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(54) Title: USE OF MIR-146A-5P AND MIR-186 AS BIOMARKERS OF OSTEOARTHRITIS

(57) Abstract: Osteoarthritis (OA) is the most frequent chronic musculoskeletal disease affecting approximately 40% of adults aged 70 years and over. The inventors investigated the associations of prevalent and incident knee osteoarthritis (OA) with the expression levels of serum miRs in subjects with and without OA. By real-time PCR, they analyzed the expression levels of 19 miRs at baseline selecting 43 women with prevalent OA (Kellgren Lawrence score of 2/3), 23 women with incident OA over a 4 years follow-up and 67 healthy subjects without prevalent or incident OA matched for age and body mass index. miR-146a-5p was significantly increased in the group of prevalent OA compared with controls (RQ: relative quantification; median [Interquartile range]: 1.12 [0.73; 1.46] vs 0.85 [0.62; 1.03], p=0.015). The likelihood of prevalent OA was significantly increased (odds-ratio [95% confidence interval (CI)]: 1.83 [1.21-2.77], p=0.004) for each quartile increase in serum miR-146a-5p. There was a significant association between baseline miR-186 levels and the risk of incident OA (Q4 vs Q1-3; odds-ratio [95% CI]: 6.13 [1.14-32.9], p=0.034). In conclusion the inventors showed for the first time that miR-146a-5p and miR-186 are significantly associated with prevalent and incident OA respectively.

USE OF MIR-146A-5P AND MIR-186 AS BIOMARKERS OF OSTEOARTHRITIS

FIELD OF THE INVENTION:

5 The present invention relates to use of miR-146a-5p and miR-186 as biomarkers of osteoarthritis.

BACKGROUND OF THE INVENTION:

Osteoarthritis (OA) is the most frequent chronic musculoskeletal disease affecting approximately 40% of adults aged 70 years and over.¹ Knee OA is usually detected only in late
10 stage by radiography and poorly sensitive clinical symptoms. Sensitive and specific blood biomarkers to detect the initial stages of osteoarthritis (OA) and to predict the future development of the disease are not available in clinical routine.² Consequently, there is a considerable interest in the identification of new markers. MicroRNAs (miRs) are small non-coding RNAs of approximately 22 nucleotides in length that can silence gene expression by
15 binding to target messenger RNA repressing the translation. Expression of miRs has been well documented in OA cartilage but data on circulating miRs is limited.³

SUMMARY OF THE INVENTION:

The present invention relates to use of miR-146a-5p and miR-186 as biomarkers of osteoarthritis. In particular, the present invention is defined by the cLOAs.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors investigated the associations of prevalent and incident knee osteoarthritis (OA) with the expression levels of serum miRs in subjects with and without OA. With a next generation sequencing approach, they compared the miRom expression of 10 women with knee OA and 10 age-matched healthy subjects. By real-time PCR, they analyzed the expression
25 levels of 19 miRs at baseline selecting 43 women with prevalent OA (Kellgren Lawrence score of 2/3), 23 women with incident OA over a 4 years follow-up and 67 healthy subjects without prevalent or incident OA matched for age and body mass index. miR-146a-5p was significantly increased in the group of prevalent OA compared with controls (RQ: relative quantification; median [Interquartile range]: 1.12 [0.73; 1.46] vs 0.85 [0.62; 1.03], p=0.015). The likelihood of
30 prevalent OA was significantly increased (odds-ratio [95% confidence interval (CI)]: 1.83 [1.21-2.77], p=0.004) for each quartile increase in serum miR-146a-5p. There was a significant association between baseline miR-186 levels and the risk of incident OA (Q4 vs Q1-3; odds-ratio [95% CI]: 6.13 [1.14-32.9], p=0.034). In conclusion the inventors showed for the first time

that miR-146a-5p and miR-186 are significantly associated with prevalent and incident OA respectively.

Accordingly, the first object of the present invention relates to a method of determining whether a subject has or is at risk of having osteoarthritis comprising i) determining the expression level of miR-146a-5p or miR-186 in sample obtained from the subject, and ii) comparing the expression level determined at step i) with a predetermined reference value wherein detecting differential between the expression level determined at step i) and the predetermined reference value is indicative of whether a subject has or is at risk of having osteoarthritis.

As used herein, the term "osteoarthritis" or "OA" has its general meaning in the art and refers to any kind of cartilage loss and subchondral bone degeneration/damage in any kind of (bone-) joints in the body of a mammal. Typically, it refers to arthritic changes of the larger and the smaller joints of the body, including the hands, wrists, feet, back, hip, and knee.

As used herein, the term "risk" in the context of the present invention, relates to the probability that an event will occur over a specific time period and can mean a subject's "absolute" risk or "relative" risk. Absolute risk can be measured with reference to either actual observation post-measurement for the relevant time cohort, or with reference to index values developed from statistically valid historical cohorts that have been followed for the relevant time period. Relative risk refers to the ratio of absolute risks of a subject compared either to the absolute risks of low risk cohorts or an average population risk, which can vary by how clinical risk factors are assessed. Odds ratios, the proportion of positive events to negative events for a given test result, are also commonly used (odds are according to the formula $p/(1-p)$ where p is the probability of event and $(1-p)$ is the probability of no event) to no- conversion. "Risk evaluation," or "evaluation of risk" in the context of the present invention encompasses making a prediction of the probability, odds, or likelihood that an event or disease state may occur, the rate of occurrence of the event or conversion from one disease state to another. Risk evaluation can also comprise prediction of future clinical parameters, traditional laboratory risk factor values, or other indices of relapse, either in absolute or relative terms in reference to a previously measured population. The methods of the present invention may be used to make continuous or categorical measurements of the risk of conversion, thus diagnosing and defining the risk spectrum of a category of subjects defined as being at risk of conversion. In the categorical scenario, the invention can be used to discriminate between normal and other subject cohorts at higher risk. In some embodiments, the present invention may be used so as to discriminate those at risk from normal.

In some embodiments, the method of diagnosing described herein is applied to a subject who presents symptoms of OA without having undergone the routine screening to rule out all possible causes for OA. The methods described herein can be part of the routine set of tests performed on a subject who presents symptoms of OA. The method of the present invention
5 can be carried out in addition of other diagnostic tools.

In some embodiments, the sample is a blood sample. As used herein the term “blood sample” means any blood sample derived from the subject. Collections of blood samples can be performed by methods well known to those skilled in the art. In some embodiments, the blood sample is a serum sample or a plasma sample.

10 As used herein, the term “miR” or “miRNA” has its general meaning in the art and refers to the miRNA sequence publicly available from the data base <http://microrna.sanger.ac.uk/sequences/> under the miRBase Accession number. miR-146a-5p or miR-186 pertaining to the invention are thus known per se.

According to the invention, measuring the expression level of miR-146a-5p or miR-186
15 in the sample obtained from the subject can be performed by a variety of techniques. For example the nucleic acid contained in the samples is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's instructions. Conventional methods and reagents for isolating RNA from a sample comprise High Pure miRNA Isolation Kit (Roche), Trizol
20 (Invitrogen), Guanidinium thiocyanate-phenol-chloroform extraction, PureLink™ miRNA isolation kit (Invitrogen), PureLink Micro-to- Midi Total RNA Purification System (Invitrogen), RNeasy kit (Qiagen), miRNeasy kit (Qiagen), Oligotex kit (Qiagen), phenol extraction, phenol-chloroform extraction, TCA/acetone precipitation, ethanol precipitation, Column purification, Silica gel membrane purification, PureYield™ RNA Midiprep
25 (Promega), PolyATtract System 1000 (Promega), Maxwell® 16 System (Promega), SV Total RNA Isolation (Promega), geneMAG-RNA / DNA kit (Chemicell), TRI Reagent® (Ambion), RNAqueous Kit (Ambion), ToTALLY RNA™ Kit (Ambion), Poly(A)Purist™ Kit (Ambion) and any other methods, commercially available or not, known to the skilled person. The expression level of miR-146a-5p or miR-186 in the sample may be determined by any suitable
30 method. Any reliable method for measuring the level or amount of miRNA in a sample may be used. Generally, miRNA can be detected and quantified from a sample (including fractions thereof), such as samples of isolated RNA by various methods known for mRNA, including, for example, amplification-based methods (e.g., Polymerase Chain Reaction (PCR), Real-Time Polymerase Chain Reaction (RT-PCR), Quantitative Polymerase Chain Reaction (qPCR),

rolling circle amplification, etc.), hybridization-based methods (e.g., hybridization arrays (e.g., microarrays), NanoString analysis, Northern Blot analysis, branched DNA (bDNA) signal amplification, in situ hybridization, etc.), and sequencing-based methods (e.g., next-generation sequencing methods, for example, using the Illumina or IonTorrent platforms). Other
5 exemplary techniques include ribonuclease protection assay (RPA) and mass spectroscopy.

In some embodiments, RNA is converted to DNA (cDNA) prior to analysis. cDNA can be generated by reverse transcription of isolated miRNA using conventional techniques. miRNA reverse transcription kits are known and commercially available. Universal primers, or specific primers, including miRNA-specific stem-loop primers, are known and commercially
10 available, for example, from Applied Biosystems. In some embodiments, miRNA is amplified prior to measurement. In some embodiments, the expression level of miRNA is measured during the amplification process. In some embodiments, the expression level of miRNA is not amplified prior to measurement. Some exemplary methods suitable for determining the expression level of miRNA in a sample are described in greater hereinafter. These methods are
15 provided by way of illustration only, and it will be apparent to a skilled person that other suitable methods may likewise be used.

Many amplification-based methods exist for detecting the expression level of miRNA nucleic acid sequences, including, but not limited to, PCR, RT-PCR, qPCR, and rolling circle amplification. Other amplification-based techniques include, for example, ligase chain reaction
20 (LCR), multiplex ligatable probe amplification, in vitro transcription (IVT), strand displacement amplification (SDA), transcription-mediated amplification (TMA), nucleic acid sequence based amplification (NASBA), RNA (Eberwine) amplification, and other methods that are known to persons skilled in the art. A typical PCR reaction includes multiple steps, or cycles, that selectively amplify target nucleic acid species: a denaturing step, in which a target
25 nucleic acid is denatured; an annealing step, in which a set of PCR primers (i.e., forward and reverse primers) anneal to complementary DNA strands, and an elongation step, in which a thermostable DNA polymerase elongates the primers. By repeating these steps multiple times, a DNA fragment is amplified to produce an amplicon, corresponding to the target sequence. Typical PCR reactions include 20 or more cycles of denaturation, annealing, and elongation. In
30 many cases, the annealing and elongation steps can be performed concurrently, in which case the cycle contains only two steps. A reverse transcription reaction (which produces a cDNA sequence having complementarity to a miRNA) may be performed prior to PCR amplification. Reverse transcription reactions include the use of, e.g., a RNA-based DNA polymerase (reverse transcriptase) and a primer. Kits for quantitative real time PCR of miRNA are known, and are

commercially available. Examples of suitable kits include, but are not limited to, the TaqMan® miRNA Assay (Applied Biosystems) and the mirVana™ qRT-PCR miRNA detection kit (Ambion). The miRNA can be ligated to a single stranded oligonucleotide containing universal primer sequences, a polyadenylated sequence, or adaptor sequence prior to reverse transcriptase and amplified using a primer complementary to the universal primer sequence, poly(T) primer, or primer comprising a sequence that is complementary to the adaptor sequence. In some embodiments, custom qRT-PCR assays can be developed for determination of miRNA levels. Custom qRT-PCR assays to measure miRNAs in a sample can be developed using, for example, methods that involve an extended reverse transcription primer and locked nucleic acid modified PCR. Custom miRNA assays can be tested by running the assay on a dilution series of chemically synthesized miRNA corresponding to the target sequence. This permits determination of the limit of detection and linear range of quantitation of each assay. Furthermore, when used as a standard curve, these data permit an estimate of the absolute abundance of miRNAs measured in the samples. Amplification curves may optionally be checked to verify that Ct values are assessed in the linear range of each amplification plot. Typically, the linear range spans several orders of magnitude. For each candidate miRNA assayed, a chemically synthesized version of the miRNA can be obtained and analyzed in a dilution series to determine the limit of sensitivity of the assay, and the linear range of quantitation. Relative expression levels may be determined, for example, according to the $2(-\Delta\Delta C(T))$ Method, as described by Livak et al., Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ Method. Methods (2001) Dec;25(4):402-8.

Rolling circle amplification is a DNA-polymerase driven reaction that can replicate circularized oligonucleotide probes with either linear or geometric kinetics under isothermal conditions (see, for example, Lizardi et al., Nat. Gen. (1998) 19(3):225-232; Gusev et al, Am. J. Pathol. (2001) 159(1):63-69; Nallur et al, Nucleic Acids Res. (2001) 29(23):E118). In the presence of two primers, one hybridizing to the (+) strand of DNA, and the other hybridizing to the (-) strand, a complex pattern of strand displacement results in the generation of over 10⁹ copies of each DNA molecule in 90 minutes or less. Tandemly linked copies of a closed circle DNA molecule may be formed by using a single primer. The process can also be performed using a matrix-associated DNA. The template used for rolling circle amplification may be reverse transcribed. This method can be used as a highly sensitive indicator of miRNA sequence and expression level at very low miRNA concentrations (see, for example, Cheng et al., Angew Chem. Int. Ed. Engl. (2009) 48(18):3268-72; Neubacher et al, Chembiochem. (2009) 10(8):1289-91).

miRNA quantification may be performed by using stem-loop primers for reverse transcription (RT) followed by a real-time TaqMan® probe. Typically, said method comprises a first step wherein the stem-loop primers are annealed to miRNA targets and extended in the presence of reverse transcriptase. Then miRNA-specific forward primer, TaqMan® probe, and reverse primer are used for PCR reactions. Quantitation of miRNAs is estimated based on measured CT values. Many miRNA quantification assays are commercially available from Qiagen (S. A. Courtaboeuf, France), Exiqon (Vedback, Denmark) or Applied Biosystems (Foster City, USA).

Nucleic acids exhibiting sequence complementarity or homology to the miRNAs of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments, it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization. A wide variety of appropriate indicators are known in the art including, fluorescent, radioactive, enzymatic or other ligands (e. g. avidin/biotin). The probes and primers are “specific” to the miRNAs they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

miRNA may be detected using hybridization-based methods, including but not limited to hybridization arrays (e.g., microarrays), NanoString analysis, Northern Blot analysis, branched DNA (bDNA) signal amplification, and in situ hybridization.

Microarrays can be used to measure the expression levels of large numbers of miRNAs simultaneously. Microarrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto glass slides, photolithography using pre- made masks, photolithography using dynamic micromirror devices, inkjet printing, or electrochemistry on microelectrode arrays. Also useful are microfluidic TaqMan Low-Density Arrays, which are based on an array of microfluidic qRT-PCR reactions, as well as related microfluidic qRT-PCR based methods. In one example of microarray detection, various oligonucleotides (e.g., 200+ 5'- amino- modified-C6 oligos) corresponding to human sense miRNA sequences are spotted on three- dimensional CodeLink slides (GE Health/ Amersham Biosciences) at a final concentration of about 20 μ M and processed according to manufacturer's recommendations. First strand cDNA synthesized from 20 μ g TRIzol-purified total RNA is labeled with biotinylated ddUTP using the Enzo BioArray end labeling kit (Enzo Life Sciences Inc.).

Hybridization, staining, and washing can be performed according to a modified Affymetrix Antisense genome array protocol. Axon B-4000 scanner and Gene-Pix Pro 4.0 software or other suitable software can be used to scan images. Non-positive spots after background subtraction, and outliers detected by the ESD procedure, are removed. The resulting signal intensity values are normalized to per-chip median values and then used to obtain geometric means and standard errors for each miRNA. Each miRNA signal can be transformed to log base 2, and a one-sample t test can be conducted. Independent hybridizations for each sample can be performed on chips with each miRNA spotted multiple times to increase the robustness of the data.

Microarrays can be used for the expression profiling of miRNAs. For example, RNA can be extracted from the sample and, optionally, the miRNAs are size- selected from total RNA. Oligonucleotide linkers can be attached to the 5' and 3' ends of the miRNAs and the resulting ligation products are used as templates for an RT-PCR reaction. The sense strand PCR primer can have a fluorophore attached to its 5' end, thereby labeling the sense strand of the PCR product. The PCR product is denatured and then hybridized to the microarray. A PCR product, referred to as the target nucleic acid that is complementary to the corresponding miRNA capture probe sequence on the array will hybridize, via base pairing, to the spot at which the capture probes are affixed. The spot will then fluoresce when excited using a microarray laser scanner. The fluorescence intensity of each spot is then evaluated in terms of the number of copies of a particular miRNA, using a number of positive and negative controls and array data normalization methods, which will result in assessment of the level of expression of a particular miRNA. Total RNA containing the miRNA extracted from the sample can also be used directly without size-selection of the miRNAs. For example, the RNA can be 3' end labeled using T4 RNA ligase and a fluorophore-labeled short RNA linker. Fluorophore-labeled miRNAs complementary to the corresponding miRNA capture probe sequences on the array hybridize, via base pairing, to the spot at which the capture probes are affixed. The fluorescence intensity of each spot is then evaluated in terms of the number of copies of a particular miRNA, using a number of positive and negative controls and array data normalization methods, which will result in assessment of the level of expression of a particular miRNA. Several types of microarrays can be employed including, but not limited to, spotted oligonucleotide microarrays, pre-fabricated oligonucleotide microarrays or spotted long oligonucleotide arrays.

Accordingly, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a detectable label). A “detectable label” is a molecule or material that can be used to produce a detectable

signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook- A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N- [3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcoumarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5''dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin

isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), Dichlorotriazinylamino fluorescein (DTAF), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649,138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals

with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., *Science* 281 :20132016, 1998; Chan et al., *Science* 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

RT-PCR is typically carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and thermal polymerase. The majority of the thermocyclers on the market now offer similar characteristics. Typically, thermocyclers involve a format of glass capillaries, plastic tubes, 96-well plates or 384-well plates. The thermocycler also involves software analysis.

miRNAs can also be detected without amplification using the nCounter Analysis System (NanoString Technologies, Seattle, WA). This technology employs two nucleic acid-based probes that hybridize in solution (e.g., a reporter probe and a capture probe). After hybridization, excess probes are removed, and probe/target complexes are analyzed in accordance with the manufacturer's protocol. nCounter miRNA assay kits are available from NanoString Technologies, which are capable of distinguishing between highly similar miRNAs with great specificity. The basis of the nCounter® Analysis system is the unique code assigned to each nucleic acid target to be assayed (International Patent Application Publication No. WO 08/124847, U.S. Patent No. 8,415,102 and Geiss et al. *Nature Biotechnology*. 2008. 26(3): 317-325; the contents of which are each incorporated herein by reference in their entireties). The code is composed of an ordered series of colored fluorescent spots which create a unique barcode for each target to be assayed. A pair of probes is designed for each oligonucleotide

target, a biotinylated capture probe and a reporter probe carrying the fluorescent barcode. This system is also referred to, herein, as the nanoreporter code system. Specific reporter and capture probes are synthesized for each target. The reporter probe can comprise at least a first label attachment region to which are attached one or more label monomers that emit light constituting a first signal; at least a second label attachment region, which is non-over-lapping with the first label attachment region, to which are attached one or more label monomers that emit light constituting a second signal; and a first target-specific sequence. Preferably, each sequence specific reporter probe comprises a target specific sequence capable of hybridizing to no more than one gene and optionally comprises at least three, or at least four label attachment regions, said attachment regions comprising one or more label monomers that emit light, constituting at least a third signal, or at least a fourth signal, respectively. The capture probe can comprise a second target-specific sequence; and a first affinity tag. In some embodiments, the capture probe can also comprise one or more label attachment regions. Preferably, the first target-specific sequence of the reporter probe and the second target-specific sequence of the capture probe hybridize to different regions of the same gene to be detected. Reporter and capture probes are all pooled into a single hybridization mixture, the "probe library". The relative abundance of each target is measured in a single multiplexed hybridization reaction. The method comprises contacting the sample with a probe library, such that the presence of the target in the sample creates a probe pair - target complex. The complex is then purified. More specifically, the sample is combined with the probe library, and hybridization occurs in solution. After hybridization, the tripartite hybridized complexes (probe pairs and target) are purified in a two-step procedure using magnetic beads linked to oligonucleotides complementary to universal sequences present on the capture and reporter probes. This dual purification process allows the hybridization reaction to be driven to completion with a large excess of target-specific probes, as they are ultimately removed, and, thus, do not interfere with binding and imaging of the sample. All post hybridization steps are handled robotically on a custom liquid-handling robot (Prep Station, NanoString Technologies). Purified reactions are typically deposited by the Prep Station into individual flow cells of a sample cartridge, bound to a streptavidin-coated surface via the capture probe, electrophoresed to elongate the reporter probes, and immobilized. After processing, the sample cartridge is transferred to a fully automated imaging and data collection device (Digital Analyzer, NanoString Technologies). The expression level of a target is measured by imaging each sample and counting the number of times the code for that target is detected. For each sample, typically 600 fields-of-view (FOV) are imaged (1376 X 1024 pixels) representing approximately 10 mm² of the binding surface.

Typical imaging density is 100- 1200 counted reporters per field of view depending on the degree of multiplexing, the amount of sample input, and overall target abundance. Data is output in simple spreadsheet format listing the number of counts per target, per sample. This system can be used along with nanoreporters. Additional disclosure regarding nanoreporters can be found in International Publication No. WO 07/076129 and WO07/076132, and US Patent Publication No. 2010/0015607 and 2010/0261026, the contents of which are incorporated herein in their entirety. Further, the term nucleic acid probes and nanoreporters can include the rationally designed (e.g. synthetic sequences) described in International Publication No. WO 2010/019826 and US Patent Publication No. 2010/0047924, incorporated herein by reference in its entirety.

Mass spectroscopy can be used to quantify miRNA using RNase mapping. Isolated RNAs can be enzymatically digested with RNA endonucleases (RNases) having high specificity (e.g., RNase T1, which cleaves at the 3'-side of all unmodified guanosine residues) prior to their analysis by MS or tandem MS (MS/MS) approaches. The first approach developed utilized the on-line chromatographic separation of endonuclease digests by reversed phase HPLC coupled directly to ESTMS. The presence of post-transcriptional modifications can be revealed by mass shifts from those expected based upon the RNA sequence. Ions of anomalous mass/charge values can then be isolated for tandem MS sequencing to locate the sequence placement of the post-transcriptionally modified nucleoside. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has also been used as an analytical approach for obtaining information about post-transcriptionally modified nucleosides. MALDI-based approaches can be differentiated from EST-based approaches by the separation step. In MALDI-MS, the mass spectrometer is used to separate the miRNA. To analyze a limited quantity of intact miRNAs, a system of capillary LC coupled with nanoESI-MS can be employed, by using a linear ion trap-orbitrap hybrid mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific) or a tandem-quadrupole time-of-flight mass spectrometer (QSTAR® XL, Applied Biosystems) equipped with a custom-made nanospray ion source, a Nanovolume Valve (Valco Instruments), and a splitless nano HPLC system (DiNa, KYA Technologies). Analyte/TEAA is loaded onto a nano-LC trap column, desalted, and then concentrated. Intact miRNAs are eluted from the trap column and directly injected into a CI 8 capillary column, and chromatographed by RP-HPLC using a gradient of solvents of increasing polarity. The chromatographic eluent is sprayed from a sprayer tip attached to the capillary column, using an ionization voltage that allows ions to be scanned in the negative polarity mode.

Additional methods for miRNA detection and measurement include, for example, strand invasion assay (Third Wave Technologies, Inc.), surface plasmon resonance (SPR), cDNA, MTDNA (metallic DNA; Advance Technologies, Saskatoon, SK), and single-molecule methods such as the one developed by US Genomics. Multiple miRNAs can be detected in a microarray format using a novel approach that combines a surface enzyme reaction with nanoparticle- amplified SPR imaging (SPRI). The surface reaction of poly(A) polymerase creates poly(A) tails on miRNAs hybridized onto locked nucleic acid (LNA) microarrays. DNA-modified nanoparticles are then adsorbed onto the poly(A) tails and detected with SPRI. This ultrasensitive nanoparticle-amplified SPRI methodology can be used for miRNA profiling at attamole levels. miRNAs can also be detected using branched DNA (bDNA) signal amplification (see, for example, Urdea, Nature Biotechnology (1994), 12:926-928). miRNA assays based on bDNA signal amplification are commercially available. One such assay is the QuantiGene® 2.0 miRNA Assay (Affymetrix, Santa Clara, CA). Northern Blot and in situ hybridization may also be used to detect miRNAs. Suitable methods for performing Northern Blot and in situ hybridization are known in the art. Advanced sequencing methods can likewise be used as available. For example, miRNAs can be detected using Illumina ® Next Generation Sequencing (e.g. Sequencing-By-Synthesis or TruSeq methods, using, for example, the HiSeq, HiScan, GenomeAnalyzer, or MiSeq systems (Illumina, Inc., San Diego, CA)). miRNAs can also be detected using Ion Torrent Sequencing (Ion Torrent Systems, Inc., Gulliford, CT), or other suitable methods of semiconductor sequencing.

The expression level of miR-146a-5p or miR-186 may be expressed as absolute expression levels or normalized expression levels. Typically, expression levels are normalized by correcting the absolute expression level of miRNAs by comparing its expression to the expression of a mRNA that is not a relevant marker for determining whether a subject suffering from acute severe colitis (ASC) will be a responder or a non-responder to a corticosteroid, infliximab and cyclosporine, e.g., a housekeeping mRNA that is constitutively expressed. This normalization allows the comparison of the expression level in one sample, e.g., a subject sample, to another sample, or between samples from different sources. In a particular embodiment, expression levels are normalized by correcting the absolute expression level of miRNAs by comparing its expression to the expression of a reference mRNA.

In some embodiments, the predetermined reference value is a threshold value or a cut-off value. Typically, a "threshold value" or "cut-off value" can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary

skilled in the art. For example, retrospective measurement of expression level of the gene in properly banked historical patient samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the level of the marker in a group of reference, one can use algorithmic analysis for the statistic treatment of the measured levels of the marker in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When AUC is between 0.5 and 0.7, the accuracy is low. When AUC is between 0.7 and 0.9, the accuracy is moderate. When AUC is higher than 0.9, the accuracy is quite high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

In some embodiments, the predetermined reference value is the level of the miRNA determined in a population of healthy individuals. Typically, it is concluded that subject the has or is at risk of having OA when the expression level of the miRNA is at least 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 100 fold higher than the expression level determined in a population of healthy individuals.

Once it is concluded that the subject has or is at risk of having OA, treatment options may be prescribed. Typical treatment position include weight management, physical activity, medications, and joint replacement surgery. Medicines for osteoarthritis are available as pills, syrups, creams or lotions, or they are injected into a joint. They include analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, ibuprofen, naproxen and celecoxib), hyaluronic acid and corticosteroids. Corticosteroids include, for example, cortisone, hydrocortisone, methylprednisolone, prednisone, prednisolone, betamethasone, beclomethasone dipropionate, budesonide, dexamethasone sodium phosphate, flunisolide, fluticasone propionate, triamcinolone acetonide, betamethasone, fluocinolone, fluocinonide, betamethasone dipropionate, betamethasone valerate, desonide, desoximetasone, fluocinolone, triamcinolone, triamcinolone acetonide, clobetasol propionate, and dexamethasone).

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Risk of prevalent and incident OA according to the median of miR-146a-5p and the quartiles of miR-186-5p respectively. Multiple logistic regression analyses to determine the ability of serum miR-146a-5p and miR-186-5p levels to predict prevalent or incident OA respectively. Prevalent OA: percentage of women with osteoarthritis under and upper the median of miR-146a-5p respectively (number of women with prevalent OA-total number of women). Incident OA: percentage of women with osteoarthritis in the first three quartiles vs the upper quartile (number of women with incident OA-total number of women). CI = Confidence Interval.

EXAMPLE:

Methods

Part I: NGS approach

Patients selected for NGS

The study group included French postmenopausal women belonging to the population-based cohort OFELY (Os des Femmes de LYon), described in greater detail elsewhere.⁴ Expression levels of all serum miRs were measured in 10 women with a knee OA (Kellgren & Lawrence score of 2 and 3) and OA at others sites, knee and lumbar spine evaluated by radiography, hip (clinical exam) and hand (questionnaire) and in 10 healthy women without OA at any site (for details, see ⁵). Both groups were matched for age (healthy: 61.9 ± 3.03 years and OA: 63.9 ± 3.4 years $p=0.17$). Prevalent knee OA was defined by a KL score higher or

equal in 2 at baseline and incident OA by a KL score higher or equal in 2 at year 4 and a KL score < 2 at baseline.

NGS experiment

According to the manufacturer's protocol (EXIQON, Denmark), RNA isolation was performed from 400 µl of serum followed by the µRNA sequencing (Illumina platform). Measurements were expressed as Tags per million (TPM).

Part II: Quantitative real-time PCR approach

Patients selected for real-time PCR

All women were postmenopausal. First, we randomly selected 43 women with prevalent knee OA (Kellgren & Lawrence score of 2 and 3; early and intermediate knee OA) with or without OA at others sites (lumbar spine, hip, hand) (age: 68.3 ± 6.6 years, body mass index (BMI): 26.6 ± 4.4 kg/m²) and 42 healthy women without OA at any site matched for age and BMI. Second, we randomly selected 23 women with incident knee OA over the next 4 years (age: 68.4 ± 8 years, BMI: 25.2 ± 4 kg/m²) and 25 healthy subjects without incident OA matched for age and BMI.

miRs selection

We have selected 19 candidate miRs for real-time PCR analysis on the basis of our NGS study (139-5p; 200a-3p; 1299) measuring all circulating miRs and on literature data (let-7e-5p; 16-5p; 29a-3p, 29b-3p; 29c-3p; 93-5p; 126-3p; 132-3p; 146a-5p; 184; 186-5p; 195-5p; 199a-3p; 345-5p; 375; 885-5p).

RNA isolation and quantitative real-time PCR

cel-miR-39-3p was added as exogenous control before total RNA extraction from 200 µl of serum using miRCURY RNA isolation kit for biofluids according to the manufacturer's protocol (EXIQON, Denmark). MiRs were reverse-transcribed with an Advanced miRNA assays kit according to the manufacturer's protocol (Applied BioSystems, CA, USA). Amplified cDNAs (15µl) were mixed with 75 µl of TaqMan Fast Advanced Mastermix (Ref. 4444963, Applied BioSystems) and water (60µl) and 100 µl of this mixture were added to the reservoir. The TaqMan array microRNA cards were designed for the quantification in duplicate of 19 miRs by real-time PCR reaction on a QuantStudio 7 flex (Applied Biosystems) according to the manufacturer's protocol.

Data normalization

We used the software Expression Suite for the delta Ct calculation (fractional cycle numbers at which fluorescence reaches the threshold). For the lack of generally accepted standards, the delta Ct was calculated using the mean Ct of miR-191-5p, 222-3p and 361-5p as

endogenous controls because they are expressed ubiquitously and unrelated to bone metabolism. Relative expression levels of miRNA were calculated using the Comparative delta delta Ct method (relative quantity, RQ).⁶

Statistical analysis

5 A miRs with a p value < 0.05 and a false discovery rate of 5% [Benjamini-Hochberg False Discovery Rate (FDR) correction for NGS approach] was considered as differentially expressed. Wilcoxon tests were used to compare miR levels between women with and without OA. We have examined the likelihood of OA, expressed as odds ratios (ORs) and 95% confidence intervals (CIs), per quartile increase in miR levels in a logistic regression model..
10 All statistical analyses were performed using Stata 12 (Stata Corp LP, College Station, Texas, USA).

Results

NGS results (Table 1)

We identified 421 miRs with an expression level ≥ 1 TPM and 241 with an expression
15 level ≥ 10 TPM. When we compared both groups, 22 miRs showed differential expression ($p < 0.05$) between controls and OA patients, 13 up-regulated and 9 down-regulated. After the Benjamini-Hochberg FDR correction, has-miR-139-5p, has-miR-1299 and has-miR-200a-3p remained significantly different between OA patients and controls ($p < 0.05$, FDR at 5%).

Real-time PCR results

Prevalent OA and miR-146a-3p (Table 2 and Figure 1A)

When considered as a continuous variable, miR-146a-5p was significantly increased in the group of prevalent OA compared with controls (RQ: relative quantification; median [Interquartile range]: 1.12 [0.73; 1.46] vs 0.85 [0.62; 1.03], $p = 0.015$). The likelihood of prevalent OA was significantly increased (odds-ratio [95% confidence interval (CI)]: 1.83
25 [1.21-2.77], $p = 0.004$) for each quartile increase in serum miR-146a-5p. Moreover, the women with miR-146a-5p levels above the median (0.851) had a higher risk of prevalent OA compared to those under the median [95% CI]: 4.62 [1.85-11.5], $p = 0.001$.

Incident OA and miR-186 (Table 2 and Figure 1B)

We found a significant association between baseline miR-186 levels and the risk of
30 incident OA for each quartile increase (odds-ratio [95% CI]: 1.71 [1.00-2.95], $p = 0.049$). Women in the upper quartile have a risk to develop radiographic OA over the next 4 years multiply by 6.13 compared to the 3 others quartiles (Q4 vs Q1-3; odds-ratio [95% CI]: 6.13 [1.14-32.9], $p = 0.034$).

Discussion:

Our NGS approach revealed that miR-139-5p, miR-1299 and miR-200a-3p had levels of expression significantly different between OA patients and controls. However, we did not validate these 3 miRs in the second step when we measured them in the entire patient samples. This discrepancy may stem from the small number of samples in the discovery step (10 OA vs 10 non OA) even if this number is comparable with those used in previous studies and/or differences in the specificity and sensitivity of the used platforms.⁷⁻⁹

The second step of our study combines the validation of our NGS results and the first attempt to replicate previously published results, a process that is lacking in the miR research field. Our study can be compared to that of Borgonio *et al*, 2014.⁷ Only two of the 10 miRs in common showed significant overexpression (146a-5p and 186) emphasizing the challenges faced with patient heterogeneity. The reasons for this discrepancy could be the ethnic origin, Mexican vs European hence the differences in exposure to environmental factors; the sex of the participants, men and women vs women only; the biological fluid used-plasma vs serum or the mean age of participants-55 vs 68 years.

Nevertheless, we found that miR 146a-5p expression was significantly increased in prevalent OA independently of age and BMI. miR-146a was found as expressed differently in joint tissues and in the circulation.^{7 10-12} However, the interpretation of this increase is difficult because of the dual role of miR-146a-5p in the pathophysiology of osteoarthritis. It may promote OA by increasing chondrocyte apoptosis and decreasing TGF- β responsiveness and cartilage anabolism.^{13 14} In contrast, miR-146a-5p reduces inflammation and increases autophagy.^{15 16} Taken together, these results suggest that miR-146a-5p could be a new biological marker for knee OA.

We report for the first time a significant association, independent of age and BMI, of serum miR-186-5p and incident OA risk in a population of postmenopausal women followed prospectively for 4 years. It has been shown that miR-186-5p was overexpressed in the plasma of early-stage OA patients compared to controls⁷ but upregulated in the synovial fluid of late-stage OA patients compared to early-stage.¹⁷ The reasons of this discrepancy are not clear but together with the serum association of miR-146a-5p and prevalent OA, these data reinforce the notion that the miRs involved in OA pathology vary according to the stage of the disease.¹⁷ *In silico* analysis has suggested that potential pathways regulated by miR-186 could be signaling by PDGF, developmental biology, membrane trafficking and collagen formation.⁷ However, miR-186 has been essentially studied in oncology. Potential targets that may be related to OA included GLUT1 (glycolysis regulation)¹⁸ and TWIST1 (osteoblast differentiation).¹⁹

Collectively, our results show that miR-146a-5p is increased in women suffering from mild to moderate OA compared to healthy women. Importantly, miR-186 is also increased in those women who will develop radiographic knee OA over the next 4 years, therefore with the potential to detect preclinical knee OA.

5

TABLES:**Table 1. Part 1: Next generation Sequencing.**

When we compared the OA vs healthy groups, 22 miRs showed differential expression ($p < 0.05$) between controls and OA patients. After Benjamini-Hochberg False Discovery Rate (FDR) correction has-miR-139-5p, has-miR-1299 and has-miR-200a-3p remained significantly different between OA patients and controls ($p < 0.05$, FDR at 5%).

10

Names	Log Fold change	p-value	FDR adjusted p-value	Healthy average TPM	OA average TPM
hsa-miR-139-5p	0.734682	0.000126	0.043432	90.1	143.3
hsa-miR-1299	-3.38328	0.000201	0.043432	12	0.8
hsa-miR-200a-3p	-1.881	0.000328	0.047335	77.2	29.4
hsa-miR-129-5p	0.810335	0.002154	0.164017	3.6	6.5
hsa-miR-429	-1.43167	0.002273	0.164017	5.5	2.5
hsa-miR-9-5p	0.829242	0.004452	0.240981	3.2	6.1
hsa-miR-375	-0.92781	0.006454	0.279453	737	411.3
hsa-miR-139-3p	0.608632	0.007165	0.282028	48.8	69.7
hsa-miR-200b-3p	-1.1824	0.00885	0.319349	28	15.7
hsa-miR-150-5p	0.721118	0.016417	0.461924	173.6	252.6
hsa-miR-192-5p	-1.0318	0.017069	0.461924	1837.8	1161.9
hsa-miR-155-5p	0.523914	0.019264	0.490676	64.9	87.5
hsa-miR-15b-3p	-0.72854	0.024665	0.586743	43.8	24.3
hsa-miR-1228-5p	0.778548	0.02948	0.591794	4.9	8.7
hsa-miR-126-3p	0.379266	0.030412	0.591794	3243.9	3976.7
hsa-miR-206	1.102002	0.03381	0.601135	51.3	95.9
hsa-miR-199b-5p	-0.57408	0.03727	0.601135	29.9	18.6
hsa-miR-1246	0.671761	0.037484	0.601135	29.9	46.9
hsa-miR-10b-5p	0.462851	0.042798	0.639025	3848.4	5039.8
hsa-miR-642a-3p	0.694674	0.044392	0.640476	4.5	7.2
hsa-miR-186-5p	-0.45246	0.047333	0.640476	777.7	534
hsa-miR-10a-5p	0.451419	0.049304	0.64693	2613.1	3361.1

Table 2. Part 2: Quantitative real-time PCR.

Differences in serum miR levels according to the prevalent or incident OA status. The relative quantity was used for the statistical comparisons. IQR = Inter-Quartile Range.

miR	Prevalent OA(-) Median (IQR 25%-75%)	Prevalent OA (+) Median (IQR 25%-75%)	<i>p-value</i>	Incident OA (-) Median (IQR 25%-75%)	Incident OA (+) Median (IQR 25%-75%)	<i>p-value</i>
126-3p	1.08 (0.85-1.36)	1.06 (0.89-1.30)	0.75	0.89 (0.80-1.04)	0.91 (0.73-1.23)	0.66
1299	0.28 (0.16-1.73)	0.33 (0.16-2.35)	0.80	0.27 (0.14-11.36)	0.34 (0.12-0.90)	0.82
132-3p	1.37 (0.71-2.12)	0.86 (0.43-1.85)	0.12	0.91 (0.63-1.19)	1.04 (0.59-1.97)	0.70
139-5p	1.50 (0.34-3.63)	2.09 (0.23-8.24)	0.27	0.28 (0.05-1.98)	0.81 (0.14-6.59)	0.16
146a-5p	0.85 (0.62-1.03)	1.12 (0.73-1.46)	0.015	0.83 (0.70-0.97)	0.88 (0.62-1.29)	0.94
16-5p	0.92 (0.72-1.82)	0.85 (0.61-1.50)	0.35	0.98 (0.59-1.24)	1.01 (0.73-1.44)	0.33
184	0.95 (0.64-2.18)	1.05 (0.58-2.38)	0.86	0.69 (0.41-1.76)	0.80 (0.31-1.24)	0.89
186-5p	1.09 (0.81-1.39)	1.03 (0.79-1.31)	0.47	0.82 (0.72-1.09)	1.05 (0.82-1.46)	0.09
195-5p	0.91 (0.75-1.42)	0.90 (0.52-1.60)	0.50	0.93 (0.65-1.24)	1.03 (0.69-1.47)	0.42
199a-3p	0.96 (0.77-1.12)	0.93 (0.82-1.47)	0.46	0.89 (0.76-0.98)	1.00 (0.71-1.39)	0.13
200a-3p	0.84 (0.56-1.93)	1.00 (0.58-1.57)	0.62	0.72 (0.49-1.06)	0.73 (0.48-1.43)	0.93
29a-3p	1.12 (0.76-1.44)	1.00 (0.68-1.38)	0.40	1.12 (0.63-1.35)	0.84 (0.61-1.50)	0.66
29b-3p	1.14 (0.81-1.74)	1.06 (0.81-1.51)	0.38	1.02 (0.71-1.42)	0.99 (0.67-1.53)	0.93
29c-3p	1.04 (0.66-1.59)	0.91 (0.64-1.59)	0.63	0.73 (0.44-1.16)	0.86 (0.45-1.56)	0.35
345-5p	3.10 (0.10-10.01)	2.81 (0.22-7.12)	0.99	0.53 (0.05-2.11)	1.14 (0.09-5.97)	0.24
375	1.57 (0.20-4.05)	1.40 (0.48-4.77)	0.56	0.98 (0.26-2.99)	0.86 (0.24-3.07)	0.91
885-5p	1.40 (0.60-2.16)	1.16 (0.28-2.40)	0.48	0.97 (0.49-2.85)	1.54 (0.70-2.38)	0.54
93-5p	1.11 (0.79-1.51)	0.91 (0.68-1.38)	0.21	1.07 (0.79-1.17)	1.19 (0.79-1.53)	0.25
let-7e-5p	1.15 (0.41-4.52)	2.28 (0.64-5.83)	0.25	1.36(0.78-2.72)	1.37 (0.36-2.36)	0.89

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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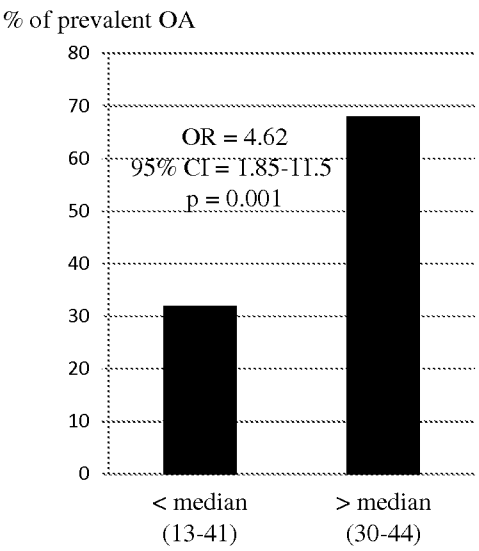
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CLAIMS:

1. A method of determining whether a subject has or is at risk of having osteoarthritis (OA) comprising i) determining the expression level of miR-146a-5p or miR-186 in sample obtained from the subject, and ii) comparing the expression level determined at step i) with a predetermined reference value wherein detecting differential between the expression level determined at step i) and the predetermined reference value is indicative of whether a subject has or is at risk of having osteoarthritis.
2. The method of claim wherein OA is knee OA.
3. The method of claim 1, which is applied to a subject who presents symptoms of OA without having undergone the routine screening to rule out all possible causes for OA.
4. The method of claim 1 wherein the predetermined reference value is the level of the miRNA determined in a population of healthy individuals.
5. The method of claim 4 wherein, it is concluded that the subject has or is at risk of having OA when the expression level of the miRNA is at least 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 100 fold higher than the expression level determined in a population of healthy individuals.
6. The method of claim 1 wherein the subject is prescribed with weight management, physical activity, medications, or joint replacement surgery when it is concluded that the subject has or is at risk of having OA.
7. The method of claim 6 wherein the medication is selected from the group consisting of analgesics, nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, ibuprofen, naproxen and celecoxib), hyaluronic acid and corticosteroids.

1A/ Prevalent OA, miR-146a-5p



1B/ Incident OA, miR-186-5p

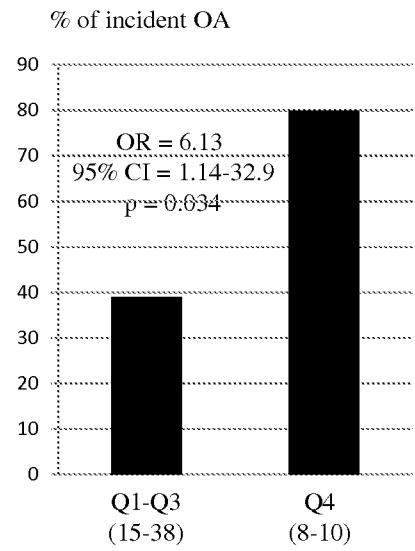


Figure 1

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2018/000676

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/6883

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/080459 A1 (SANOFI SA [FR]; HEERMEIER KATHRIN [DE]; SCHNEIDER JOHANNES CHRISTOF [D] 21 June 2012 (2012-06-21) page 15, paragraph 2 page 18, lines 5-7 page 19, paragraph 2 abstract the whole document	1-7
X	WO 2012/080460 A1 (SANOFI SA [FR]; HEERMEIER KATHRIN [DE]; SCHNEIDER JOHANNES CHRISTOF [D] 21 June 2012 (2012-06-21) page 1, lines 5-9 page 15, paragraph 1 page 19, paragraph 2 abstract the whole document ----- -/--	1-7

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

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 October 2018

Date of mailing of the international search report

15/10/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Helliot, Bertrand

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2018/000676

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/080461 A1 (SANOFI SA [FR]; HEERMEIER KATHRIN [DE]; SCHNEIDER JOHANNES CHRISTOF [D] 21 June 2012 (2012-06-21) page 15 - page 16 page 18 - page 19 page 19 - page 20 abstract the whole document	1-7
X	----- VERÓNICA M. BORGONIO CUADRA ET AL: "Altered Expression of Circulating MicroRNA in Plasma of Patients with Primary Osteoarthritis and In Silico Analysis of Their Pathways", PLOS ONE, vol. 9, no. 6, 5 June 2014 (2014-06-05), page e97690, XP055504911, DOI: 10.1371/journal.pone.0097690 figure 1 page 3, column 2, paragraph 1 abstract the whole document	1-7
X	----- RUINA KONG ET AL: "Combination of circulating miR-19b-3p, miR-122-5p and miR-486-5p expressions correlates with risk and disease severity of knee osteoarthritis", AMERICAN JOURNAL OF TRANSLATIONAL RESEARCH, vol. 9, no. 6, 15 June 2017 (2017-06-15), pages 2852-2864, XP055504887, US ISSN: 1943-8141 page 2861, column 2, paragraph 3 - page 2862, column 1, paragraph 1 abstract the whole document	1-7
X	----- TRACHANA VARVARA ET AL: "Studying microRNAs in osteoarthritis: Critical overview of different analytical approaches", MECHANISMS OF AGEING AND DEVELOPMENT, ELSEVIER SEQUOIA, LAUSANNE, CH, vol. 171, 27 February 2018 (2018-02-27), pages 15-23, XP085368858, ISSN: 0047-6374, DOI: 10.1016/J.MAD.2018.02.006 page 18, column 1, paragraph 4 page 18, column 2, paragraph 2 abstract the whole document	1-7
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INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2018/000676

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUGENT M ED - VAN DEN BERG WIM ET AL: "MicroRNAs: exploring new horizons in osteoarthritis", OSTEOARTHRITIS AND CARTILAGE, vol. 24, no. 4, 4 April 2016 (2016-04-04), pages 573-580, XP029448196, ISSN: 1063-4584, DOI: 10.1016/J.JOCA.2015.10.018 table 1 abstract the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2018/000676

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

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

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

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

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

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7(partially)

A method of determining whether a subject has or is at risk of having osteoarthritis (OA) comprising determining the expression level of miR-146a-5p in sample obtained from the subject.

2. claims: 1-7(partially)

A method of determining whether a subject has or is at risk of having osteoarthritis (OA) comprising determining the expression level of miR-186 in sample obtained from the subject.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2018/000676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012080459 A1	21-06-2012	AR 084332 A1	08-05-2013
		TW 201238973 A	01-10-2012
		UY 33807 A	31-07-2012
		WO 2012080459 A1	21-06-2012
WO 2012080460 A1	21-06-2012	AR 084334 A1	08-05-2013
		TW 201238974 A	01-10-2012
		WO 2012080460 A1	21-06-2012
WO 2012080461 A1	21-06-2012	AR 084331 A1	08-05-2013
		TW 201239097 A	01-10-2012
		WO 2012080461 A1	21-06-2012