Table I in pre-disease patient samples v. disease free samples (control) results showed a sensitivity of 83.3 (training sensitivity of 85.0; validation sensitivity of 81.3) and a specificity of 95.5 (training specificity of 85.7; validation specificity of 100.0).

Table Ib: preferred microRNAs used for evaluation of the risk of manifesting a pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

Comparing the preferred miRNAs listed in Table Ib in pre-disease patient samples v. disease free samples (control) results showed a sensitivity of 80.6 (training sensitivity of 80.0; validation sensitivity of 81.3) and a specificity of 95.5 (training specificity of 85.7; validation specificity of 100.0).
Table II: microRNAs used for evaluation of the risk of manifesting an aggressive pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-140-5p</td>
</tr>
<tr>
<td>hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-197</td>
</tr>
<tr>
<td>hsa-miR-221</td>
</tr>
<tr>
<td>hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-28-3p</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
</tr>
<tr>
<td>hsa-miR-15b</td>
</tr>
<tr>
<td>hsa-miR-30c</td>
</tr>
<tr>
<td>hsa-miR-30b</td>
</tr>
<tr>
<td>hsa-miR-101</td>
</tr>
<tr>
<td>hsa-miR-21</td>
</tr>
<tr>
<td>hsa-miR-140-3p</td>
</tr>
<tr>
<td>hsa-miR-142-3p</td>
</tr>
</tbody>
</table>

Comparing the miRNAs listed in Table II in pre-disease patient samples of aggressive lung cancer v. pre-disease samples of indolent lung cancer and disease free samples (control) results showed a sensitivity of 94.5 (training sensitivity of 90.9; validation sensitivity of 100.0) and a specificity of 97.6 (training specificity of 100.0; validation specificity of 96.0).

Table IIb: preferred microRNAs used for evaluation of the risk of manifesting an aggressive pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-221</td>
</tr>
</tbody>
</table>
Comparing the miRNAs listed in Table IIb in pre-disease patient samples of aggressive lung cancer v. pre-disease samples of indolent lung cancer and disease free samples (control) results showed a sensitivity of 94.5 (training sensitivity of 90.9; validation sensitivity of 100.0) and a specificity of 95.0 (training specificity of 100.0; validation specificity of 92.0).

Table III: ratios among measured values of expression of pairs of microRNAs used for evaluating a risk of manifesting a pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).
Table IIIb: ratios among measured values of expression of preferred pairs of microRNAs used for evaluating a risk of manifesting a pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-30b / hsa-miR-320</td>
</tr>
<tr>
<td>hsa-miR-30b / hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-30b / hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-197 / hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-197 / hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-197 / hsa-miR-320</td>
</tr>
<tr>
<td>hsa-miR-30c / hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-30c / hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-30c / hsa-miR-320</td>
</tr>
</tbody>
</table>

Table IV: ratios among measured values of expression of pairs of microRNAs used for determining a risk of manifesting an aggressive pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1=hsa-miR-221/hsa-miR-660</td>
</tr>
<tr>
<td>Q2=hsa-miR-221/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q3 = hsa-miR-221/hsa-miR-451</td>
</tr>
<tr>
<td>Q4 = hsa-miR-140-3p/hsa-miR-221</td>
</tr>
<tr>
<td>Q5 = hsa-miR-21/hsa-miR-221</td>
</tr>
<tr>
<td>Q6 = hsa-miR-101/hsa-miR-221</td>
</tr>
<tr>
<td>Q7 = hsa-miR-197/hsa-miR-660</td>
</tr>
<tr>
<td>Q8 = hsa-miR-197/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q9 = hsa-miR-140-5p/hsa-miR-660</td>
</tr>
<tr>
<td>Q10 = hsa-miR-140-5p/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q11 = hsa-miR-140-5p/hsa-miR-19b</td>
</tr>
<tr>
<td>Q12 = hsa-miR-142-3p/hsa-miR-660</td>
</tr>
<tr>
<td>Q13 = hsa-miR-148a/hsa-miR-660</td>
</tr>
<tr>
<td>Q14 = hsa-miR-148a/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q15 = hsa-miR-148a/hsa-miR-19b</td>
</tr>
<tr>
<td>Q16 = hsa-miR-148a/hsa-miR-451</td>
</tr>
<tr>
<td>Q17 = hsa-miR-15b/hsa-miR-660</td>
</tr>
<tr>
<td>Q18 = hsa-miR-15b/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q19 = hsa-miR-15b/hsa-miR-19b</td>
</tr>
<tr>
<td>Q20 = hsa-miR-19b/hsa-miR-221</td>
</tr>
<tr>
<td>Q21 = hsa-miR-19b/hsa-miR-30c</td>
</tr>
<tr>
<td>Q22 = hsa-miR-28-3p/hsa-miR-660</td>
</tr>
<tr>
<td>Q23 = hsa-miR-28-3p/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q24 = hsa-miR-30b/hsa-miR-660</td>
</tr>
<tr>
<td>Q25 = hsa-miR-30b/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q26 = hsa-miR-30c/hsa-miR-660</td>
</tr>
<tr>
<td>Q27 = hsa-miR-30c/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q28 = hsa-miR-19b/hsa-miR-28-3p</td>
</tr>
</tbody>
</table>

[000386] Table IVb: ratios among measured values of expression of preferred pairs of microRNAs used for determining a risk of manifesting an aggressive pulmonary tumour (within 1-3 years from collecting the sample of biological fluid).

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-221/hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-28-3p/hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-19b/ hsa-miR-221</td>
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<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>hsa-miR-19b/ hsa-miR-28-3p</td>
</tr>
<tr>
<td>hsa-miR-148a/ hsa-miR-19b</td>
</tr>
<tr>
<td>hsa-miR-148a/ hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-28-3p/ hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-221/ hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-148a/ hsa-miR-660</td>
</tr>
</tbody>
</table>

[000387] In connection with the risk of manifesting a pulmonary tumor, Figure 5 shows (on the left hand side) the ROC curve when using the 15 miRNAs of Table I to create the 30 ratios of Table III and (on the right end side) the ROC curve when using the 6 miRNAs of Table Ib to create the 9 ratios of Table IIIb.

[000388] In connection with the risk of manifesting an aggressive pulmonary tumor, Figure 6 shows (on the left hand side) the ROC curve when using the 16 miRNAs of Table II to create the 28 ratios of Table IV and (on the right end side) the ROC curve when using the 6 miRNAs of Table IIb to create the 9 ratios of Table IVb.

[000389] **Table V:** microRNAs used for determining the actual presence of a pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA</th>
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<tbody>
<tr>
<td>hsa-miR-106a</td>
</tr>
<tr>
<td>hsa-miR-140-3p</td>
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<tr>
<td>hsa-miR-17</td>
</tr>
<tr>
<td>hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-15b</td>
</tr>
<tr>
<td>hsa-miR-92a</td>
</tr>
<tr>
<td>hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
</tr>
<tr>
<td>hsa-miR-28-3p</td>
</tr>
<tr>
<td>hsa-miR-133a</td>
</tr>
<tr>
<td>hsa-miR-101</td>
</tr>
<tr>
<td>hsa-miR-197</td>
</tr>
<tr>
<td>hsa-miR-145</td>
</tr>
</tbody>
</table>
Comparing the miRNAs listed in Table V in the plasma of patients at surgery v. disease free samples (control) results showed a sensitivity of 80.5 (training sensitivity of 84.2; validation sensitivity of 76.5) and a specificity of 95.5 (training specificity of 100.0; validation specificity of 93.3).

Table Vb: preferred microRNAs used for determining the actual presence of a pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-106a</td>
</tr>
<tr>
<td>hsa-miR-17</td>
</tr>
<tr>
<td>hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-92a</td>
</tr>
<tr>
<td>hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-197</td>
</tr>
</tbody>
</table>

Comparing the miRNAs listed in Table Vb in the plasma of patients at surgery v. disease free samples (control) results showed a sensitivity of 77.8 (training sensitivity of 84.2; validation sensitivity of 70.6) and a specificity of 90.9 (training specificity of 85.7; validation specificity of 93.3).

Table VI: microRNAs used for determining the actual presence of an aggressive pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-142-3p</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
</tr>
<tr>
<td>hsa-miR-15b</td>
</tr>
<tr>
<td>hsa-miR-21</td>
</tr>
<tr>
<td>hsa-miR-221</td>
</tr>
<tr>
<td>hsa-miR-660</td>
</tr>
</tbody>
</table>
Comparing the miRNAs listed in Table VI in patient samples of aggressive lung cancer v. pre-disease samples of indolent lung cancer and disease free samples (control) results showed a sensitivity of 86.7 (training sensitivity of 80.0; validation sensitivity of 100.0) and a specificity of 93.2 (training specificity of 94.0; validation specificity of 92.6).

Table VIb: preferred microRNAs used for determining the actual presence of an aggressive pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-142-3p</td>
</tr>
<tr>
<td>hsa-miR-21</td>
</tr>
<tr>
<td>hsa-miR-221</td>
</tr>
<tr>
<td>hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
</tr>
<tr>
<td>hsa-miR-486-5p</td>
</tr>
</tbody>
</table>

Comparing the miRNAs listed in Table VIb in patient samples of aggressive lung cancer v. pre-disease samples of indolent lung cancer and disease free samples (control) results showed a sensitivity of 80.0 (training sensitivity of 80.0; validation sensitivity of 80.0) and a specificity of 93.2 (training specificity of 94.0; validation specificity of 92.6).

Table VII: ratios among measured values of expression of pairs of microRNAs used for determining an actual presence of pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1=hsa-miR-17/hsa-miR-451</td>
</tr>
<tr>
<td>Q2=hsa-miR-106a/hsa-miR-451</td>
</tr>
<tr>
<td>Q3=hsa-miR-133a/hsa-miR-451</td>
</tr>
<tr>
<td>Q4=hsa-miR-17/hsa-miR-660</td>
</tr>
<tr>
<td>Q5=hsa-miR-106a/hsa-miR-660</td>
</tr>
<tr>
<td>Q6=hsa-miR-197/hsa-miR-451</td>
</tr>
<tr>
<td>Q7=hsa-miR-133a/hsa-miR-660</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Q8=hsa-miR-145/hsa-miR-451</td>
</tr>
<tr>
<td>Q9=hsa-miR-28-3p/hsa-miR-451</td>
</tr>
<tr>
<td>Q10=hsa-miR-17/hsa-miR-92a</td>
</tr>
<tr>
<td>Q11=hsa-miR-106a/hsa-miR-92a</td>
</tr>
<tr>
<td>Q12=hsa-miR-197/hsa-miR-660</td>
</tr>
<tr>
<td>Q13=hsa-miR-133a/hsa-miR-92a</td>
</tr>
<tr>
<td>Q14=hsa-miR-145/hsa-miR-660</td>
</tr>
<tr>
<td>Q15=hsa-miR-28-3p/hsa-miR-660</td>
</tr>
<tr>
<td>Q16=hsa-miR-15b/hsa-miR-451</td>
</tr>
<tr>
<td>Q17=hsa-miR-19b/hsa-miR-451</td>
</tr>
<tr>
<td>Q18=hsa-miR-30b/hsa-miR-451</td>
</tr>
<tr>
<td>Q19=hsa-miR-17/hsa-miR-320</td>
</tr>
<tr>
<td>Q20=hsa-miR-106a/hsa-miR-320</td>
</tr>
<tr>
<td>Q21=hsa-miR-17/hsa-miR-21</td>
</tr>
<tr>
<td>Q22=hsa-miR-106a/hsa-miR-21</td>
</tr>
<tr>
<td>Q23=hsa-miR-197/hsa-miR-92a</td>
</tr>
<tr>
<td>Q24=hsa-miR-101/hsa-miR-106a</td>
</tr>
<tr>
<td>Q25=hsa-miR-133a/hsa-miR-320</td>
</tr>
<tr>
<td>Q26=hsa-miR-101/hsa-miR-17</td>
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<td>Q28=hsa-miR-28-3p/hsa-miR-92a</td>
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<tr>
<td>Q29=hsa-miR-106a/hsa-miR-140-3p</td>
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<tr>
<td>Q30=hsa-miR-15b/hsa-miR-660</td>
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<tr>
<td>Q31=hsa-miR-19b/hsa-miR-660</td>
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<tr>
<td>Q32=hsa-miR-30b/hsa-miR-660</td>
</tr>
<tr>
<td>Q33=hsa-miR-126/hsa-miR-451</td>
</tr>
<tr>
<td>Q34=hsa-miR-140-5p/hsa-miR-451</td>
</tr>
<tr>
<td>Q35=hsa-miR-133a/hsa-miR-21</td>
</tr>
<tr>
<td>Q36=hsa-miR-140-3p/hsa-miR-17</td>
</tr>
</tbody>
</table>

[000398] **Table VIIb**: ratios among measured values of expression of preferred pairs of microRNAs used for determining an actual presence of pulmonary tumour in an individual.
### Table VIII: ratios among measured values of expression of pairs of microRNAs used for determining an actual presence of aggressive pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-106a/ hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-106a/ hsa-miR-92a</td>
</tr>
<tr>
<td>hsa-miR-106a/ hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-17/ hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-17/ hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-17/ hsa-miR-92a</td>
</tr>
<tr>
<td>hsa-miR-197/ hsa-miR-451</td>
</tr>
<tr>
<td>hsa-miR-197/ hsa-miR-92a</td>
</tr>
<tr>
<td>hsa-miR-197/ hsa-miR-660</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1=hsa-miR-142-3p/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q2=hsa-miR-21/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q3=hsa-miR-221/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q4=hsa-miR-19b/hsa-miR-21</td>
</tr>
<tr>
<td>Q5=hsa-miR-19b/hsa-miR-221</td>
</tr>
<tr>
<td>Q6=hsa-miR-142-3p/hsa-miR-19b</td>
</tr>
<tr>
<td>Q7=hsa-miR-148a/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q8=hsa-miR-15b/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q9=hsa-miR-30b/hsa-miR-486-5p</td>
</tr>
<tr>
<td>Q10=hsa-miR-142-3p/hsa-miR-660</td>
</tr>
<tr>
<td>Q11=hsa-miR-221/hsa-miR-660</td>
</tr>
<tr>
<td>Q12=hsa-miR-148a/hsa-miR-19b</td>
</tr>
<tr>
<td>Q13=hsa-miR-15b/hsa-miR-19b</td>
</tr>
<tr>
<td>Q14=hsa-miR-19b/hsa-miR-30b</td>
</tr>
<tr>
<td>Q15=hsa-miR-16/hsa-miR-486-5p</td>
</tr>
</tbody>
</table>
Table VIIIb: ratios among measured values of expression of preferred pairs of microRNAs used for determining an actual presence of aggressive pulmonary tumour in an individual.

<table>
<thead>
<tr>
<th>miRNA Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-142-3p/hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-142-3p/hsa-miR-19b</td>
</tr>
<tr>
<td>hsa-miR-21/hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-221/hsa-miR-660</td>
</tr>
<tr>
<td>hsa-miR-19b/hsa-miR-21</td>
</tr>
<tr>
<td>hsa-miR-19b/hsa-miR-221</td>
</tr>
<tr>
<td>hsa-miR-142-3p/hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-221/hsa-miR-486-5p</td>
</tr>
<tr>
<td>hsa-miR-21/hsa-miR-486-5p</td>
</tr>
</tbody>
</table>

In connection with the determination of the actual presence of a pulmonary tumour in an individual, Figure 7 shows (on the left hand side) the ROC curve when using the 18 miRNAs of Table V to create the 36 ratios of Table VII and (on the right end side) the ROC curve when using the 6 miRNAs of Table Vb to create the 9 ratios of Table VIIIb.

In connection with the determination of the actual presence of an aggressive pulmonary tumour in an individual, Figure 8 shows (on the left hand side) the ROC curve when using the 10 miRNAs of Table VI to create the 16 ratios of Table VIII and (on the right end side) the ROC curve when using the 6 miRNAs of Table VIb to create the 9 ratios of Table VIIIb.
We claim:

1. A method comprising:
   determining the level of expression of at least six miRNA from the miRNA listed in Tables I, II, V, or VI in a biological sample from a subject, and
   comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample.

2. A method comprising:
   determining the level of expression of the six miRNA listed in Table Ib in a biological sample from a subject, and
   comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample,
   wherein a change or deviation in the level of expression of said at least six miRNA in said biological sample from said control biological sample identifies a subject at risk of manifesting a tumor.

3. The method of claim 2, wherein said tumor cannot be detected by CT spiral scan.

4. A method comprising:
   determining the level of expression of the six miRNA listed in Table Iib in a biological sample from a subject, and
   comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample,
   wherein a change or deviation in the level of expression of said at least six miRNA in said biological sample from said control biological sample identifies a subject at risk of manifesting an aggressive tumor.

5. A method comprising:
   determining the level of expression of the six miRNA listed in Table Vb in a biological sample from a subject, and
   comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample,
wherein a change or deviation in the level of expression of said at least six miRNA in said biological sample from said control biological sample determines the presence of a tumor in said subject.

6. The method of claim 5, wherein said determination of the presence of said tumor confirms detection by CT spiral scan.

7. A method comprising:
   determining the level of expression of the six miRNA listed in Table VIb in a biological sample from a subject, and
   comparing the level of expression of said miRNA from said sample from said subject to the level of expression of said miRNA from a control biological sample,
   wherein a change or deviation in the level of expression of said at least six miRNA in said biological sample from said control biological sample determines the presence of an aggressive tumor in said subject.

8. The method of claim 7, wherein said determination provides a prognosis of disease-free survival following surgical intervention.

9. The method of claim 1, further comprising
   calculating a plurality of real quotients by determining a ratio between the level of expression of at least one pair of miRNA from at least six miRNA listed in Tables I, II, V, or VI;
   comparing each of the real quotients with a respective control value; and
   determining the real quotients which deviate from the respective control quotient value.

10. The method of claim 2, further comprising
    calculating a plurality of real quotients by determining a ratio between the level of expression of at least one pair of miRNA from at least six miRNA listed in Table Ib;
    comparing each of the real quotients with a respective control value; and
    determining the real quotients which deviate from the respective control quotient value.
11. The method of claim 4, further comprising
   calculating a plurality of real quotients by determining a ratio between the level of
   expression of at least one pair of miRNA from at least six miRNA listed in Table IIb;
   comparing each of the real quotients with a respective control value; and
   determining the real quotients which deviate from the respective control quotient
   value.

12. The method of claim 5, further comprising
   calculating a plurality of real quotients by determining a ratio between the level of
   expression of at least one pair of miRNA from at least six miRNA listed in Table Vb;
   comparing each of the real quotients with a respective control value; and
   determining the real quotients which deviate from the respective control quotient
   value.

13. The method of claim 7, further comprising
   calculating a plurality of real quotients by determining a ratio between the level of
   expression of at least one pair of miRNA from at least six miRNA listed in Table VIb;
   comparing each of the real quotients with a respective control value; and
   determining the real quotients which deviate from the respective control quotient
   value.

14. The method of claim 9, further comprising determining a number or percentage of
   real quotients which deviate from the respective control value.

15. The method of claim 14, further comprising defining as an individual at risk an
   individual for whom at least a predetermined number or a predetermined percentage of the
   real quotients deviates with respect to the respective control quotient value.

16. The method of claim 9, wherein for each of the calculated quotients a respective
   control quotient is associated, represented by a ratio of the levels of expression for the
   miRNA in a control biological sample relative to a biological sample of a same type.

17. The method of claim 14, further comprising correlating the deviation of a
   predetermined number or a predetermined percentage of levels of expression with respect to
the corresponding control criteria to a presence or absence of risk that the individual might clinically present with a tumor in a predetermined time.

18. The method of claim 17, wherein the individual might clinically present an aggressive tumor in a predetermined time.

19. The method of claim 17, wherein the predetermined time is between one and three years.

20. The method of claim 17, wherein the predetermined time is within 28 months.

21. The method of claim 9, wherein calculating the plurality of real quotients comprises using the expression level of at least six miRNA from the miRNA listed in Tables I, II, V, or VI.

22. The method of claim 10, wherein said at least six miRNA are listed in Table Ib.

23. The method of claim 11, wherein said at least six miRNA are listed in Table IIb.

24. The method of claim 12, wherein said at least six miRNA are listed in Table Vb.

25. The method of claim 13, wherein said at least six miRNA are listed in Table VIb.

26. The method of claim 9, wherein calculating the plurality of real quotients comprises determining a predetermined number or a predetermined percentage of quotients from among the levels of expression, wherein the quotients are selected from at least one of the quotients as listed in Tables III, IV, VII, or VIII.

27. The method of claim 26, wherein the quotients are selected from at least six of the quotients as listed in Tables III, IV, VII, or VIII.

28. The method of claim 26, wherein at least 20% of the real quotients listed in Tables III, IV, VII, or VIII are determined.
29. The method of claim 26, wherein at least 30% of the real quotients listed in Tables III, IV, VII, or VIII are determined.

30. The method of claim 26, wherein at least 50% of the real quotients listed in Tables III, IV, VII, or VIII are determined.

31. The method of claim 26, wherein 100% of the real quotients listed in Tables III, IV, VII, or VIII are determined.

32. The method of claim 26, wherein the quotients are selected from the quotients as listed in Tables IIIb, IVb, VIIb, or VIIIb.

33. The method of claim 9, further comprising defining as an individual at risk of a tumor, an individual for whom at least 20% of the real quotients calculated deviate with respect to the respective control quotient value.

34. The method of claim 33, wherein the individual is at risk of a tumor between one to three years from a collection of the biological sample.

35. The method of claim 33, wherein at least 30% of the real quotients calculated deviate with respect to the respective control quotient value.

36. The method of claim 33, wherein at least 50% of the real quotients calculated deviate with respect to the respective control quotient value.

37. The method of claim 33, wherein the tumor is an aggressive tumor.

38. The method of claim 9, further comprising defining as an individual presenting a tumor, an individual for whom at least 20% of the real quotients calculated deviate with respect to the respective control quotient value.

39. The method of claim 38, wherein at least 50% of the real quotients calculated deviate with respect to the respective control quotient value.
40. The method of claim 38, wherein at least 60% of the real quotients calculated deviate with respect to the respective control quotient value.

41. The method of claim 38, wherein the tumor is an aggressive tumor.

42. The method of any one of claims 1, 2, 4, 5 or 7, wherein the tumor is a pulmonary tumor.

43. The method of claim 42, wherein the pulmonary tumor is small-cell lung cancer (SCLC), non small-cell lung cancer (NSCLC), pulmonary adenocarcinoma (ADC), bronchio-alveolar carcinoma (BAC), squamous-cell lung carcinoma (SCC) or large-cell carcinoma (LC).

44. The method of any one of claims 1, 2, 4, 5 or 7, wherein the biological sample is a biological fluid.

45. The method of claim 44, wherein the biological fluid is whole blood, a fraction of blood, plasma or serum.

46. The method of any one of claims 1, 2, 4, 5 or 7, wherein the biological sample originates from a smoker individual who, at the moment of the collection of the sample, does not present a pulmonary tumor if subjected to imaging diagnostic methods, in particular the smoker individual not presenting nodules of dimensions of greater than 5mm if subjected to a spiral CT scan.

47. The method of any one of claims 1, 2, 4, 5 or 7, wherein said control biological sample is a biological sample from a disease-free subject.

48. The method of any one of claims 1, 2, 4, 5 or 7, wherein said control biological sample is a biological sample obtained from said subject at a previous time.

49. The method of claim 48, wherein said control biological sample is obtained from said subject up to three years preceding diagnosis.
50. The method of any one of claims 1, 2, 4, 5 or 7, wherein said control biological sample is a biological sample obtained from a different tissue from said subject.

51. An article comprising:
a support having a plurality of sites, wherein each site is capable of receiving a quantity of a biological sample,
wherein each of the sites comprises at least one reagent capable of binding with at least one miRNA listed in Tables I, II, V, or VI.

52. The article of claim 51, wherein each of the sites comprises at least one reagent capable of binding with at least six miRNA listed in Tables I, II, V, or VI.

53. The article of claim 51, wherein the reagent is selected from group consisting of:
a polynucleotide comprising a nucleotide sequence of at least one miRNA from the miRNA listed in Tables I, II, V, or VI;
a polynucleotide comprising a nucleotide sequence which is complementary to a sequence of at least one miRNA from the miRNA listed in Tables I, II, V, or VI; and
a molecular probe configured such as to recognize a sequence of at least one miRNA from the miRNA listed in Tables I, II, V, or VI.

54. An article comprising:
a support having a plurality of sites, wherein each site is capable of receiving a quantity of a biological sample,
wherein each of the sites comprises at least one reagent capable of binding with at least six miRNA listed in Table Ib.

55. An article comprising:
a support having a plurality of sites, wherein each site is capable of receiving a quantity of a biological sample,
wherein each of the sites comprises at least one reagent capable of binding with at least six miRNA listed in Table IIb.

56. An article comprising:
a support having a plurality of sites, wherein each site is capable of receiving a quantity of a biological sample,
wherein each of the sites comprises at least one reagent capable of binding with at least six miRNA listed in Table Vb.

57. An article comprising:
a support having a plurality of sites, wherein each site is capable of receiving a quantity of a biological sample,
wherein each of the sites comprises at least one reagent capable of binding with at least six miRNA listed in Table VIb.

58. An apparatus comprising:
at least one unit capable of receiving at least one of the articles of claim 51;
means for determining the level of expression of at least one miRNA from the miRNA listed in Tables I, II, V, or VI, and
means for calculating the real quotients from among the levels of expression of at least one pair of miRNA from the pairs of miRNA listed in Tables III, IV, VII, or VIII.

59. The apparatus of claim 58, wherein the means for determining the value of the level of expression is selected from the group consisting of Quantitative Real-time PCR, Microfluidic cards, Microarrays, RT – PCR, quantitative or semi-quantitative, Northern blot, Solution Hybridization, and Sequencing.

60. The method of claim 2, further comprising altering the level of expression of at least one miRNA for which the level of expression changes or deviates, thereby reducing or eliminating the risk of developing a tumor in said subject.

61. The method of claim 60, further comprising altering the level of expression of at least six miRNA for which the level of expression changes or deviates.

62. The method of claim 4, further comprising altering the level of expression of at least one miRNA for which the level of expression changes or deviates, thereby reducing or eliminating the risk of developing an aggressive tumor in said subject.
63. The method of claim 62, further comprising altering the level of expression of at least six miRNA for which the level of expression changes or deviates.

64. The method of claim 5, further comprising altering the level of expression of at least one miRNA for which the level of expression changes or deviates, thereby treating a tumor in said subject.

65. The method of claim 64, further comprising altering the level of expression of at least six miRNA for which the level of expression changes or deviates.

66. The method of claim 7, further comprising altering the level of expression of at least one miRNA for which the level of expression changes or deviates, thereby treating an aggressive tumor in said subject.

67. The method of claim 66, further comprising altering the level of expression of at least six miRNA for which the level of expression changes or deviates.

68. The method of claims 60-67, wherein altering the level of expression of said at least one miRNA comprises
   administering to said subject a therapeutically effective amount of at least one miRNA listed in Tables I, II, V, or VI, or a chemically synthesised miRNA mimetic or recombinant thereof, if the level of expression of said at least one miRNA is lower than the control level of expression or
   administering to said subject a therapeutically effective amount of a compound capable of inhibiting the expression of at least one miRNA listed in Tables I, II, V, or VI, if the level of expression of said at least one miRNA is higher than the control level of expression.

69. The method of claim 68, comprising increasing the level of expression of said at least one miRNA, which is under-expressed with respect to the control level of expression.

70. The method of claim 68, comprising administering a therapeutically effective amount of a composition comprising at least one miRNA listed in Tables I, II, V, or VI, or a chemically synthesised miRNA mimetic or recombinant thereof.
71. The method of claim 68, comprising administering a therapeutically effective amount of a composition comprising at least one miRNA listed in Tables Ib, IIb, Vb, or VIIb, or a chemically synthesized miRNA mimetic or recombinant thereof.

72. The method of claim 68, comprising decreasing the level of expression of said at least one miRNA, which is over-expressed with respect to the control level of expression.

73. The method of claim 68, comprising administering a therapeutically effective amount of a composition comprising an inhibitor of at least one miRNA listed in Tables I, II, V, or VI.

74. The method of claim 68, comprising administering a therapeutically effective amount of a composition comprising an inhibitor of at least one miRNA listed in Tables Ib, IIb, Vb, or VIIb.

75. The method of claim 68, wherein the inhibitor comprises double-filament RNA, short interfering RNA (siRNA), antisense nucleic acids, anti-miRNA oligonucleotides (AMOs), molecules of enzymatic RNA, or ribozymes.

76. A pharmaceutical compound comprising:
   at least one miRNA listed in Tables I, II, V, or VI, chemically synthesized miRNA mimetic or recombinant thereof, or
   an inhibitor of the expression of at least one miRNA listed in Tables I, II, V, or VI and a pharmaceutically acceptable carrier.

77. A pharmaceutical compound comprising:
   at least six miRNA listed in Tables I, II, V, or VI, chemically synthesized miRNA mimetic or recombinant thereof, or
   an inhibitor of the expression of at least six miRNA listed in Tables I, II, V, or VI and a pharmaceutically acceptable carrier.

78. A pharmaceutical compound comprising:
at least one miRNA listed in Tables Iib, IIb, Vb, or VIb, chemically synthesized
miRNA mimetic or recombinant thereof, or
an inhibitor of the expression of at least one miRNA listed in Tables Iib, IIb, Vb, or
VIb and a pharmaceutically acceptable carrier.

79. A pharmaceutical compound comprising:
at least six miRNA listed in Tables Iib, IIb, Vb, or VIb, chemically synthesized
miRNA mimetic or recombinant thereof, or
an inhibitor of the expression of at least six miRNA listed in Tables Iib, IIb, Vb, or
VIb and a pharmaceutically acceptable carrier.

80. A pharmaceutical compound comprising:
at least six miRNA listed in Table Iib, chemically synthesized miRNA mimetic or
recombinant thereof, or
an inhibitor of the expression of at least six miRNA listed in Table Iib and a
pharmaceutically acceptable carrier.

81. A pharmaceutical compound comprising:
at least six miRNA listed in Table IIb, chemically synthesized miRNA mimetic or
recombinant thereof, or
an inhibitor of the expression of at least six miRNA listed in Table IIb and a
pharmaceutically acceptable carrier.

82. A pharmaceutical compound comprising:
at least six miRNA listed in Table Vb, chemically synthesized miRNA mimetic or
recombinant thereof, or
an inhibitor of the expression of at least six miRNA listed in Table Vb and a
pharmaceutically acceptable carrier.

83. A pharmaceutical compound comprising:
at least six miRNA listed in Table VIb, chemically synthesized miRNA mimetic or
recombinant thereof, or
an inhibitor of the expression of at least six miRNA listed in Table VIb and a
pharmaceutically acceptable carrier.
ABSTRACT OF THE DISCLOSURE

[000403] A procedure and an apparatus are described for identifying individuals at risk of pulmonary tumour and/or for diagnosing a pulmonary tumour using the study of levels of expression of miRNA in the blood or another biological fluid. Also described are a method and a compound for reducing or eliminating a risk of pulmonary tumour by rebalancing the miRNAs that are underexpressed or overexpressed.
### Figure 1

<table>
<thead>
<tr>
<th></th>
<th>Training set</th>
<th>Validation set</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tr>
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<td></td>
<td>N=18</td>
<td>N=22</td>
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<tr>
<td><strong>Gender</strong></td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>11 (61.1%)</td>
<td>16 (72.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (38.9%)</td>
<td>6 (27.3%)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>57.5 ± 5.6 (s.d.)</td>
<td>61.9 ± 7 (s.d.)</td>
</tr>
<tr>
<td><strong>Smoking habit (Pack-Years index)</strong></td>
<td>60.3 ± 23.8 (s.d.)</td>
<td>55 ± 21 (s.d.)</td>
</tr>
<tr>
<td><strong>Screening year of disease detection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st year</td>
<td>1 (5.6%)</td>
<td>5 (22.7%)</td>
</tr>
<tr>
<td>2nd year</td>
<td>6 (33.3%)</td>
<td>9 (40.9%)</td>
</tr>
<tr>
<td>3rd - 5th year</td>
<td>11 (61.1%)</td>
<td>13 (57.1%)</td>
</tr>
<tr>
<td>Interval cancers</td>
<td>3 (13.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC (adenocarcinoma)</td>
<td>13 (72.2%)</td>
<td>14 (63.6%)</td>
</tr>
<tr>
<td>SCC (squamous carcinoma)</td>
<td>3 (16.7%)</td>
<td>4 (18.2%)</td>
</tr>
<tr>
<td>other</td>
<td>2 (11.1%)</td>
<td>4 (18.2%)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia-Ib</td>
<td>11 (61.1%)</td>
<td>15 (68.2%)</td>
</tr>
<tr>
<td>II-III-IV</td>
<td>13 (38.9%)</td>
<td>7 (31.8%)</td>
</tr>
<tr>
<td><strong>Median Follow up (months)</strong></td>
<td>67*</td>
<td>14 (min = 4, max = 46)</td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease free</td>
<td>10 (55.6%)</td>
<td>16 (72.7%)</td>
</tr>
<tr>
<td>Alive with disease</td>
<td>1 (4.6%)</td>
<td>1 (4.6%)</td>
</tr>
<tr>
<td>Dead</td>
<td>8 (44.4%)</td>
<td>5† (22.7%)</td>
</tr>
<tr>
<td><strong>Control Pools‡</strong></td>
<td>5 (5-7 samples)</td>
<td>10 (5-7 samples)</td>
</tr>
</tbody>
</table>

* an outlier has a follow-up of 35 months.
† a subject died for clinical complications.
‡ disease free individuals pooled by sex, age and smoking habit to best match with patient’s characteristics.
Figure 2

Survival probability (%)

Time (months)

RAD+ (n=17)

RAD- (n=20)

p=0.0006
Figure 3

Survival probability (%)

- PAD+ (n=13)
- PAD- (n=18)

Time (months)

p=0.0001
Figure 5: risk of manifesting a pulmonary tumor - Validation set

Using all 15 miRNAs of Table I

30 Ratios of Table III

ROC curve

AUC=0.87

Using 6 best miRNAs of Table Ib

9 Ratios of Table IIIb

ROC curve

AUC=0.86
**Figure 6:** risk of manifesting an aggressive pulmonary tumor - Validation set

Using all 16 miRNAs of Table II

28 Ratios of Table IV

ROC curve

AUC = 0.99

Using 6 best miRNAs of Table IIb

9 Ratios of Table IVb

ROC curve

AUC = 0.98
Figure 7: risk of manifesting an aggressive pulmonary tumor - Validation set

Using 6 best miRNAs of Table V

ROC curve

AUC=0.90

9 Ratios of Table VIIb

ROC curve

AUC=0.86

36 Ratios of Table VII
Figure 8: risk of manifesting an aggressive pulmonary tumor - Validation set

Using 6 best miRNAs of Table VIIb

ROC curve

9 Ratios of Table VIIIb

AUC=0.91

Using all 10 miRNAs of Table VI

ROC curve

16 Ratios of Table VIII

AUC=0.92
Application Data Sheet

Application Information

Application Type:: Provisional
Subject Matter:: Utility
Suggested Group Art Unit:: N/A
CD-ROM or CD-R?:: None
Sequence submission?:: None
Computer Readable Form (CRF)?:: No
Title:: Micro-RNA Biomarkers and Methods of Using Same
Attorney Docket Number:: 42823-501P01US
Request for Early Publication?:: No
Request for Non-Publication?:: No
Small Entity?:: Yes
Petition included?:: No
Secrecy Order in Parent Appl.?:: No

Applicant Information

Applicant Authority Type:: Inventor
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Family Name:: Pastorino
City of Residence:: Milan
Country of Residence: Italy
Applicant Authority Type: Inventor
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Country of Residence: Italy

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Country of Residence: Italy

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Representative Information
Representative Customer Number: 30623

Domestic Priority Information