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(54) Title: METHOD FOR PREDICTING WHETHER A SUBJECT WILL ACHIEVE A LYMPHODEPLETION WITH AN ANTIBODY SPECIFIC FOR CD20

(57) Abstract: The present invention relates to a method for predicting whether a subject will achieve a lymphodepletion with an antibody specific for CD20. The prediction of therapeutic response of patients is difficult because underlying anti-CD20 antibodies in vivo mechanisms of action remain however incompletely understood. The inventors identify circulating miR-125b and miR-532-3p as potential predictive biomarkers for treatment efficacy of anti-CD20 antibodies. In particular, the present invention relates to a method for predicting whether a subject will achieve a lymphodepletion with an antibody specific for CD20 comprising determining the expression level of miR-532-3p or miR-125b in a blood sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference level and iii) and concluding that the subject will achieve a lymphodepletion when the level determined at step i) is lower than the predetermined reference level or concluding that the subject will not achieve a lymphodepletion when the level determined at step i) is higher than the predetermined expression level.



METHOD FOR PREDICTING WHETHER A SUBJECT WILL ACHIEVE A LYMPHODEPLETION WITH AN ANTIBODY SPECIFIC FOR CD20

5 FIELD OF THE INVENTION:

The present invention relates to a method for predicting whether a subject will achieve a lymphodepletion with an antibody specific for CD20.

BACKGROUND OF THE INVENTION:

10 MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level and play an important regulatory role in many cellular processes.¹ Increased or decreased expression of oncogenic or tumor-suppressor miRNAs has been associated with cancer.^{2,3} Dysregulation of miRNAs expression has clearly been linked to hematological malignancies, especially to B-cell lymphomas.⁴⁻⁸ In most of
15 these studies, peripheral blood mononuclear cells or B-cell subsets from healthy volunteers were compared with cell counterparts from B-chronic lymphocytic leukemia (B-CLL) patients.^{9,10} Microenvironment that delivers signals favoring clonal expansion and drug resistances, as well as genetic heterogeneity of B-CLL cells, are main prognostic factors of B-CLL.^{11,12} A better understanding of miRNAs role in clonal expansion and drug resistance in
20 CLL disease should allow developing targeted treatments, providing greater degrees of success and optimizing diagnosis/prognosis for a personalized medicine. Several studies correlated miRNAs with clinical characteristics or outcome of B-CLL-patients, leading to the identification of B-CLL subgroups with worst outcome.¹³⁻¹⁶ Some of these miRNAs contribute to deregulate pathways involved in B-CLL, such as PI3K/Akt (miR-22), NF-κB
25 (miR-9 family), or toll-like receptor 9 (miR-17~92 family).¹⁷⁻²⁰ BCR signaling pathway was recently shown to be directly regulated by miR-34, miR-150, and miR-155 in CLL^{21,22}, as well as BCL2 (miR-15a/16), TCL1 (miR-29 and miR-181), P53 (miR-15a/miR-16-1 cluster, miR-17-5p, miR-29c and miR-34a), or PTEN (miR-26a and miR-214).²³⁻²⁸ Response to a CLL treatment could also be regulated by miRNAs. Thus, patients refractory to fludarabine
30 exhibited significantly higher expression levels of miR-21, miR-148a and miR-222 than fludarabine-sensitive patients.²⁹ Activation of the P53-reponsive genes was only found in fludarabine responsive patients, suggesting a possible link between abnormal miRNA expression and P53 dysfunctional pathway in non-responder patients. Links between miRNAs, fludarabine-refractory CLL and genomic abnormalities were further demonstrated;

underlying the crucial role of *MYC* and *P53* regulatory networks in determining cell response to fludarabine in CLL.³⁰ Finally, in a prospective clinical trial aiming at evaluating the contribution of 17p deletion and *TP53* mutation in fludarabine-refractory CLL, miR-34a expression at baseline was lower than in a control cohort of CLL without refractory disease.³¹

5 Many publications have reported significant levels of miRNAs in serum and other body fluids in physiological and pathological conditions, raising the possibility that miRNAs may be probed in the circulation and can serve as diagnostic or prognostic outcome biomarkers.³²⁻³⁵ Visone et al. found that blood expression levels of miR-181b decreased in progressive B-CLL patients but not in patients with a stable disease.³⁶ Recently, the inventors
10 have shown that miR-125b concentration can be used to predict clinical response to rituximab treatment in patients with rheumatoid arthritis.³⁷

The exact *in vivo* mechanisms of rituximab (MabThera®, Rituxan®) action remain incompletely understood and could differ depending on the subtype of B-lymphoproliferative disorders. *In vitro* data demonstrated that rituximab is able to induce apoptosis, complement-
15 mediated lysis (CDC), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADPC). Although, the influence of FcγRIIIa-V158V on rituximab response in follicular lymphoma patients strongly suggests that ADCC occurs *in vivo*, there is a lack of evidence for contribution of the other immune mechanisms.³⁸ One of the main barriers to improve our knowledge is the scarcity of clinical situation where rituximab is used
20 alone. Thus, chemotherapy associated with rituximab pollutes any definitive conclusions in studies attempting to analyze *in vivo* rituximab mechanisms of action.

SUMMARY OF THE INVENTION:

The present invention relates to a method for predicting whether a subject will achieve
25 a lymphodepletion with an antibody specific for CD20. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Anti-CD20 antibodies, such as Rituximab (RTX) have dramatically improved the
30 outcome of chronic lymphocytic leukemia (CLL) patients. The underlying *in vivo* mechanisms of action remain however incompletely understood. Several factors influencing response to rituximab have been described including histology, tumor burden and rituximab pharmacokinetics. Circulating miRNAs represent a novel class of molecular biomarkers which expression is altered in hematological disorders. The inventors' study aimed at

identifying circulating miRNAs implicated in the rituximab monotherapy treatment response in CLL patients. Using a hierarchical clustering of miRNA expression profiles discriminating 10 patients with low or high lymphocyte counts before RTX treatment, the inventors found 26 miRNAs differentially expressed and identified two clusters. Using individual RT-qPCR, the expression levels of miRNAs representative of these two clusters were further validated on a larger cohort (n=61). MiR-125b and miR-532-3p were correlated with the lymphodepletion induced by RTX therapy ($p=0.0042$ and $p<0.0001$, respectively) and with the CD20 expression on CD19+ cells ($p=0.0018$ and $p=0.0054$, respectively). Using miRWalk software, they identified 26 putative target genes common to miR-125b and miR-532-3p. In a RTX context, eight of these common genes were associated with the IL-10 pathway. The results identify circulating miR-125b and miR-532-3p as novel miRNAs implicated in the lymphodepletion provided by RTX, and suggest that these two miRNAs are potential predictive biomarkers for treatment efficacy of anti-CD20 antibodies.

Accordingly, the present invention relates to a method for predicting whether a subject will achieve a lymphodepletion with an antibody specific for CD20 comprising i) determining the expression level of miR-532-3p or miR-125b in a blood sample obtained from the subject, ii) comparing the expression level determined a step i) with a predetermined reference level and iii) and concluding that the subject will achieve a lymphodepletion when the level determined at step i) is lower than the predetermined reference level or concluding that the subject will not achieve a lymphodepletion when the level determined at step i) is higher than the predetermined expression level.

In some embodiments, the subject suffers from a B-cell malignancy. As used herein, the term "B-cell malignancy" includes any type of leukemia or lymphoma of B cells. B-cell malignancies include, but are not limited to, non-Hodgkin's lymphoma, Burkitt's lymphoma, small lymphocytic lymphoma, primary effusion lymphoma, diffuse large B-cell lymphoma, splenic marginal zone lymphoma, MALT (mucosa-associated lymphoid tissue) lymphoma, hairy cell leukemia, chronic lymphocytic leukemia, B-cell prolymphocytic leukemia, B cell lymphomas (e.g. various forms of Hodgkin's disease, B cell non-Hodgkin's lymphoma (NHL) and related lymphomas (e.g. Waldenstrom's macroglobulinaemia (also called lymphoplasmacytic lymphoma or immunocytoma) or central nervous system lymphomas), leukemias (e.g. acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL; also termed B cell chronic lymphocytic leukemia BCLL), hairy cell leukemia and chronic myeloblastic leukemia) and myelomas (e.g. multiple myeloma). Additional B cell malignancies include small lymphocytic lymphoma, B cell prolymphocytic leukemia, lymphoplasmacytic

lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma, mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, grey zone lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder.

In some embodiments, the subject suffers from an inflammatory disorder. As used herein, the term “inflammatory disorder” refers to any disease, disorder, or condition in which the immune system is abnormally activated. The inflammatory disorder may be, e.g., ulcerative colitis, Crohn's disease, inflammatory bowel disease, rheumatoid arthritis, spondyloarthritis, myositis, multiple sclerosis, neuromyelitis optica, atherosclerosis, psoriasis, systemic lupus erythematosus (e.g., lupus of the central nervous system or lupus nephritis), nephritis, glomerulonephritis, autoimmune hepatobiliary disease (e.g., autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis), graft-versus-host disease, atopic dermatitis, asthma, neurodegenerative disease (e.g., Alzheimer's disease), demyelinating polyradiculopathy (e.g., Guillain-Barre syndrome or chronic inflammatory demyelinating polyradiculopathy), neuropathic pain, visceral pain of cancer, atherosclerosis, age-related macular degeneration, diabetic nephropathy, sarcoidosis-originated uveitis, or diabetes mellitus.

As used herein, the term “lymphodepletion” refers to the depletion (i.e. the elimination) of B cell populations. The term “B cell” has its general meaning in the art. B cells are lymphocytes that play a large role in the humoral immune response (as opposed to the cell-mediated immune response, which is governed by T cells). Said depletion could be achieved with an antibody specific for CD20. Such depletion may be achieved via various mechanisms such antibody-dependent cell mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC), inhibition of B cell proliferation and/or induction of B cell death (e.g. via apoptosis).

As used herein, the expression “predicting whether a subject will achieve a lymphodepletion” means determining the likelihood that a subject will achieve an elimination of B lymphocytes with an antibody specific for CD20. According to the present invention said determination is performed before the administration to the subject of the antibody specific for CD20.

In some embodiments, the method of the present invention is particularly suitable for predicting that the patient will achieve at least 80%, 90% or 95% of lymphodepletion after 3

weeks of treatment when the level determined at step i) is lower than the predetermined reference level.

As used herein, the term "CD20" has its general meaning in the art and refers to the B-lymphocyte antigen CD20 that is an activated-glycosylated phosphoprotein expressed on the surface of all B-cells beginning at the pro-B phase (CD45R+, CD117+) and progressively increasing in concentration until maturity. Human CD20 has the amino acid sequence of UniProt P011836.

As used herein the term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen-binding site that specifically binds to an antigen. As such, the term antibody encompasses not only whole antibody molecules, but also antibody fragments as well as variants (including derivatives) of antibodies and antibody fragments. In natural antibodies, two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (1) and kappa (k). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The light and heavy chains of an immunoglobulin each have three complementarity determining regions (CDRs), designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. The CDRs of the heavy chain variable domain are located at residues 31-35B (H-CDR1), residues 50-65 (H-CDR2) and residues 95-102 (H-CDR3) according to the Kabat numbering system. The CDRs of the light chain variable domain are located at residues 24-34 (L-CDR1), residues 50-56 (L-CDR2) and residues 89-97 (L-CDR3) according to the Kabat numbering system.

In some embodiments, the antibody of the present invention is a chimeric antibody, typically a chimeric mouse/human antibody. The term "chimeric antibody" refers to a monoclonal antibody which comprises a VH domain and a VL domain of an antibody derived from a non-human animal, a CH domain and a CL domain of a human antibody. As the non-human animal, any animal such as mouse, rat, hamster, rabbit or the like can be used.

In some embodiments, the antibody is a humanized antibody. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the

CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference.

5 In some embodiments, the antibody is a fully human antibody. Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference.

10 In some embodiments, the Fc region is modified to increase the ability of the antibody to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fc receptor by modifying one or more amino acids. In this manner the capabilities of the antibody to deplete B cells can be increased. This approach is described further in PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human
15 IgG1 for FcγRI, FcγRII, FcγRIII and FcγRn have been mapped and variants with improved binding have been described (see Shields, R. L. et al, 2001 J. Biol. Chem. 276:6591-6604, WO2010106180). In some embodiments, the glycosylation of an antibody is modified either by Fc-mutation or Fc-glycoengineering strategy. For example, an aglycosylated antibody can be made (i.e., the antibody lacks glycosylation). Additionally or alternatively, an antibody can
20 be made that has an altered type of glycosylation, such as a hypofucosylated or non-fucosylated antibody having reduced amounts of or no fucosyl residues or an antibody having increased bisecting GlcNAc structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies.

Examples of antibodies having specificity for CD20 include: “C2B8” which is now
25 called “Rituximab” (“RITUXAN®”) (U.S. Pat. No. 5,736,137, expressly incorporated herein by reference), a chimaeric pan-B antibody targeting CD20; the yttrium-[90]-labeled 2B8 murine antibody designated “Y2B8” or “Ibritumomab Tiuxetan” ZEVALIN® (U.S. Pat. No. 5,736,137, expressly incorporated herein by reference), a murine IgG1 kappa mAb covalently linked to MX-DTPA for chelating to yttrium-[90]; murine IgG2a “BI,” also called
30 “Tositumomab,” optionally labeled with radioactive ¹³¹I to generate the “¹³¹I-B1” antibody (iodine ¹³¹ tositumomab, BEXXAR™) (U.S. Pat. No. 5,595,721, expressly incorporated herein by reference); murine monoclonal antibody “1F5” (Press et al. Blood 69 (2):584-591 (1987) and variants thereof including “framework patched” or humanized 1F5 (WO03/002607, Leung, S.; ATCC deposit HB-96450); murine 2H7 and chimeric 2H7

antibody (U.S. Pat. No. 5,677,180, expressly incorporated herein by reference); humanized 2H7, also known as ocrelizumab (PRO-70769); Ofatumumab (Arzerra), a fully human IgG1 against a novel epitope on CD20 huMax-CD20 (Genmab, Denmark; WO2004/035607 (U.S. Ser. No. 10/687,799, expressly incorporated herein by reference)); AME-133 (ocaratuzumab; Applied Molecular Evolution), a a fully-humanized and optimized IgG1 mAb against CD20; A20 antibody or variants thereof such as chimeric or humanized A20 antibody (cA20, hA20, respectively) (U.S. Ser. No. 10/366,709, expressly incorporated herein by reference, Immunomedics); and monoclonal antibodies L27, G28-2, 93-1B3, B-CI or NU-B2 available from the International Leukocyte Typing Workshop (Valentine et al, In: Leukocyte Typing III (McMichael, Ed., p. 440, Oxford University Press (1987))). Further, suitable antibodies include e.g. antibody GA101 (obinutuzumab), a third generation humanized anti-CD20-antibody of Biogen Idec/Genentech/Roche. Moreover, BLX-301 of Biolex Therapeutics, a humanized anti CD20 with optimized glycosylation or Veltuzumab (hA20), a 2nd-generation humanized antibody specific for CD20 of Immunomedics or DXL625, derivatives of veltuzumab, such as the bispecific hexavalent antibodies of IBC Pharmaceuticals (Immunomedics) which are comprised of a divalent anti-CD20 IgG of veltuzumab and a pair of stabilized dimers of Fab derived from milatuzumab, an anti-CD20 mAb enhanced with InNexus' Dynamic Cross Linking technology, of Inexus Biotechnology both are humanized anti-CD20 antibodies are suitable. Further suitable antibodies are BM-ca (a humanized antibody specific for CD20 (Int J. Oncol. 2011 February; 38(2):335-44)), C2H7 (a chimeric antibody specific for CD20 (Mol Immunol. 2008 May; 45(10):2861-8)), PRO131921 (a third generation antibody specific for CD20 developed by Genentech), Reditux (a biosimilar version of rituximab developed by Dr Reddy's), PBO-326 (a biosimilar version of rituximab developed by Probiomed), a biosimilar version of rituximab developed by Zenotech, TL-011 (a biosimilar version of rituximab developed by Teva), CMAB304 (a biosimilar version of rituximab developed by Shanghai CP Guojian), GP-2013 (a biosimilar version of rituximab developed by Sandoz (Novartis)), SAIT-101 (a biosimilar version of rituximab developed by Samsung BioLogics), a biosimilar version of rituximab developed by Intas Biopharmaceuticals, CT-P10), a biosimilar version of rituximab developed by Celltrion), a biosimilar version of rituximab developed by Biocad, Ublituximab (LFB-R603, a transgenically produced mAb targeting CD20 developed by GTC Biotherapeutics (LFB Biotechnologies)), PF-05280586 (presumed to be a biosimilar version of rituximab developed by Pfizer), Lymphomun (Bi-20, a trifunctional anti-CD20 and anti-CD3 antibody, developed by Trion Pharma), a biosimilar version of rituximab developed by Natco Pharma, a biosimilar

version of rituximab developed by iBio, a biosimilar version of rituximab developed by Gedeon Richter/Stada, a biosimilar version of rituximab developed by Curaxys, a biosimilar version of rituximab developed by Coherus Biosciences/Daiichi Sankyo, a biosimilar version of rituximab developed by BioXpress, BT-D004 (a biosimilar version of rituximab developed by Protheon), AP-052 (a biosimilar version of rituximab developed by Aprogen), a biosimilar version of ofatumumab developed by BioXpress, MG-1106 (a biosimilar version of rituximab developed by Green Cross), IBI-301 (a humanized monoclonal antibody against CD20 developed by Innovent Biologics), BVX-20 (a humanized mAb against the CD20 developed by Vaccinex), 20-C2-2b (a bispecific mAb-IFN α that targets CD20 and human leukocyte antigen-DR (HLA-DR) developed by Immunomedics), MEDI-552 (developed by MedImmune/AstraZeneca), the anti-CD20/streptavidin conjugates developed by NeoRx (now Poniard Pharmaceuticals), the 2nd generation anti-CD20 human antibodies developed by Favril (now MMRGlobal), TRU-015, an antibody specific for CD20 fragment developed by Trubion/Emergent BioSolutions, as well as other preclinical approaches by various companies and entities. All aforementioned publications, references, patents and patent applications are incorporated by reference in their entireties. All antibodies disclosed in therein may be used within the present invention.

In some embodiments, the antibody specific for CD20 is rituxan. Rituxan comprises an HCDR1 region of sequence SYNMH, an HCDR2 region of sequence AIYPGNGDTSYNQKFKG, an HCDR3 region of sequence STYYGGDWYFNV, an LCDR1 region of sequence RASSSVSYIH, an LCDR2 region of sequence ATSNLAS, and an LCDR3 region of sequence QQWTSNPPT. Rituxan comprises a variable heavy chain of the sequence:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAI
YPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYG
GDWYFNVWGAGTTVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE
VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK,

and a variable light chain of the sequence:

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKPWYATSNLA
 SGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEKRT
 VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES
 VTEQDSKDSSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC.

5 In some embodiments, the antibody specific for CD20 is an antibody which cross-competes with rituxan.

As used herein, the term “blood sample” means any blood sample (for instance whole blood sample, plasma sample or serum sample) derived from the subject that contains nucleic acids. According to the invention, the blood sample is obtained from the subject before any
 10 administration of the antibody specific for CD20.

Conventional methods and reagents for isolating RNA from a sample comprise High Pure miRNA Isolation Kit (Roche), Trizol (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
 15 (Qiagen), Oligotex kit (Qiagen), phenol extraction, phenol-chloroform extraction, TCA/acetone precipitation, ethanol precipitation, Column purification, Silica gel membrane purification, PureYield™ RNA Midiprep (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
 20 RNA™ Kit (Ambion), Poly(A)Purist™ Kit (Ambion) and any other methods, commercially available or not, known to the skilled person.

The term "miRNAs" refers to mature microRNA (non-coding small RNAs) molecules that are generally 21 to 22 nucleotides in length, even though lengths of 19 and up to 23 nucleotides have been reported. miRNAs are each processed from longer precursor RNA
 25 molecules (“precursor miRNA”: pri-miRNA and pre-miRNA). Pri-miRNAs are transcribed either from non-protein-encoding genes or embedded into protein-coding genes (within introns or non-coding exons). The “precursor miRNAs” fold into hairpin structures containing imperfectly base-paired stems and are processed in two steps, catalyzed in animals by two Ribonuclease III-type endonucleases called Drosha and Dicer. The processed miRNAs (also
 30 referred to as “mature miRNA”) are assembled into large ribonucleoprotein complexes (RISCs) that can associate them with their target mRNA in order to repress translation. All the miRNAs pertaining to the invention are known *per se* and sequences of them are publicly available from the data base http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa.

The expression level of one or more miRNA in the sample may be determined by any suitable 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 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. Examples of suitable kits include, but are not limited to the mirVana TaqMan® miRNA transcription kit (Ambion, Austin, TX), and the TaqMan® miRNA transcription kit (Applied Biosystems, Foster City, CA). Universal primers, or specific primers, including miRNA-specific stem-loop primers, are known and commercially 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 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, multiplex ligatable probe amplification, in vitro transcription (IVT), strand displacement amplification, transcription-mediated amplification, 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 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 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.

In some embodiments, two or more miRNAs are amplified in a single reaction volume. For example, multiplex q-PCR, such as RT-qPCR, enables simultaneous amplification and quantification of at least two miRNAs of interest in one reaction volume by

using more than one pair of primers and/or more than one probe. The primer pairs comprise at least one amplification primer that specifically binds each miRNA, and the probes are labelled such that they are distinguishable from one another, thus allowing simultaneous quantification of multiple miRNAs.

5 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
10 to the (-) strand, a complex pattern of strand displacement results in the generation of over 10^9 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
15 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).

miRNAs may be detected using hybridization-based methods, including but not limited to hybridization arrays (e.g., microarrays), NanoString analysis, Northern Blot
20 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
25 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
30 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.

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 DNA or RNA 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 a 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 tumor 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 posttranscriptional 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.

In some embodiments, the expression levels of miR-532-3p and miR-125b are determined in the blood sample of the present invention.

Typically, 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 the expression level of the selected miRNA in properly banked historical subject 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 expression level of the selected miRNA in a group of reference, one can use algorithmic analysis for the statistic treatment of the expression levels determined 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 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.

A further object of the invention relates to a kit for performing the method of the present invention, wherein said kit comprises means for measuring the expression levels of

the miRNAs of the invention in the sample obtained from the patient. The kits may include probes, primers macroarrays or microarrays as above described. For example, the kit may comprise a set of miRNA probes as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, probes may be unlabelled and the ingredients for labelling may be included in the kit in separate containers. The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards. Alternatively the kit of the invention may comprise amplification primers (e.g. stem- loop primers) that may be pre-labelled or may contain an affinity purification or attachment moiety. The kit may further comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

In some embodiments, labels, dyes, or labelled probes and/or primers are used to detect amplified or unamplified miRNAs. The skilled artisan will recognize which detection methods are appropriate based on the sensitivity of the detection method and the abundance of the target. Depending on the sensitivity of the detection method and the abundance of the target, amplification may or may not be required prior to detection. One skilled in the art will recognize the detection methods where miRNA amplification is preferred. A probe or primer may include standard (A, T or U, G and C) bases, or modified bases. Modified bases include, but are not limited to, the AEGIS bases (from Eragen Biosciences), which have been described, e.g., in U.S. Pat. Nos. 5,432,272, 5,965,364, and 6,001,983. In certain aspects, bases are joined by a natural phosphodiester bond or a different chemical linkage. Different chemical linkages include, but are not limited to, a peptide bond or a Locked Nucleic Acid (LNA) linkage, which is described, e.g., in U.S. Pat. No. 7,060,809. In a further aspect, oligonucleotide probes or primers present in an amplification reaction are suitable for monitoring the amount of amplification product produced as a function of time. In certain aspects, probes having different single stranded versus double stranded character are used to detect the nucleic acid. Probes include, but are not limited to, the 5'-exonuclease assay {e.g., TaqMan™} probes (see U.S. Pat. No. 5,538,848), stem-loop molecular beacons (see, e.g., U.S. Pat. Nos. 6,103,476 and 5,925,517), stemless or linear beacons (see, e.g., WO 9921881, U.S. Pat. Nos. 6,485,901 and 6,649,349), peptide nucleic acid (PNA) Molecular Beacons (see, e.g., U.S. Pat. Nos. 6,355,421 and 6,593,091), linear PNA beacons (see, e.g. U.S. Pat. No. 6,329,144), non- FRET probes (see, e.g., U.S. Pat. No. 6,150,097), Sunrise™/AmplifluorB™ probes (see, e.g., U.S. Pat. No. 6,548,250), stem-loop and duplex Scorpion™ probes (see, e.g., U.S. Pat. No. 6,589,743), bulge loop probes (see, e.g., U.S. Pat. No. 6,590,091), pseudo

knot probes (see, e.g., U.S. Pat. No. 6,548,250), cyclicons (see, e.g., U.S. Pat. No. 6,383,752), MGB Eclipse™ probe (Epoch Biosciences), hairpin probes (see, e.g., U.S. Pat. No. 6,596,490), PNA light-up probes, antiprimer quench probes (Li et al., Clin. Chem. 53:624-633 (2006)), self-assembled nanoparticle probes, and ferrocene-modified probes described, for example, in U.S. Pat. No. 6,485,901. In some embodiments, one or more of the primers in an amplification reaction can include a label. In yet further embodiments, different probes or primers comprise detectable labels that are distinguishable from one another. In some embodiments a nucleic acid, such as the probe or primer, may be labeled with two or more distinguishable labels. In some aspects, a label is attached to one or more probes and has one or more of the following properties: (i) provides a detectable signal; (ii) interacts with a second label to modify the detectable signal provided by the second label, e.g., FRET (Fluorescent Resonance Energy Transfer); (iii) stabilizes hybridization, e.g., duplex formation; and (iv) provides a member of a binding complex or affinity set, e.g., affinity, antibody- antigen, ionic complexes, hapten-ligand (e.g., biotin-avidin). In still other aspects, use of labels can be accomplished using any one of a large number of known techniques employing known labels, linkages, linking groups, reagents, reaction conditions, and analysis and purification methods. Labels include, but are not limited to: light-emitting, light-scattering, and light-absorbing compounds which generate or quench a detectable fluorescent, chemiluminescent, or bioluminescent signal (see, e.g., Kricka, L., Nonisotopic DNA Probe Techniques, Academic Press, San Diego (1992) and Garman A., Non-Radioactive Labelling, Academic Press (1997)). A dual labeled fluorescent probe that includes a reporter fluorophore and a quencher fluorophore is used in some embodiments. It will be appreciated that pairs of fluorophores are chosen that have distinct emission spectra so that they can be easily distinguished. In some embodiments, labels are hybridization-stabilizing moieties which serve to enhance, stabilize, or influence hybridization of duplexes, e.g., intercalators and intercalating dyes (including, but not limited to, ethidium bromide and SYBR-Green), minor-groove binders, and cross-linking functional groups (see, e.g., Blackburn et al., eds. "DNA and RNA Structure" in Nucleic Acids in Chemistry and Biology (1996)).

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. MicroRNAs expression profile discriminating CLL patients with low or high lymphocyte count at D0.

(A) The profiles of 26 microRNAs significantly differently expressed ($p < 0.01$) between high and low lymphocyte concentration samples isolated from 10 CLL patients were visualized using a supervised heatmap (average linkage and Pearson's correlation). Relative miRNAs expression was calculated using the comparative threshold cycle (CT) method. For normalization, the mean CT value of all miRNA targets was used. Dendrograms indicated the correlation between miRNAs that was generated by the perfect clustering of both patients' groups. (B-C) Expression levels of 4 miRNAs representative of each cluster, miR-193 and miR-125b for cluster 1 (B), and miR-652 and miR-532-3p for cluster 2 (C), were measured for 61 CLL patients treated in the Dense FCR arm, using RT-qPCR. A significant inverse correlation was observed depending on the lymphocytes count for miR-193b ($r^2 = -0.19$), miR-125b ($r^2 = -0.39$), miR-652 ($r^2 = -0.30$) and miR-532-3p ($r^2 = -0.34$).

Figure 2. Lymphodepletion after 4 total doses (6500mg) of rituximab treatment correlated with miRNAs.

(A) Univariate analysis of miRNAs with lymphodepletion in CLL patients. Abbreviations: 95%, 90%, 85%, 80%, lymphodepletion between D22 and D0. (B-C) miRNA expression levels were inversely correlated with the percentage of lymphodepletion between D22 and D0, miR-125b ($r^2 = -0.42$) (B) and miR-532-3p ($r^2 = -0.49$) (C). Abbreviation: ns, not significant.

Figure 3. Inverse correlation between miR-125b and miR-532-3p expression levels and CD20 expression on CD19⁺ cells in CLL blood patients.

CD20 expression levels on CD19⁺ lymphocytes were quantified using flow cytometry. PBMC were collected and miR-125b ($r^2 = -0.37$) (A) and miR-532-3p ($r^2 = -0.29$) (B) were quantified using RT-QPCR. Abbreviation: MESF, Molecule of equivalent soluble fluorescence.

EXAMPLE:

Material & Methods

CLL2010FMP protocol

A prospective, randomized, open-label, phase II study (CLL2010FMP, NCT01370772) have included 140 treatment-naïve patients (aged 18-65 years) diagnosed with confirmed chronic lymphocytic leukemia according to IWCLL 2008 criteria and Binet stage C or with active Binet stage A or B.⁴¹ An additional inclusion criteria was the absence

of 17p deletion, assessed by FISH (<10% positive nuclei). Each patient provided a written informed consent before enrolment. Patients were stratified according to *IGVH* mutational status, FISH analysis (11q deletion or not) and were randomly assigned to receive either 6 cycles of chemo-immunotherapy FCR (rituximab 375 mg/m² for the first course, D1 and 500 mg/m² for the others, fludarabine 40 mg/m²/d D2-4, cyclophosphamide 250 mg/m²/d D2-4) every 28 days or Dense-FCR with an intensified rituximab pre-phase (500 mg on D0, and 2000 mg on D1, D8 and D15) before the standard treatment FCR. The main objective was to increase complete response rate with undetectable minimal residual disease three months after treatment and was previously published.³⁹ In the present study we have explored miRNA signature only in the cohort of patients receiving rituximab pre-phase before immune-chemotherapy.

miRNA analysis

mRNA extraction

Blood was collected before treatment in PAXgene Blood RNA Tubes according to the instructions in the PAXgene Blood RNA Tube Product Circular. Total RNAs, including small RNAs, were extracted using the PAXgene Blood miRNA Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instruction with minor modifications due to the B cell amount. The procedure (one column) was performed for no more than 40 G/L lymphocytes. For patients with more than 40 G/L lymphocytes, we divided sample as often as necessary to load about 40 G/L lymphocytes in a column.

RNA concentration and purity were assessed using the NanoDropTM spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RNA quality were assessed using the 2100 Bioanalyzer assay (Agilent, Les Ulis, France), and according to the criteria of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments MIQE guidelines, only samples with a RIN>8 were used.

miRNA TaqMan Low-Density Array

Ten patients were analyzed with the TaqMan Low-Density Array (TLDA) technology is divided in two groups according to the lymphocyte concentration at Day 0 (D0). One group is composed of 5 patients with lymphocyte count inferior to quartile 1 (11.67 G/L) of the lymphocyte concentration at D0 for all patients: to 4.6 G/L to 10.7 G/L (low). The other group is composed of 5 patients with lymphocyte count superior to quartile 3 (93.93 G/L) of the lymphocyte concentration at D0 for all patients: to 97 G/L to 223 G/L at D0 (high).

Total RNAs (70 ng) were converted to cDNAs using MegaplexTM RT Primers (human pool A v2.1 or pool B v3.0) and TaqMan® MicroRNA Reverse Transcription kit.

Collectively the pools cover 754 unique miRNAs. A pre-amplification step using Megaplex™ PreAmp Primers (human pool A v2.1 or pool B v3.0) and TaqMan® PreAmp Master Mix was performed. MicroRNA profiling was achieved using the TaqMan® Human MicroRNA Array Cards A v2.0 and B v3.0 and TaqMan® Fast Advanced Master Mix. The 384-well format TLDA were run on an ABI 7900 HT fast real-time PCR system (Applied Life Technologies, Saint Aubin, France). All reagents were supplied by Applied Life Technologies, Saint Aubin, France.

miRNA array analysis

RT-qPCR raw data were analyzed using SDS 2.3 and RQ Manager Software (Applied Life Technologies, Saint Aubin, France). Each miRNA for each sample was normalized to the mean Ct value of all expressed miRNAs and RNU48. Relative miRNA expression was calculated using the comparative threshold cycle (Ct) method. Using a fold change (FC) ± 1.5 ($P \leq 0.05$), and a two-dimensional hierarchical clustering analysis, we selected a set of miRNAs correlated with the lymphocyte concentration at D0.

miRNA validation

MicroRNA validation consisted in a RT-qPCR on the Dense-FCR arm of the protocol with the set of miRNAs previously selected with TLDA assay. We performed a multiplex RT and pre-amplification from 60 ng of RNA, using respectively TaqMan® MicroRNA Reverse Transcription kit and TaqMan® PreAmp Master Mix. PCR for each miRNA was done for each patient using TaqMan® Fast Advanced Master Mix. The 96-well format plates were run on an ABI 7900 HT fast real-time PCR system (Applied Life Technologies, Saint Aubin, France). All reagents were supplied by Applied Life Technologies, Saint Aubin, France. RNU48 was used for normalization to obtain relative miRNA expression.

FCGR3A genotyping

Single-step multiplex allele-specific PCR assays were performed as described by Dall'Ozzo et al. with minor modifications.⁴² The 25 μ L reaction mixture contained 10ng of genomic DNA, 400 nM of forward primer (5'-TCCAAA AGCCACACTCAAAGTC-3'), 400nM of reverse V allele primer (5'-AGACACATTTTACTCCCATC -3') and 200nM reverse F allele primer (5'-GCGGGCAGGGCGGCGGGGGCGGGGCCGGTGATGTTTACAGTCTCTGATCACACA TTTTACTCCCAT-3'), 400 μ M of each dNTP, 2mM MgCl₂ and 0.5U of Taq DNA polymerase in its buffer (Promega, Madison, USA). PCR conditions consisted in 3.5 min at 95°C followed by 35 cycles, each consisting in 95°C for 20 sec, 56°C for 20 sec, 72°C for 30 sec. After amplification PCR products (137bp fragment for F allele and 81bp for V allele)

were resolved using 8% acrylamide gel (Invitrogen, Carlsbad, USA) and visualized after ethidium bromide staining.

IL-10 competent B-CLL cells identification

IL-10 competent B-CLL cells counts were determined by flow cytometry analysis of IL-10 production. Peripheral blood mononuclear cells (PBMCs) were purified from peripheral blood samples of 68 arm B patients with CLL using Ficoll-Hypaque density gradients (Eurobio, Courtaboeuf, France). 3 PBMCs were resuspended (9×10^6 cells/mL) in medium (RPMI 1640 media (Biotech GmbH, Aidenbach, Germany) containing 10% fetal calf serum (Eurobio, Courtaboeuf, France), 2 mM L-glutamine (Eurobio, Courtaboeuf, France), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2.5 μ g/mL amphotericin (all antibiotics from Tebu-bio, Le Perray-en-Yvelines, France). Clonal activation of lymphocytes B (LB) were stimulated with CpG (ODN 2006, 10 μ g/mL; InvivoGen, San Diego, USA), CD40L (50 ng/mL; R&D Systems, Minneapolis, MN, USA) and anti-polyHistidine (500 ng/mL; R&D Systems, Minneapolis, MN, USA) for 48 h at 37°C in a 5% CO₂–95% air humidified atmosphere. PMA (50 ng/mL; Sigma-Aldrich, Saint Louis, MO, USA) and ionomycin (1 μ g/mL; Sigma-Aldrich, Saint Louis, MO, USA) were added on cells to stimulate IL-10 production. After 4 h at 37°C in a 5% CO₂–95% air humidified atmosphere, brefeldin A (1 X solution/mL; BioLegend, San Diego, CA, USA) blocked IL-10 secretion to determine B10pro + B10 cell population. 3-5 Antihuman antibody included: CD19 BV421 (HIB 19), CD69 PE/Cy7 (FN 50), CD38 APC (HIT 2), IL-10 PE (JES3-9D7) from BioLegend (San Diego, CA, USA), and CD45 KO (J.33) and CD5 FITC (BL1a) from Beckman Coulter (Brea, CA, USA). Clonal CLL cells were identified as CD19⁺ CD5⁺ CD20^{int} lymphocytes. Analyses were performed on CyAn™ ADP flow cytometer (Beckman Coulter, Brea, CA, USA).

Quantification of CD20 expression:

CD20 expression was quantified using the commercial kit QuantiBRITETM CD20PE according to manufacturer's recommendations ((BD Biosciences, Le Pont-de-Claix, France). This kit uses an antiCD20 reagent certified with a PE to mAb ratio of 1:1. An initial cytometer setup was performed to allow the study of CD20 expression on T-lymphocytes as negative control, normal B-cells as positive control and CLL cells. Then fluorescence target values were determined for PE channel using 8-peak Rainbow bead calibration particles (Spherotech, Lake Forest IL, USA) and a calibration curve for CD20 QuantiBRITETM assay was established based upon these settings. Before the realization of any new calibration curve, the cytometer setup was adjusted to reproduce the initial settings using the same lot of 8-peak Rainbow bead calibration particles. By using a calibration curve, the measure of CD20

fluorescence intensity on CLL cells allows calculating of the number of equivalent CD20 molecules present at the cell surface.

Statistical analysis

Distributions of data were tested with the Shapiro-Wilk test. X^2 or Fisher test was used for categorical data. For numerical data, comparisons of medians were performed using Student T or Mann-Whitney test. All variables with a p value of <0.10 in univariate analysis were included in an intermediate model. The final model variables were determined by backward selection using Student T test ($p < 0.05$ as significant model). A probability $p < 0.05$ was considered statistically significant. Pearson's correlation test was used to assess the association between two numerical data. All statistical analyses were performed at the conventional two-tailed α level of 0.05 using R software version 3.0.2.10.

Results

Patients' characteristics.

Sixty-eight patients were allocated to receive rituximab pre-phase. MicroRNA extraction was performed on sixty one patients. Patients' characteristics are presented in Table 1. Median age was 58 years (interquartile range (IQR): 53-61), 28% were female and 77% were Binet stage A or B. Cytogenetic analysis demonstrated del11q in 20% of patients and median lymphocyte count was 89 G/L (IQR: 43-123).

Differentially expressed miRNAs in B-CLL patients with high and low lymphocytosis.

To identify baseline B-CLL cells miRNA signature, we used a real-time PCR-based high-throughput miRNA array (TaqMan Low-Density Array or TLDA). Analysis was performed using 5 B-CLL patients with high lymphocyte count (up to 93.93 G/L, Q3) compared with 5 B-CLL patients with low lymphocyte count (lower than 11.67 G/L, Q1). Q1 and Q3 were interquartile values of lymphocyte count at D0 for all patients of the Dense-FCR arm. After fold change filtering (fold-change ≥ 1.5) on the differentially expressed miRNAs, we found 5 miRNAs down regulated and 21 miRNAs up regulated in CLL patients with low lymphocyte count compared with those with high lymphocyte count ($P < 0.05$) (Table 2). The heat map showed results of the unsupervised hierarchical clustering based on the significantly differentially expressed miRNAs. Two patterns of miRNA expression named cluster 1 and cluster 2 were clearly identified according to lymphocyte counts (Fig 1A). To confirm these patterns, RT-qPCR was performed for all patients included in Dense-FCR arm ($n=61$) to quantify the expression levels of 2 representative miRNA per cluster, namely miR-193b and

miR-125b for cluster 1, and miR-652 and miR-532-3p for cluster 2. Scatter plots (Fig 1B) confirmed that increased lymphocyte count was inversely correlated with the expression levels of miR-193b and miR-125b ($p=0.03$ and $p=0.0001$, respectively) for cluster 1, and miR-652 and miR-532-3p ($p=0.0017$ and $p=0.0001$, respectively) for cluster 2. These results demonstrated that all 4 miRNAs tested were markedly down regulated in B-CLL patients with high lymphocyte count compared with B-CLL patients with low lymphocyte count.

miR-125b and miR-532-3p expression levels correlated with lymphodepletion observed after rituximab treatment.

Lymphocyte depletion after rituximab monotherapy was assessed at D22, after four rituximab infusions (6500 mg of rituximab). We hypothesized that the lymphocyte depletion observed after rituximab infusions was related to *in vivo* rituximab activity. We thus analyzed the correlation between the miRNA expression profile before rituximab (D0) and the efficacy of lymphocyte depletion measured at D22 in B-CLL patients included in the experimental arm ($n=61$). Median lymphocyte counts was 88.81 G/L (range: 3.74-350.50) before the four infusions of rituximab (D0) and 2.45 G/L (range: 0.14-189.40) at the end of rituximab pre-phase (D22). The median lymphocyte depletion after rituximab pre-phase (D22) was 95.9% (range: -5.0-99.6). No significant correlation was found between individual miRNAs (miR-125b, miR-193b, miR-532-3p, miR-652) and clinical (age, Binet stage, ECOG) or biological (*IGHV* mutation, cytogenetic abnormalities (FISH 11q, FISH 13q, FISH trisomy 12, frequency of IL-10-competent B cells, or FcγRIIIa-158V/F polymorphism) parameters.

Among the 4 miRNAs validated, only two of them, miR-125b and miR-532-3p, were significantly correlated to all lymphodepletion rates, namely 95, 90, 85 and 80%. The correlation for miR-652 was only found significant for 95 and 90% lymphodepletion rate. No correlation was found between miR-193b and all depletion rates tested (Fig. 2A). Scatter plots in Figure 2B showed that the lymphodepletion rate was inversely correlated with the increase of expression levels of miR-125b and miR-532-3p ($p=0.0042$ and $p<0.0001$, respectively).

Logistic regression analyses showed that only the frequency of IL-10-competent B cells and miR-532-3p were associated with 90% lymphodepletion after rituximab pre-phase (odds ratio (OR) = 0.87; confidence interval (CI): 0.76-0.97; $p=0.014$, and OR = 0.0002; 95% CI: $<10^{-4}$ -0.34; $p=0.029$, respectively). No significant association was found between lymphodepletion and FcγRIIIa-158V/F polymorphism or miR-125b. Receiver operating characteristic curve (ROC) using IL-10-competent B cells frequencies and miR-532-3p expression levels showed a highly discriminative power (AUC=0.795; 95% CI: 0.652-0.939),

leading to predict patients who will have more than 90% of lymphodepletion after rituximab pre-phase.

Putative and validated target genes of miR-125b and miR-532-3p.

Using the miRWalk database, a tool that compares miRNAs binding sites resulting from 5 main existing miRNA-target prediction programs (DIANA, RNA22, Pictar, miRanda and Targetscan), we investigated putative target genes of miRNAs associated with rituximab-induced lymphodepletion.⁴³ Two lists of putative target genes were obtained: 5053 genes for miR-125b and 6652 for miR-532-3p. The Venny program, an interactive tool for comparing lists identified 3151 common genes targeted by both miR-125b and miR-532-3p.⁴⁴ We then compared with transcriptomic datasets available for IL-10-competent B cells gene expression profiles.⁴⁵ Among the 104 genes differentially expressed in the study that compared IL10⁺ and IL-10⁻ human regulatory B cells, 33 and 46 genes overlapped with miR-125b and miR-532-3p putative targeted genes, respectively.⁴⁵ Importantly, 26 genes were common targets of both miRNAs. Then, in the context of rituximab that is known to target the pan-B-cell marker CD20/MS4A1, we wondered whether this gene could also be targeted by miR-125b and miR-532-3p. We found that both miRNAs were predicted to target MS4A1.

Pathway enrichment analysis was performed using the web-based bioinformatics application Ingenuity Pathway Analysis (IPA Ingenuity Systems, <http://www.ingenuity.com>) based on the in silico 26 predicted target genes common to miR-125b, miR-532-3p and differentially regulated in human IL10⁺ regulatory B cells, as well as MS4A1. A hierarchical layout was built with only miRNA/mRNA interactions displaying high-predicted scores and for which the correlation was experimentally observed in humans. All the 9 genes presented in this figure were associated with the IL-10 pathway (EGR3, IL1A, IL10, IL10RA, IRF4, IRF5, MS4A1, TLR7 and TSC22D3).

CD20 expression on B-CLL cells inversely correlated with miR-125b and miR-532-3p.

We analyzed the association between miR-125b and miR-532-3p expression levels and the CD20 expression on CD19⁺/CD5⁺ B-CLL cells. For both cases, a significant inverse correlation was observed between CD20 and miRNA expression levels. A high CD20 expression by B-CLL cells correlated with a low expression of either miR-125b or miR-532-3p (p=0.0018 and p=0.005, respectively) (Figure 3).

Discussion:

In the present study, we investigated whether miRNAs play a role in rituximab treatment outcome in CLL patients. The first step of our study was to identify a miRNA profile dependent on lymphocytosis level in CLL patients before treatment initiation. We found that 26 miRNAs were significantly deregulated in CLL patients with low lymphocytosis compared with patient with high lymphocytosis. For the 4 miRNAs validated (miR-125b, miR-193b, miR-652 and miR-532-3p), we found an inverse correlation between the miRNA expression levels and the lymphocyte concentration at D0. In the second phase of the study, lymphocyte depletion after rituximab monotherapy was assessed at D22 to confirm influence of miRNA profile on *in vivo* rituximab efficacy, and we found that only miR-125b and miR-532-3p were negatively correlated with lymphodepletion at D22.

These miRNAs have already been described in leukemia disorders. miR-125b is implicated in specific subtypes of leukemia induced by chromosomal translocation such as B-cell acute lymphoblastic leukemia (ALL).⁴⁶ This highly conserved miRNA consists of two homologs hsa-miR-125b-1 and hsa-miR-125-2. The miR-125b-1 maps at 11q24, a chromosomal region close to the epicenter of 11q23 deletions in chronic lymphocytic leukemias, and miR-125b expression reduction was described both in aggressive and indolent CLL patients.⁴⁷ No correlation was found between miR-125b expression and patients with 11q deletion ($p=0.11$). Recently, it has been shown that miR-125b induces myeloid leukemia and B-cell leukemia through the IRF4 silencing or the genetic deletion, respectively.⁴⁸ In a recent study investigating miRNAs changes upon B cell receptor stimulation in distinguish CLL patients' subclasses, miR532-3p was increased at 48 hours, exclusively in CLL patients with stable disease.¹⁵ Like miR-125b, mir-532-3p role and implication in CLL is established. Importantly, it was strongly associated with progression-free survival in CLL.¹⁰

Gagez et al. showed that the frequency of IL-10-competent B CLL cells adversely impacted on 90% lymphodepletion observed after rituximab pre-phase ($p=0.004$) and also correlate with clinical response assessed 3 months after immuno-chemotherapy by FCR (complete response (CR) vs no-CR, $p=0.04$).⁴⁹ In our present study, logistic regression analysis showed that only the frequency of IL-10-competent B CLL cells and miR-532-3p were associated with 90% lymphodepletion after rituximab pre-phase, but not significantly for miR-125b. Moreover, we did not find correlation between miRNAs and the clinical response assessed 3 months after immuno-chemotherapy by FCR. Subsequently, we investigated putative target genes of these three variables associated to the prediction of the lymphodepletion due to rituximab monotherapy. Using software and database available, we identified 3151 common putative target genes for miR-125b and miR-532-3p, representing

respectively over 62% and 50% of common targets for miR-125b and miR532-3p. Interestingly, lack of sequence homology between miR-125b and miR-532-3p (seed or all sequence) does not explain such large number of overlapping putative targets. These two miRNAs rather target distinct regions of the same genes, suggesting a synergistic action of both miRNAs.

Because IL-10-competent regulatory B cells suppress inflammation in both mice and humans, we investigated which genes can be dependent on CD19⁺ IL10⁺ cells. Lin et al. used a microarray analysis of human B cells stimulated or not by CpG and anti-Ig and shown that only ~0.7% of genes are differentially expressed between IL-10⁺ and IL-10⁻ cells.⁴⁵ Among the 104 genes differentially expressed between IL-10⁺ and IL-10⁻ cells, we found over 50% of genes putatively targeted by miR-125b and/or miR-532-3p, among which 26 genes are common for both miRNAs. Pathway enrichment analysis of these 26 overlapping genes identified 9 genes associated with the IL-10 pathway in the rituximab context. Some target genes presented in the Figure 3 are already validated targets for miR-125b. Rossi et al. showed that miR-125b was implicated in T cell differentiation, being upregulated in human naive CD4⁺ T cells as compared with CD4⁺ memory T cells. *In vitro* studies found that miR-125b directly regulated several genes including IL-10 receptor- α (*IL10RA*). Moreover, deletion of the miR-125b-responsive elements in the UTRs *IL10RA* resulted in abrogation of the inhibition effect of miR-125b. These results indicated that miR-125b expression in naive CD4⁺ T cells contributed to the maintenance of cells in this state and thereby suggested that its down regulation was associated with the acquisition of an effector-memory phenotype.⁵⁰ As we have already mentioned, miR-125b was described to repress interferon regulatory factor 4 (IRF4) in B lymphocytes, diffuse large B-cell lymphomas and myeloma cell lines, but also to induce myeloid and B-cell leukemias in mice.^{48,21,52} Ruiz-Lafuente et al. showed an indirect implication of miR-532-3p on TLR7 target gene via an up-regulation of IL-4, in peripheral blood samples from CLL patients. MiR-532-3p induced IL-4 secretion and was induced by IL-4 cytokine itself, which regulates TLR7 expression.^{53,54} The dysregulation of miR-532-3p was also evidenced in Binet A stage CLL patients as compared with normal B-cell subset population. Among the miRNAs tested in relation with clinical data, miR-532-3p was part of miRNAs that were strongly associated with progression-free survival.¹⁰

Analysis showed that miR-125b, miR-532-3p and the frequency of the IL-10-competent B cells were implicated in the efficacy of monotherapy rituximab treatment.

Moreover, these two miRNAs targeted putatively MS4A1, the CD20 gene, and their relative expressions were correlated with CD20 expression on CD19⁺ cells. However, the

frequency of the IL-10-competent B cells or IL-10 plasmatic levels were not correlated with CD20 expression on CD19⁺ cells ($p=0.9678$, $p=0.9983$, respectively). Thus, all of these elements were in favor of an implication of the IL-10 pathway in the mediated inhibitory effects on rituximab efficacy treatment in CLL patients.

5 Our results suggest that miR-125b and miR-532-3p are potential non-invasive circulating biomarkers, detectable in the blood of CLL patients before treatment, which predict rituximab efficacy, and can help the clinician to offer a personalized medicine. To conclude, strategies using miRNAs as companion test should be considered to improve rituximab efficacy.

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Table 1. Patients' characteristics for the experimental arm (Dense-FCR) of the protocol CLL2010FMP. Abbreviation: IQR, interquartile range.

	Dense FCR (n=61)	
	n (%)	Median (IQR)
Age (years)	-	58 (53-61)
Women	17 (28)	-
Binet stage AB	47 (77)	-
ECOG 0	45 (74)	-
<i>IGHV</i> unmutated	36/60 (60)	-
Cytogenetic abnormalities		
Del(13q)	25/48 (52)	-
Del(11q)	12/59 (20)	-
Trisomy 12	4/42 (10)	-
Lymphocyte count (G/L)	-	89 (43-123)
$\beta 2$ microglobulin (mg/L)	59 (97)	3 (2-4)
IL-10 competent B cells	44 (72)	3 (1-10)
<i>FCGR3A</i>	58 (95)	
V/V	6 (10)	-
V/F	29 (50)	-
F/F	23 (40)	-

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Table 2. The *P* value and fold change of 26 differential miRNAs ($P<0.05$).

id	CT values	Fold Change	Pvalues
hsa-miR-29c-000587	19,6	11,15	0,002
hsa-miR-29a-002112	18,9	18,85	0,002
hsa-miR-16-000391	16,6	4,93	0,044
hsa-miR-26a-000405	17,6	6,72	0,031
hsa-miR-184-000485	29,1	8,19	0,041
hsa-miR-99b-000436	26,5	0,18	0,003
hsa-miR-328-000543	22,9	0,25	0,015
hsa-miR-323-3p-002227	31,8	0,32	0,022
hsa-miR-532-3p-002355	22,1	0,44	0,01
hsa-miR-125b-000449	28,6	0,45	0,033
hsa-miR-326-000542	31,3	0,46	0,001
hsa-miR-486-001278	18,9	0,47	0,013
hsa-miR-365-001020	31,6	0,49	0,004
hsa-miR-92a-000431	17,2	0,51	0,001
hsa-miR-193b-002367	25,6	0,52	0,009
hsa-miR-223-002295	14,5	0,54	0,034
hsa-miR-324-3p-002161	22,2	0,55	0,004
hsa-miR-211-000514	28,0	0,58	0,012
hsa-miR-494-002365	28,4	0,58	0,022
hsa-miR-339-5p-002257	23,3	0,65	0,01
hsa-miR-423-5p-002340	22,8	0,71	0,013
hsa-miR-484-001821	17,4	0,71	0,028
hsa-miR-212-000515	26,4	0,78	0,012
hsa-miR-30c-000419	16,6	0,85	0,029
hsa-miR-652-002352	21,2	0,87	0,038
hsa-miR-193a-5p-002281	26,5	0,90	0,02

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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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CLAIMS:

1. A method for predicting whether a subject will achieve a lymphodepletion with an antibody specific for CD20 comprising i) determining the expression level of miR-532-3p or miR-125b in a blood sample obtained from the subject, ii) comparing the expression level determined a step i) with a predetermined reference level and iii) and concluding that the subject will achieve at least 80% of lymphodepletion when the level determined at step i) is lower than the predetermined reference level or concluding that the subject will achieve less than 80% of lymphodepletion when the level determined at step i) is higher than the predetermined expression level.
2. The method of claim 1 wherein the subject suffers from a B-cell malignancy.
3. The method of claim 1 wherein the subject suffers from a B-cell malignancy selected from the group consisting of non-Hodgkin's lymphoma, Burkitt's lymphoma, small lymphocytic lymphoma, primary effusion lymphoma, diffuse large B-cell lymphoma, splenic marginal zone lymphoma, MALT (mucosa-associated lymphoid tissue) lymphoma, hairy cell leukemia, chronic lymphocytic leukemia, B-cell prolymphocytic leukemia, B cell lymphomas (e.g. various forms of Hodgkin's disease, B cell non-Hodgkin's lymphoma (NHL) and related lymphomas (e.g. Waldenstrom's macroglobulinaemia (also called lymphoplasmacytic lymphoma or immunocytoma) or central nervous system lymphomas), leukemias (e.g. acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL; also termed B cell chronic lymphocytic leukemia BCLL), hairy cell leukemia and chronic myeloblastic leukemia), myelomas (e.g. multiple myeloma), small lymphocytic lymphoma, B cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B cell lymphoma, mediastinal (thymic) large B cell lymphoma, intravascular large B cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, grey zone lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder.

4. The method of claim 1 wherein the subject suffers from an inflammatory disorder.
5. The method of claim 1 wherein the subject suffers from an inflammatory disorder selected from the group consisting of ulcerative colitis, Crohn's disease, inflammatory bowel disease, rheumatoid arthritis, myositis, multiple sclerosis, neuromyelitis optica,
5 atherosclerosis, psoriasis, systemic lupus erythematosus (e.g., lupus of the central nervous system or lupus nephritis), nephritis, glomerulonephritis, autoimmune hepatobiliary disease (e.g., autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis), graft-versus-host disease, atopic dermatitis, asthma, neurodegenerative disease (e.g., Alzheimer's disease), demyelinating
10 polyradiculopathy (e.g., Guillain-Barre syndrome or chronic inflammatory demyelinating polyradiculopathy), neuropathic pain, visceral pain of cancer, atherosclerosis, age-related macular degeneration, diabetic nephropathy, sarcoidosis-originated uveitis, and diabetes mellitus.
6. The method of claim 1 for predicting that the patient will achieve at least 80%, 90% or
15 95% of lymphodepletion after 3 weeks of treatment with the antibody specific for CD20 when the level determined at step i) is lower than the predetermined reference level.
7. The method of claim 1 wherein the antibody specific for CD20 is rituxan.
8. The method of claim 1 wherein the antibody specific for CD20 is an antibody which
20 cross-competes with rituxan.
9. The method of claim 1 wherein the expression levels of miR-532-3p and miR-125b are determined in the blood sample.
10. A method of treating a B-cell malignancy or an inflammatory disorder in a subject in need thereof comprising i) predicting whether a subject will achieve a
25 lymphodepletion with an antibody specific for CD20 by performing the method of claim 1 and ii) administering to the subject a therapeutically effective amount of an antibody specific for CD20 when it is concluded that the subject will achieve a lymphodepletion.

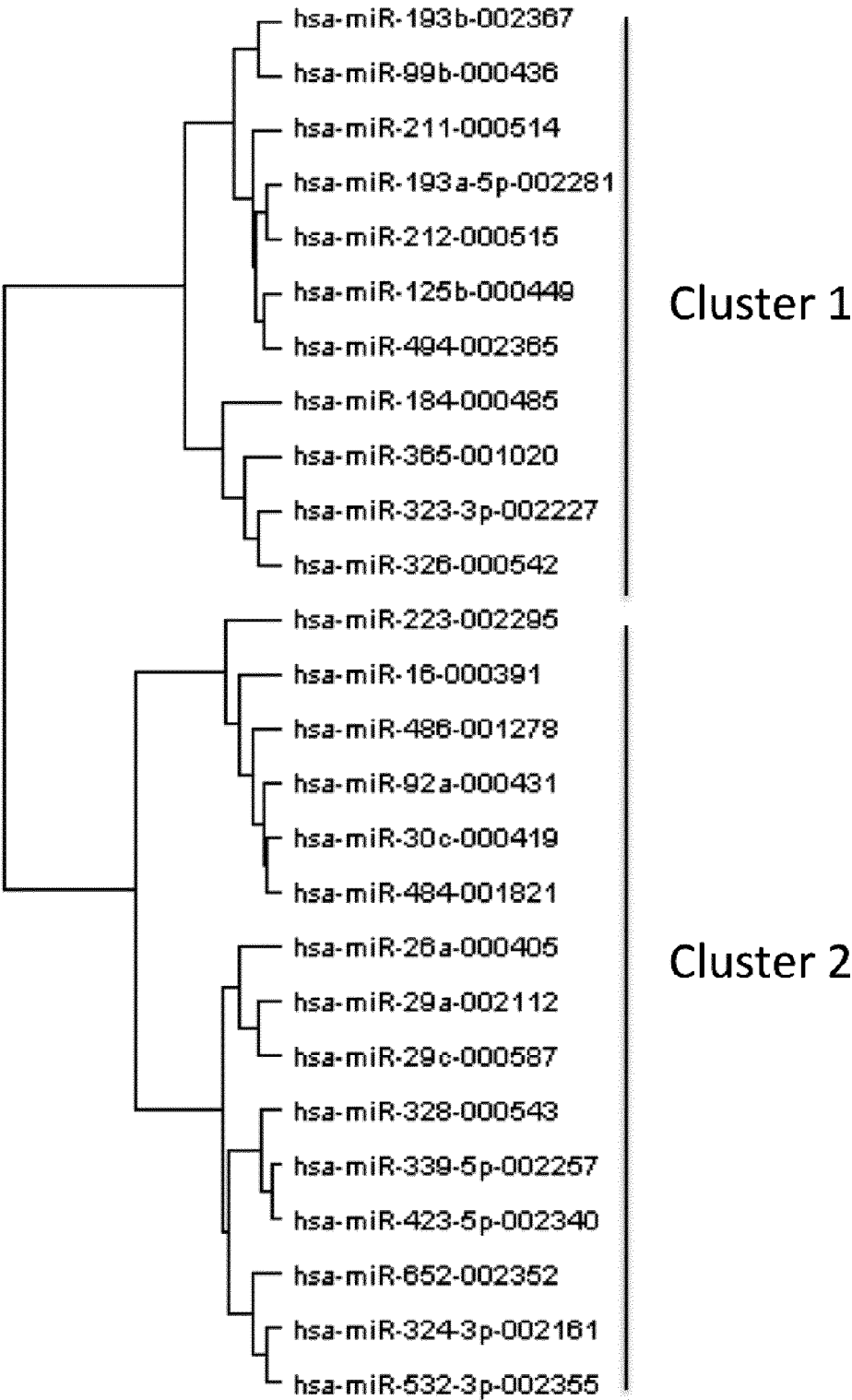


Figure 1A

B

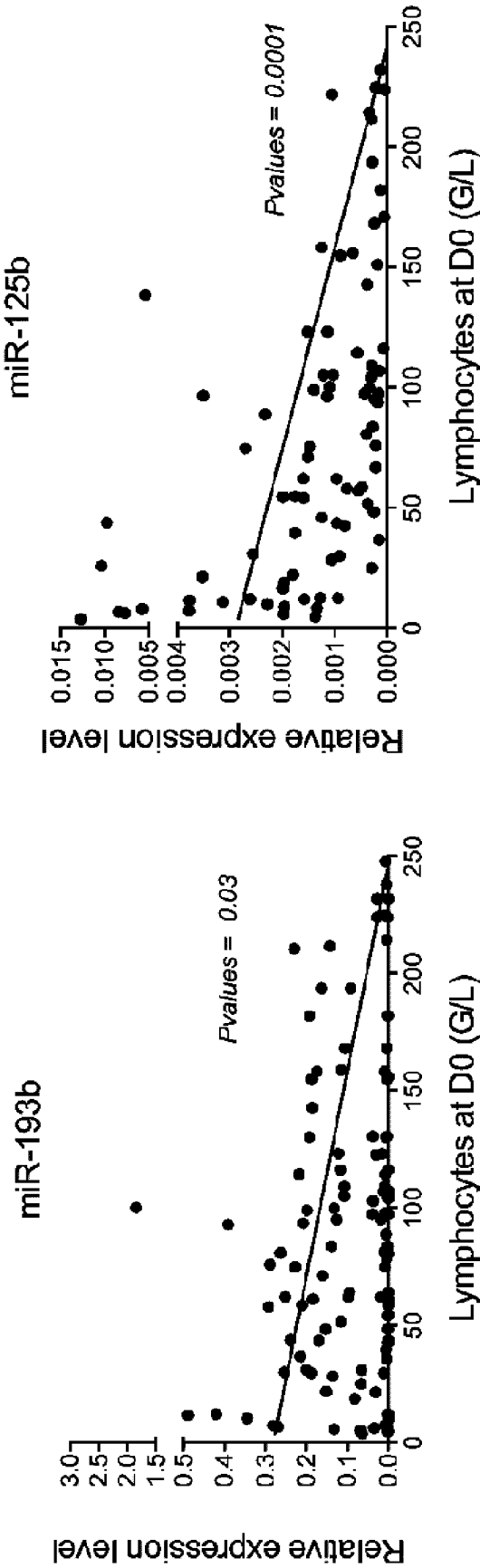


Figure 1B

C

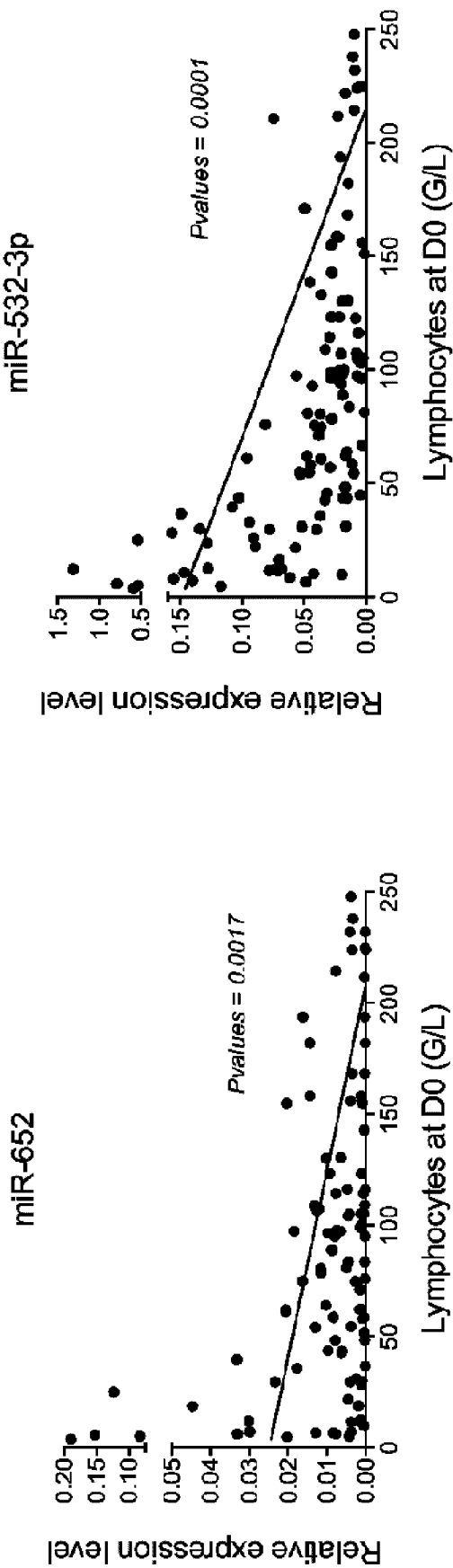
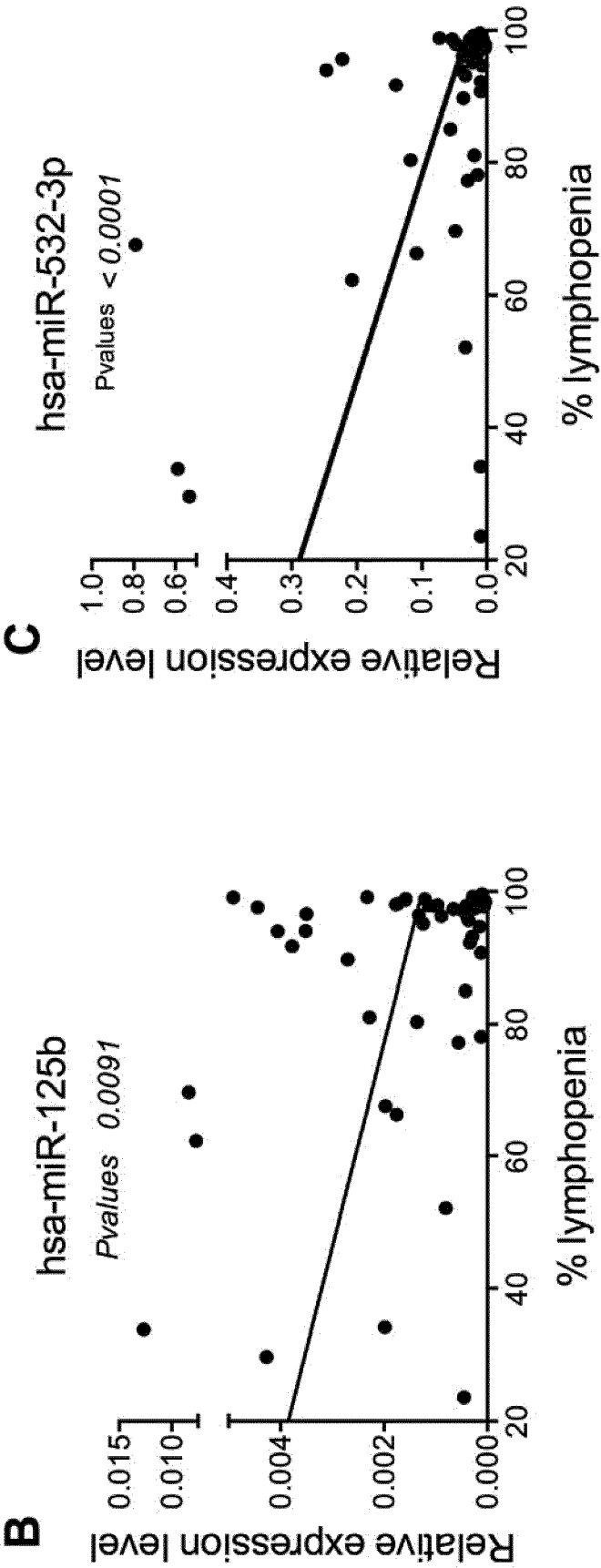


Figure 1C

A

Lymphodepletion (D22-D0)				
Variables	95%	90%	85%	80%
miR-125b	0.0203	0.0107	0.0157	0.0394
miR-193b	ns	ns	ns	ns
miR-532-3p	0.0024	0.0021	0.0174	0.0295
miR-652	0.0195	0.0445	ns	ns

Figure 2A



Figures 2B & 2C

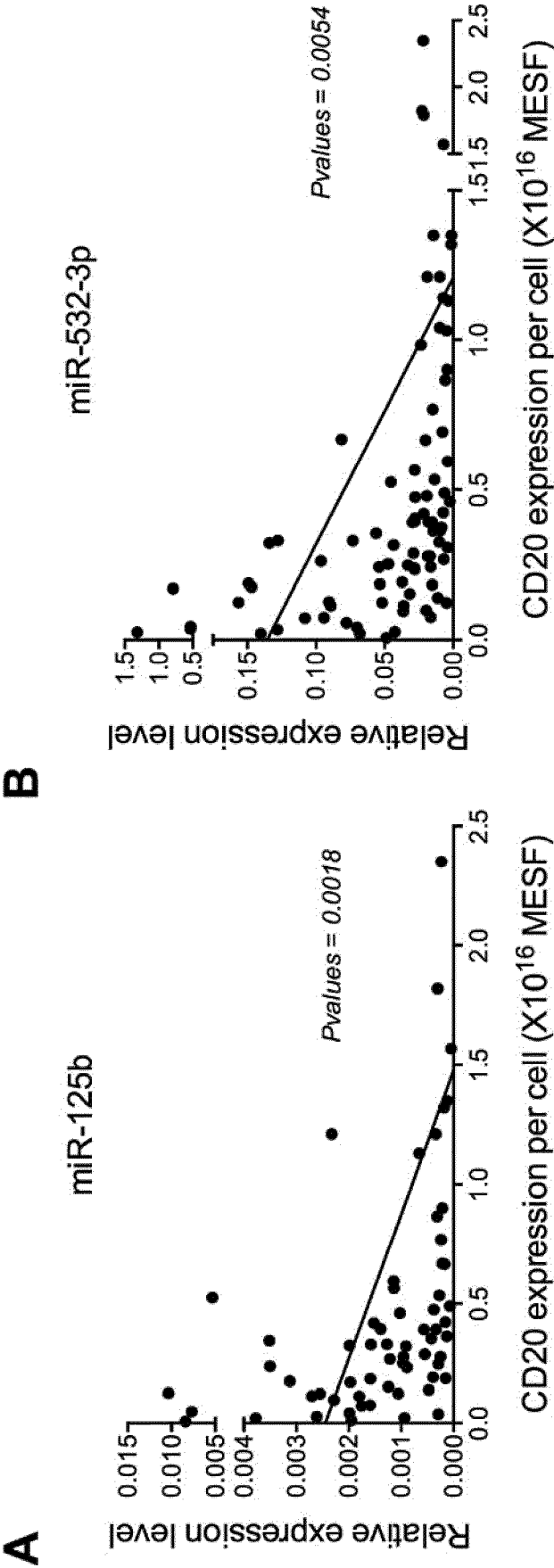


Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/053947

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ISABELLE DUROUX-RICHARD ET AL: "Circulating miRNA-125b Is a Potential Biomarker Predicting Response to Rituximab in Rheumatoid Arthritis", MEDIATORS OF INFLAMMATION., vol. 17, no. 4, 1 January 2014 (2014-01-01), pages 1244-9, XP055288185, GB ISSN: 0962-9351, DOI: 10.1002/art.30233 the whole document	1-10
X	----- WO 2010/133562 A1 (INST NAT SANTE RECH MED [FR]; APPARAILLY FLORENCE [FR]; RICHARD ISABEL) 25 November 2010 (2010-11-25) the whole document ----- -/--	1-10



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

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"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

24 March 2017

Date of mailing of the international search report

31/03/2017

Name and mailing address of the ISA/

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Authorized officer

Cornelis, Karen

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/053947

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/135459 A2 (MEDICAL PROGNOSIS INST A S [DK]; KNUDSEN STEEN [DK]) 3 November 2011 (2011-11-03) claims 17,18	1-10
X	----- W. WANG ET AL: "MicroRNA profiling of follicular lymphoma identifies microRNAs related to cell proliferation and tumor response", HAEMATOLOGICA, THE HEMATOLOGY JOURNAL : OFFICIAL ORGAN OF THE EUROPEAN HEMATOLOGY ASSOCIATION, vol. 97, no. 4, 1 April 2012 (2012-04-01), pages 586-594, XP055288519, IT ISSN: 0390-6078, DOI: 10.3324/haematol.2011.048132 the whole document	1-10
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/053947

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WO 2011135459 A2	03-11-2011	AU 2011246976 A1 CA 2800557 A1 EP 2563936 A2 US 2013053275 A1 WO 2011135459 A2	08-11-2012 03-11-2011 06-03-2013 28-02-2013 03-11-2011