(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 September 2011 (15.09.2011)

PCT

(10) International Publication Number
WO 2011/110644 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/EP2011/053649

(22) International Filing Date:
10 March 2011 (10.03.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/313,059 11 March 2010 (11.03.2010) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

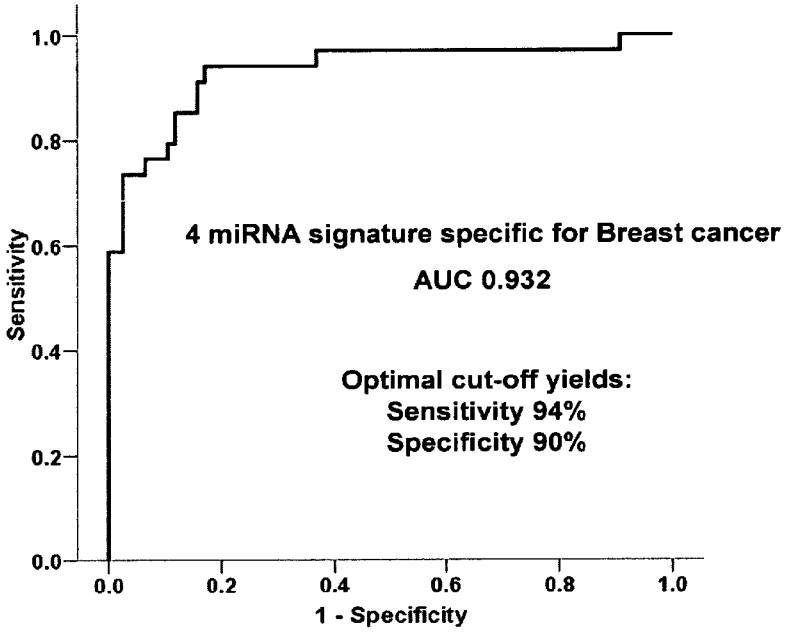
Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) Title: DETECTION AND QUANTIFICATION OF MICRORNAS IN THE CIRCULATION AND THE USE OF CIRCULATING MICRORNAS AS BIOMARKERS FOR CANCER

Figure 9



(57) Abstract: The present invention relates to the identification of circulating miRNAs as biomarkers suitable for use in the diagnosis and prognosis of a number of cancers, in particular breast cancer. In addition, the invention relates to improved methods for the identification and quantification of such biomarkers in samples taken from patients.

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Title

Detection and quantification of microRNAs in the circulation and the use of circulating microRNAs as biomarkers for cancer

Field of the Invention

The present invention relates to the identification of biomarkers suitable for use in the diagnosis and prognosis of a number of cancers. In addition, the invention relates to improved methods for the identification and quantification of such biomarkers in samples taken from patients.

Background to the Invention

Mi(cro)RNAs are short RNA molecules that regulate gene expression across a wide spectrum of biological and pathological processes. The discovery that mi(cro)RNA expression is frequently dysregulated in many disease processes has uncovered a new repertoire of molecular factors upstream of gene expression, which play critical regulatory roles in various cellular processes. In relation to cancer, aberrant miRNA expression has been shown to promote tumourigenesis, metastasis, and associate with other tumor characteristics. The finding that miRNA expression profiles have the capacity to accurately classify tumours according to existing clinicopathological variables has highlighted their potential as reliable prognostic indicators and cancer biomarkers.

The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia [1]. Loss of miR-15a and miR-16-1 from this locus results in increased expression of the antiapoptotic gene BCL2. Intensifying research in this field, using a range of techniques including miRNA cloning, quantitative PCR, microarrays and bead-based flow cytometric miRNA expression profiling, has resulted in the identification and confirmation of abnormal miRNA expression in almost every human malignancy including breast cancer (Table 1 [2]).

Table 1. MiRNAs with altered expression in malignancy

Tissue/tumor type	Increased expression	Decreased expression
Breast	miR-21, miR-29b-2	miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b
Ovarian	miR-141, miR-200(a-c), miR-221	let-7f, miR-140, miR-145, miR199a, miR-424
Endometrial	miR-103, miR-107, miR-185, miR-205, miR-210, miR-449	miR-99b, miR-152, miR-193, miR-204, miR-221, let-7i
Glioblastoma	miR-221, miR-21	miR-181a, miR-181b, miR-181c
Chronic lymphocytic leukemia		miR-15, miR-16
Lymphoma	miR-155, miR-17-92cluster	miR-15a
Colorectal	miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31	miR-143, miR-145, let-7
Thyroid	miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346	
Hepatocellular	miR-18, miR-224	miR-199a, miR-195, miR-200a, miR-125a
Testicular	miR-372, miR-373	
Pancreatic	miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1	miR-375
Cholangiocarcinoma	miR-21, miR-141, miR-200b	
Prostate	let-7d, miR-195, miR-203	miR-128a
Gastric	miR-223, miR-21, miR-103-2	miR-218-2
Lung	mir-17-92 cluster, miR-17-5p	let-7 family

MiRNA expression has been observed to be up-regulated or down-regulated in tumours compared with normal tissue, supporting their dual role in carcinogenesis as either 'oncomirs' or tumour suppressors respectively. The focus of miRNA research to date has been at tumour tissue level; however recent reports on the detection of these molecules in the circulation has generated significant interest in the concept that systemic microRNAs hold potential as novel minimally invasive biomarkers for cancer and other disease processes. Methods of extracting miRNAs from the circulation, and subsequent quantification of systemic miRNA levels, are ill-defined. The techniques are variable and

difficult to reproduce. Several questions permeate this field – which circulating medium is preferable for systemic miRNA investigations, which purification technique retrieves superior concentration of quality miRNA from blood, which concentration of miRNA is optimal for RQ-PCR analysis and which endogenous controls are appropriate for circulating miRNA studies.

The purpose of this investigation was to define a protocol for optimal extraction, quantification and analysis of miRNA expression in human blood samples. This was achieved using a breast cancer case-control cohort, to compare different methods for the extraction of microRNA from blood samples.

Current challenges in the management of breast cancer include a continuing search for sensitive and specific minimally invasive biomarkers that can be exploited to detect early neoplastic changes, thus facilitating the detection of breast cancer at an early stage, as well as for monitoring the progress of patients with breast cancer and their response to treatments. Existing diagnostic tools and biomarkers for breast cancer have many inherent deficiencies. Mammography is currently the gold standard diagnostic tool however it is not without limitations, including its use of ionizing radiation and a false negative rate of 8-10%. To date, only two markers are established in the routine evaluation of breast tumors: ER (for predicting response to endocrine therapies) and HER2/*neu* (for predicting response to Trastuzumab) [3]. Although these markers are assessed routinely, ER and HER2/*neu* assessment is far from perfect. A number of circulating tumor markers (e.g., carcinoembryonic antigen [CEA] and carbohydrate antigen 15-3 [CA 15-3]) are widely used in the management of breast cancer, but the sensitivity of these markers is low, and so they are not useful as screening tools although they have long been in clinical use as prognostic markers and to monitor for disease progression or recurrence [4-6].

MiRNA expression studies in breast cancer indicate their importance and potential use as disease classifiers and prognostic tools in this field. A relevant and important feature of miRNAs is their remarkable stability. They are known to be well preserved in tissue samples even after years of formalin-fixation and paraffin-embedding, and can be efficiently extracted from and quantified in such specimens [7]. Investigation of cancer-specific miRNAs in the circulation is an emerging and exciting field of study. It is hypothesized that if miRNAs are present in the circulation of cancer patients, their unique stability and resilience should allow their detection and quantification to be practicable. The first report of circulating miRNAs, by Lawrie et al, described elevated serum levels of

miR-21 in patients with diffuse large B-cell lymphoma [8]. Subsequently, circulating miRNAs have been postulated as novel biomarkers for cancer, and other disease processes. However this concept needs investigation to validate the theory. To date there has been no report on the role of circulating miRNAs in breast cancer.

The primary aim of our study was to investigate whether cancer specific miRNAs are detectable and altered in the circulation of breast cancer patients compared to age matched healthy controls, and if so, whether significantly altered systemic miRNAs reflected the tumor miRNA expression profile. We also aimed to identify the circulating medium which best represented miRNA levels (serum, plasma or whole blood).

Previous studies, although few in number, have reported discrepancies between serum and plasma miRNA expression levels, or the investigators have chosen either medium alone for use in their studies [9-11]. Finally a potential relationship between circulating miRNA levels and existing clinicopathological features of breast cancer such as tumor subtype, stage of disease, nodal status or hormone receptor status, was investigated.

Object of the Invention

A first object of the invention is to provide a simple protocol for the extraction and quantification of microRNAs in the circulation. In particular it is an aim to provide an optimal extraction technique, which is most effective on whole blood specimens. A further object is to provide novel biomarkers for the detection of breast cancers. The ideal biomarker should be easily accessible such that it can be sampled relatively non-invasively, sensitive enough to detect early presence of tumors in almost all patients and absent or minimal in healthy tumor free individuals. A further object is that the biomarker be capable of indicating the presence of early stage breast cancers.

Summary of the Invention

According to the present invention there is provided a diagnostic kit to detect cancers including breast cancer, diabetes, cardiovascular disease, hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, or to stratify patients according to expected prognosis comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-16 (as endogenous control), miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, or miR-375 biomarkers .

A kit to detect cancers including breast cancer may comprise at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c or let-7a biomarkers. The circulating miRNA may be selected from the group comprising miR-16, miR-195, miR342, miR181c and let-7a biomarkers. Such markers a particularly useful as markers for early breast cancer. In particular embodiments the circulating miRNA may be miRNA-195 or let7a biomarkers. A kit for the detection of cancers including breast cancer may comprise at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-342 or miR-181c biomarkers.

A kit to detect or stratify metabolic diseases may comprise oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-145, miR-375.

The kit may be adapted for performance of an assay selected from a real-time PCR assay, a micro-array assay, a histochemical assay or an immunological assay. For LRG assays cytochrome C may be used as a capturing ligand for building an ELISA. All such assays are well known to those of skill in the art. Where the assay is a histochemical assay, the antibody may be labelled with a suitable label. Suitable labels include coloured labels, fluorescent labels and radioactive labels.

The kit may be adapted to carry out a multiplex assay, in which a number of miRNAs are detected. The multiplex assay may be adapted to detect the miR-16 (as endogenous control), miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, and miR-375 biomarkers .

Alternatively the assay may be for the miR-16, miR-195, miR342, miR181c and let-7a markers. In another embodiment the assay may detect the miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c and let-7a biomarkers. In an alternative embodiment the multiplex assay may be for the detection of the miRNA-195 or let7a markers, or for the miR-342 or miR-181c markers. In still another embodiment the multiplex assay may be for the determination of the miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-145, miR-375 markers.

The kit is capable of detecting breast cancer, even in its earliest stage. The kit allows one to obtain prognostic information on the patient from their blood miRNA analysis – this information is currently obtained from the patient's clinical and/or pathological details, for example the size and grade of their tumour, hormone receptor status, presence of nodal or distant metastases. This information is then used to guide further treatment regimens. However the current methods of prognostication and stratification of breast cancers are far from perfect, so the miRNA blood test of the invention has the potential to improve the current system and be more accurate and specific in determining the patient's prognosis, and guiding adjuvant treatment regimens.

This novel diagnostic kit has potential for the following clinical applications:

(i) Breast screening: to identify changes in blood miRNA levels in cancer patients compared to normal cancer-free individuals, in an asymptomatic group.

(ii) Prognostication and prediction of outcome. This kit could identify differences between patients who present with early or late stage cancers, as well as stratify patients into molecular subtypes. This information can then aid in strategic planning of an individual patients therapeutic regimen.

(iii) Monitoring of response to treatments, through serial blood miRNA measurements using this kit; particularly in the neoadjuvant chemotherapy and metastatic disease settings.

The miRNAs identified and incorporated into this kit may also serve as novel therapeutic targets for breast cancer. The invention further provides a method of identifying a therapeutic agent capable of preventing or treating cancers including breast cancer, diabetes, cardiovascular disease, hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, comprising testing the ability of the potential therapeutic agent to reduce or enhance the expression of at least one protein selected from the group comprising miR-16 (as endogenous control), miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, or miR-375 biomarkers.

In another aspect the invention provides use of a circulating miRNA selected from the group comprising comprising miR-16 (as endogenous control), miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143 or miR-375 biomarkers to detect cancers including breast cancer, diabetes, cardiovascular disease,

hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, or to stratify patients according to expected prognosis.

The detection may be carried out on a blood sample or a sample derived from blood. The kit may be adapted for performance of an assay selected from a real-time PCR assay, a micro-array assay, a histochemical assay or an immunological assay. For LRG assays cytochrome C may be used as a capturing ligand for building an ELISA. All such assays are well known to those of skill in the art. Where the assay is a histochemical assay, the antibody may be labelled with a suitable label. Suitable labels include coloured labels, fluorescent labels and radioactive labels.

The invention also provides a method of detecting or screening for early stage breast cancers, comprising analysing a sample of blood taken from a patient for the presence of one or more biomarkers selected from the group comprising miR-16 (as endogenous control), miR-195, miR-342, miR-181c or let-7a, the increased expression of at least one of these miRNAs in the sample indicating the presence of breast cancer.

Also provided is a method of detecting small RNAs (that is molecules of less than about 200 nucleotides) in the circulation comprising:-

(i) Treating whole blood with TRIzol reagent at a ratio of one part blood to three parts Trizol reagent,

(ii) Adding 1-bromo-4-methoxybenzyene (also known as bromoanisole,

(iii) Centrifuging the mixture at at least 12000g for at least 5 min at less than 10°C

(iv) Precipitating RNA from the aqueous phase

(v) quantifying the miRNAs precipatated.

The ratio of 1-bromo-4-methoxybenzyene to blood or blood –derived sample is suitably in the range of 0.2 - 0.8ml 1-bromo-4-methoxybenzyene to 1ml blood or blood-derived sample. Preferably 0.2 ml 1-bromo-4-methoxybenzyene to 1ml blood or blood-derived sample is used.

It is important to separate phases at a cool temperature (4-10°C] – centrifugation performed at elevated temperatures may sequester DNA into the aqueous phase. Thus preferably the mixture is centrifuged at 4°C for 15 min.

Optionally, following the addition of 1-bromo-4-methoxybenzyene, 8 - 32 microlitres of polyacryl carrier may be added to the blood/trizol/1-bromo-4-methoxybenzyene solution. This augments visualization of the RNA pellet once it is precipitated.

Any RNA precipitation method could be used (e.g. Sodium acetate, Isopropanol, etc) as would be known to those skilled in the art. Particularly preferred is use of one part Isopropanol to one part aqueous RNA solution, centrifuged for 8 minutes at 12,000g, at a higher temp of 18 degree Celsius.

The precipitated RNA may then be washed with ethanol, preferably twice. The second wash with ethanol does improve the purity of the RNA isolated.

Quantification of miRNAs may be carried out via NanoDrop® spectrophotometry set at a conversion factor of 33µg/ml or equivalent spectrophotometry, or Agilent quantification method.

The method may further comprise synthesising cDNA on 1-1000ng of small RNA, as quantified by the method described above. The small RNA is reverse transcribed using stem loop RT primers, specific for each miRNA target and diluted with nuclease –free water to give 50µM concentration per reaction, according to standard terms and conditions. The DNA may then be stored at about –20°C.

The miRNA expression levels may be quantified (relative quantification) by real-time PCR, using the expression level of miR-16 and/or another stably expressed small RNA(s) to normalise the expression level of the target miRNA. All reactions may be performed in triplicate and using an interassay control. The data may be analysed using $2^{-}\Delta\Delta CT$ to determine relative quantities of the target miRNA.

The kits, assays and methods of the invention may comprise determining the level of at least 2 miRNA biomarkers from the group, or at least 3 biomarkers, or at least 4 biomarkers, or at least 5 biomarkers, or at least 6 biomarkers from the group. In other embodiments the invention provides determining the level of all biomarkers from the group, or at least 10 biomarkers or at least 12 biomarkers from the group.

A kit to be applied to the detection of metabolic diseases would require quantification of miRNAs which represent biomarkers of metabolic diseases including miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-145, miR-375.

The development of a miRNA primer/probe signature panel to rapidly test blood samples for expression levels of candidate metabolic miRNAs allows the kit to be used to predict obese patients who are at high risk of developing the metabolic syndrome, those who would benefit most from bariatric surgery as well as blood lipid and glucose levels. This in turn allows the development of therapeutic strategies using miRNA

replacement or antagonism for the treatment of obesity and the metabolic syndrome. In particular, replacement of *miR-17-5p* in obese patients may have the potential to restore catabolic activity and thus aid in weight loss. Similarly, replacement of miR-143 in obese diabetic patients may revert their glycaemic indices to normal, and thus cure obese (and possibly non-obese) patients of diabetes.

A kit to be applied to the detection of cancers would require quantification of miRNAs which represent biomarkers of miR-16 (as endogenous control), miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c or let-7a.

Circulating miR-195 is a marker for early stage breast cancer; increased miR-195 levels in blood are observed in breast cancer patients. More significantly increased circulating miR-195 levels are a marker of more advanced breast cancers (compared with early stage tumours) so circulating miR-195 levels may be an indicator of stage of disease. MiR-195 in combination with let-7a, miR-342 and miR-181c may be an indicator of subtype of breast cancer, although alone, miR-195 does not indicate subtype of breast cancer. Circulating miR-195 levels decreased 2 weeks following tumour resection, therefore circulating miR-195 may be a marker for determining complete surgical resection/response to therapy/ recurrence of disease. Thus an inhibitor of miR-195 may also have a therapeutic application.

Circulating let-7a is a marker for early stage breast cancer as increased levels of this marker are seen in circulation of breast cancer patients. Circulating let-7a is a marker for pre-invasive breast cancer, which is the earliest stage of breast cancer. The present inventors have found that let-7a, one of the most well established and defined cancer associated miRNAs, was significantly elevated in the circulation of patients with several visceral malignancies (breast, prostate, colon and renal cancers) compared to controls. The findings are in keeping with existing evidence which supports let-7a as a protagonist in many cancers, particularly lung, breast, colon, gastric and ovarian. However the present study observed a paradoxical effect in the circulation (i.e. a significant increase in systemic let-7a levels in cancer patients compared to controls), to that described previously at tumour tissue level where let-7a is most commonly found to be under-expressed in tumour tissue compared to normal tissue, for these individual cancers.

Circulating miR-21 & miR-10b are increased in oestrogen-receptor negative disease so these have potential as markers for a subtype of disease, in addition to measuring miR-

195, let-7a, miR-342 and mir-181c levels. Circulating miR-21 serves as a marker for disease progression, and advanced or aggressive disease.

Brief Description of the Drawings

Figure 1: *MiR-16* expression in whole blood samples of 127 subjects: 83 samples were from women with histologically confirmed breast cancer and from whom the blood sample was taken preoperatively, whilst 44 samples were from disease free and age matched control females. This illustrates the finding that miR-16 is stably expressed across all analyzed blood samples and thus is a suitable endogenous control.

Figure 2: Phase separation of whole blood using Tri Reagent® BD copurification protocol. Following centrifugation of a homogeneous mixture of blood (1ml, unclotted), Trizol (3ml) and Bromoanisole (200µl), the mixture separates into 3 phases: the upper clear aqueous phase containing RNA exclusively, a DNA interphase, and the lower organic phase containing protein and lipids.

Figure 3: Expression levels of *miR-195* (a) and *let-7a* (b) in preoperative (n=83) and postoperative (n=29) blood samples from breast cancer patients and controls (n=44). At two weeks postoperatively a significant decrease in mean circulating *miR-195* and *let-7a* levels was observed, reaching levels comparable with control subjects.

Figure 4: *MiR-195* expression in breast cancer tissues (n=65), tumor associated normal (n=18), preoperative invasive breast cancer blood samples (n=73) and healthy control bloods (n=44). *Mir-195* expression in tumor tissue differed significantly to TAN ($p<0.001$) and similarly its expression in blood from breast cancer patients differed significantly to healthy control blood ($p < 0.001$).

Figure 5: *MiR-195* expression according to stage of disease, in invasive breast cancer tissue specimens (n=65) and invasive breast cancer preoperative blood sample (n=73) and healthy control blood (n=44). Tumor *miR-195* expression was significantly higher in Stage IV compared to Stages I & II tumors ($p= 0.006$ and 0.039 respectively ANOVA and Tukey post hoc analysis). Whilst there was no significant difference between stage of disease and *miR-195* expression in blood from breast cancer patients, there was a similar trend for increasing *miR-195* levels with advancing stage of disease.

Figure 6: Correlation of *miR-195* levels in blood (n=65) and tumor tissue (n=65) from stage-matched invasive breast cancer patients. [Stage 1 (n=13), Stage 2 (n=29), Stage 3 (n=17), Stage 4 (n= 6)]. There was no significant correlation between blood and tumor

miR-195 levels, for stages of disease. (Pearson's correlation coefficient r = 0.126, p=0.323).

Figure 7: *MiR-195* expression in invasive breast cancer tissue (n=65), according to HER2/*neu* status. HER2/*neu* positive tumors (n=20) expressed higher *miR-195* than HER2/*neu* negative tumors (n=45), p=0.002.

Figure 8: Decreased circulating levels of miR-195 in 2-week post-operative blood samples from 29 patients. The box-plot indicates pre-operative miR-195 levels which dropped to levels comparable with the control group following curative tumour resection.

Figure 9: miRNA signature of *miR-195, miR-181c, miR-342* and *let-7a* which are specific for breast cancer.

Detailed Description of the Drawings

Methods

Blood collection: Venous blood samples (non-fasting) were collected as follows: from each participant, whole blood was collected in two Vacuette EDTA K3E blood bottles (Grenier Bio-one); one processed for plasma and the other unprocessed; and a third sample was collected in Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum. Upon obtaining, samples for serum collection were allowed to clot for 30 min and then all samples destined for serum and plasma collection were centrifuged at 2000 rpm @ 4°C for 10 minutes in a Sorvall RT6000D centrifuge. Plasma/serum was removed, aliquoted and stored at -20°C until required. The unprocessed whole blood sample was stored at 4°C until required.

RNA isolation:

Protocol 1.

Separate purification of miRNA and larger RNAs (>200nt) using column based method: Micro RNA was extracted from whole blood, serum and plasma samples as follows: In brief 250µl of thawed serum/plasma was mixed with 1ml QIAzol® lysis reagent and 250µl of Chloroform, and then centrifuged at 12000g for 15 min at 4°C. Large RNA fractions (> 200 nucleotides) and small RNA fractions (< 200 nucleotides) were isolated separately using the QIAGEN RNeasy Minikits and the protocol supplied by the manufacturer.

Protocol 2. Copurification of total RNA:

Total RNA was extracted from whole blood, serum and plasma samples using a modification of the Tri Reagent® BD (Molecular Research Centre, Inc., Cincinnati, OH)

copurification protocol, as follows: Using 1ml of whole blood, or its derivatives, phase separation was performed by the addition of 3 ml of TRIzol® reagent (Invitrogen, Carlsbad, CA) and 200 microlitres of 1-bromo-4-methoxybenzene to augment the RNA phase separation process (Figure 2). The homogenous blood/Trizol mixture was split between two 2ml collection tubes and then centrifuged at 12000g for 15 min, at 4 °Celsius. The clear aqueous phase (approximately 1ml) from each tube was then removed, transferred to fresh collection tubes respectively and RNA precipitated by the addition of 1ml of Isopropanol and centrifugation of the solution at 12000g for 8min at 18°C. Following removal of the supernatant, the RNA pellet was then washed with 1ml of 75% ethanol. We performed an additional ethanol wash to improve the purity of RNA isolated, which was reflected in an improved 260/280 ratio. Each RNA pellet was briefly air dried and then solubilised using 30 microlitres of nuclease free water. Hence each 1ml of whole blood yielded 60 microlitres of total RNA when the two matched RNA pellets were solubilised, mixed together again, and finally transferred to storage tubes prior to storage at -80 °Celsius.

RNA isolation

Total RNA was extracted from 1ml of blood/serum/plasma respectively using TRI Reagent® BD (Molecular Research Centre, Inc., USA). RNA was extracted from breast tissue as described above. RNA concentration and integrity were determined using by NanoDrop spectrophotometry (NanoDrop® ND-1000 Technologies Inc, DE, USA) and an Agilent Bioanalyzer (Agilent Technologies, Germany), respectively.

Assessing concentration and integrity of RNA

RNA concentration was determined using the Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). The wavelength-dependent extinction coefficient '33' was taken to represent the micro component of the RNA solution. In general we obtained concentrations ranging between 30-300 nanograms per microlitre of miRNA. Integrity was assessed using the RNA 6000 Nano LabChip Series II Assay with the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Analysis of miRNA gene expression

We chose to study a panel of nine miRNAs in the circulation of breast cancer patients (miR-10b, miR-21, miR-145, miR-155, miR-195, miR-181c, miR-342, let 7a, and miR-16). These were chosen for their potential as breast cancer biomarkers based on their

documented association with breast cancer in existing literature, and from ongoing work in our Department of Surgery investigating the role of microRNAs in breast cancer. RQ-PCR quantification of miRNA expression was performed using TaqMan MicroRNA® Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Total RNA (100 ng) was reverse-transcribed using the MultiScribe™-based High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). RT-negative controls were included in each batch of reactions. PCR reactions were carried out in final volumes of 10 µl using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, reactions consisted of 0.7 µl cDNA, 5 µl TaqMan® Universal PCR Fast Master Mix, 0.2 µM TaqMan® primer–probe mix (Applied Biosystems, Foster City, CA, USA). Reactions were initiated with 10-minute incubation at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. An interassay control derived from a breast cancer cell line (ZR-75-1) was included on each plate and all reactions were performed in triplicate. *MiR-16* was used as endogenous controls to standardize miRNA expression. The threshold standard deviation for intra-assay and inter-assay replicates was 0.3. The percentage PCR amplification efficiencies (*E*) for each assay were calculated, using the slope of the semi-log regression plot of cycle threshold versus log input of cDNA (10-fold dilution series of five points), with the following equation:

$$E = (10 - 1 / \text{slope} - 1) \times 100$$

A threshold of 10% above or below 100% efficiency was applied. The relative quantity of miRNA expression was calculated using the comparative cycle threshold (ΔΔCt) method [12], normalised to miR-16 levels, and the lowest expressed sample was used as a calibrator.

Study cohort

Following ethical approval and written informed consent, blood samples (whole blood, serum and plasma) were collected prospectively from 127 females, including 83 consecutive breast cancer patients and 44 healthy age-matched female volunteers who served as controls for this study. All patients had histologically confirmed pre-invasive or invasive breast cancer and their relevant demographic and clinicopathological details were obtained from our prospectively maintained breast cancer database. The histological tumor profile of patients in this study reflects that of a typical breast cancer cohort, with the majority of invasive tumors being of ductal type, and Luminal A

epithelial subtype (Table 1). In addition, repeat blood samples were collected from a subset of this cohort, at their initial clinical review two weeks postoperatively (n=29). The control blood samples were collected from healthy women with no current or previous malignancy, or inflammatory condition. A similar cohort of age and stage-matched breast cancer patients (n=65) were identified from whom tumor and tumor associated normal (TAN) tissues were prospectively collected (Table 1).

MiRNA targets

The expression of a panel of 9 cancer associated miRNAs was chosen on the basis of their reported relevance to breast cancer (Table 2) [10 13-16].

Statistical Analysis

Data were analysed using the software package SPSS 17.0 for Windows. Due to the magnitude and range of relative miRNA expression levels observed, results data were log transformed for analysis. Data are presented as Mean ± SD. There was no evidence against Normality for the log transformed data as confirmed using the Kolmogorov-Smirnov test. The two-sample t-test was used for all two sample comparisons and ANOVA, followed by Tukey HSD Post Hoc test, to compare the mean response between the levels of the between subject factors of interest. All tests were two tailed and results with a $p<0.05$ were considered statistically significant.

Table 2. Candidate miRNAs for investigation in the circulation of breast cancer patients.

miRNA of interest	Previous association with breast cancer
miR-10b	Decreased expression in breast tumour tissue compared to normal breast tissue
miR-21	Increased expression in breast tumour tissue compared to normal breast tissue. Also increased in other solid cancers: colorectal, pancreas, gastric, lymphomas
miR-145	Decreased expression in breast tumour tissue compared to normal breast tissue
miR-155	Increased expression in breast tumour tissue compared to normal breast tissue
miR-195	Reported by *Mattie et al* to be associated with hormone receptor status, as part of a 'miRNA signature'
let 7a	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *miR-16*
miR-16	Reliable endogenous controls for analysis of miRNA by RQ-PCR in human breast tissue, with *let-7a* and used as a single endogenous control for investigating serum miRNA levels in recent studies.
miR-342	Increased expression in breast tumour tissue compared to normal breast tissue, and expression correlates with hormone receptor status (ER)

miR-181c	Increased expression in breast tumour tissue compared to normal breast tissue and expression correlates with hormone receptor status (ER)

Results

Comparison of techniques: Separate miRNA purification vs. Total RNA copurification

The standard protocol for isolation of miRNA from whole blood, serum and plasma, using QIAGEN RNeasy Minikits, yielded low concentrations of miRNA (Range 1.9 – 86.1ng/ µl, for 25 samples). Applying the modified Tri Reagent® BD (Molecular Research Centre, Inc., Cincinnati, OH) copurification protocol to patients matched blood, serum and plasma samples, we obtained consistently higher concentrations of miRNA with this method, in particular for whole blood (Range 20.3 – 221.6 ng/µl, for 110 samples, Table 3). The concentration and integrity of the miRNA in these samples was confirmed using the RNA 6000 Nano LabChip Series II Assay with an Agilent Bioanalyzer. Additionally, RT-PCR quantitation of miRNA extracted from blood (whole, serum and plasma) samples using the latter technique, indicated that earlier amplification, therefore higher yields, of miRNAs were observed in whole blood compared to matched serum and plasma (Table 4).

Table 3. Comparison of RNA isolation techniques according to miRNA yield (ng/µl)

	Whole blood miRNA	Serum miRNA	Plasma miRNA
Qiagen separate purification			
Patient #3038	6.75	16.79	48.41
Trizol copurification			
Patient #3038	139.3	121.3	135.7

Table 4. CT values for miR-16 expression in matched whole blood, serum and plasma (100ng miRNA per reaction)

	Whole blood	Serum	Plasma
Patient 1	15.7	31.3	31.6
Patient 2	17.9	33.1	30.9
Patient 3	17.4	32.7	32.9

Whole blood holds advantages over serum or plasma, as a medium for RNA isolation; most notably that no additional processing of the sample is required prior to RNA extraction and therefore is less labour intensive to work with.

It is not known why the separate purification of miRNAs from blood and its derivatives results in lesser yields of miRNA. Whilst this method works effectively for tissue miRNA isolation, results are less successful when it is applied to fluid media such as blood, serum or plasma. One potential explanation may be that the silica-membrane of

the RNeasy spin columns, with its maximal binding capacity of 100 μg RNA [17], may not have the capacity to bind the maximal miRNA component in a small volume of a dilute circulating medium such as blood. Perhaps modification of the column membrane may alter its affinity for miRNA molecules in fluid specimens and improve the ability of this technique to yield higher quantities of quality miRNA. Another potential explanation of why the separate purification protocol using QIAGEN RNeasy Minikits yields low miRNA concentration, is that the recommended volume of blood/serum/plasma (250μl) to be filtered through the columns contains only dilute miRNA amounts. If miRNAs in the circulation represent what is shed from the tumour or released from lysed tumour cells, then one may hypothesize that the miRNA complement in the entire blood volume is at much lower concentration than at tissue level. By sampling only a tiny volume of the circulation such as 250μl, the miRNA concentration within such a small volume could be expected to be very low. Perhaps if the columns and their silica membranes were modified to hold larger blood volumes such as 1ml, then the resulting miRNA yield could be increased.

Whole blood miRNA yield superior from fresher samples

Our comparative analysis of whole blood samples which had been stored for varying lengths of time, indicated that fresher blood samples yielded higher concentrations than blood stored for several weeks or months (up to 12 months) (Table 5).

Table 5. Concentrations (ng/ul) of miRNA in fresh and stored whole blood

	Fresh*	Stored for 6 weeks	Stored for 6 months
Patient 1	81.59	28.4	23.1
Patient 2	122.4	23.88	19.8
Patient 3	170.9	66.46	28.5

*RNA isolated from sample within 3 days of blood collection

Whilst miRNAs are remarkably stable molecules in tissue and circulation [10], it would still appear that storing the blood samples for lengthy periods of time allows a minor degree of miRNA degradation. In order to maximize the yields from a small volume of blood, we recommend isolation of the miRNA shortly after the sample as been obtained. Nevertheless, it must be noted that even in whole blood samples stored at 4°C for several months; it is still possible to accurately and consistently quantify the miRNA complement of the sample by RQ-PCR. The primary advantage then of prompt RNA

isolation from blood is a greater quantity of miRNA which allows a greater number of miRNA targets to be measured from a single 1ml aliquot of blood.

Concentration of miRNA per PCR reaction

The concentration of miRNA per cDNA synthesis reaction which returned superior RQ-PCR amplification was 100ng per reaction, compared to when the starting miRNA concentration per was 5ng, 10ng or 50ng (Table 6).

Table 6: miR-16 amplification (CT cycle) using differing concentrations of miRNA per reaction

	100ng	50ng	10ng	5ng
Patient 1	15.7	16.9	23.1	24.6
Patient 2	17.9	19.3	23.8	24.9

Over the last few years, tissue miRNA quantitation by RQ-PCR has developed substantially. High throughput technology and currently available primers and probes have advanced dramatically so that the technique is sensitive enough to quantify miRNAs from a minute starting volume of RNA. This is hugely beneficial when the specimen is limited in size, as is routinely the case with small tumour biopsy specimens. Hence it has become routine to use RNA quantities as low as 5ng for tissue miRNA quantitation. However the same limitation does not apply to blood based miRNA investigations, as the sample is easier to obtain in larger quantities, and can be resampled at various time-points without great difficulty or inappropriate distress to the patient. Therefore it is more feasible to use starting quantities as high as 100ng per reaction for circulating miRNA investigations, and in order to return superior miRNA expression results, this is to be recommended based on the data presented here. The concentrations of miRNA used in existing reports of circulating miRNA investigations have varied between 5-50 ng. The authors have not described their reasoning behind choosing specific concentrations, or indeed if they made any effort to determine which concentration is optimal. This is the first study to address this specific and important methodological concern.

Endogenous control for circulating miRNA investigations

MiR-16 was found to be abundantly expressed in all samples included in this study; in cancer patients and healthy controls alike. Thus *miR-16* was used as the endogenous control to normalise RQ-PCR data (Fig 1). There is no consensus in the published reports on blood-based miRNA studies as to what the ideal endogenous control is for

these investigations. However *miR-16* has been shown to be abundantly expressed in normal healthy individuals and levels in the circulation have been documented several times to be unaltered in the presence of malignancy. Hence circulating *miR-16* is the most commonly used miRNA reference gene in this context to date [8-10].

Detection of MiRNA in the circulation of breast cancer patients

Expression of nine miRNAs, chosen for their established relevance to breast cancer, (*miR-10b, miR-21, miR-145, miR-155, miR-195, miR-181c, miR-342, miR-16* and *let-7a*) were detectable in whole blood, serum and plasma samples from breast cancer patients (n=83) as well as healthy controls (n=44) (Table 7). Analysis of miRNA expression levels in whole blood, serum and plasma from a random sample of patients indicated that higher yields of miRNAs by RQ-PCR were obtained from whole blood, compared to either serum or plasma. Based on these findings, whole blood was identified as the preferred medium for investigation of miRNAs in circulation.

Table 7. Mean RQ expression levels (SD) of target miRNAs in blood from breast cancer patients compared with blood from healthy controls.

Target miRNA	**Breast Cancer blood samples (n=83)**	**Control blood samples (n=44)**	**Mean fold change in miRNA expression in breast cancer blood compared to controls**	*p-value*
miR-10b	1.05 (3.03)	0.83 (0.83)	1.27	*0.449*
miR-21	3.52 (10.30)	2.69 (7.47)	1.31	*0.606*
miR-145	3.58 (7.29)	1.65 (4.14)	2.17	*0.062*
miR-155	2.92 (6.23)	1.77 (4.48)	1.65	*0.280*
miR-195	6.91 (12.17)	0.36 (0.43)	19.25	*<0.001*
let 7a	5.05 (24.33)	0.45 (0.9)	11.20	*<0.001*
miR-181c	133.2 (3.1)	18.3 (2.5)	7.28	*<0.001*
miR-342	307.9 (3.7)	23.4 (3.2)	13.16	*<0.001*

Expression profiles of blood miRNAs in breast cancer patients

To explore the potential of using circulating miRNAs as novel biomarkers for breast cancer, we investigated the levels of 8 target miRNAs (miR-16 used as endogenous control) in the circulation of 83 consecutive breast cancer patients and compared with those of 44 normal subjects. The levels of four tumor-associated miRNAs (*miR-195, miR-181c, miR-342* and *let-7a*) were significantly higher, on average, in the breast cancer cohort than in healthy controls ($p < 0.001$), corresponding to average fold-changes of 19.2, 7.3, 13.2 and 11.2 respectively (Table 7, Figure 1). Elevated levels of this 4-miRNA signature increased discriminatory power of this test for breast cancer (all types, including non-invasive breast cancer (DCIS) to 94% ($p<0.001$), as shown in Figure 9.

Within this breast cancer cohort, a subset of patients had postoperative blood samples collected (n=29) to assess the effect of curative tumor resection on circulating miRNA levels. Thus it was found that *miRNA* expression in the blood had decreased significantly to levels comparable with control subjects ($p<0.001$). Expression of preoperative circulating *miR-10b, miR-21, miR-145* and *miR-155* did not differ significantly between the breast cancer cohort and controls ($p = 0.142, 0.587, 0.162, 0.366$ respectively).

Relationship of systemic and tumor miRNA profiles

Breast tumor expression of *miR-195* was significantly higher compared to that in TAN: 1.23(0.43) *vs.* 0.49(0.37), $p<0.001$ (Figure 4). Tumor *miR-195* expression was also

significantly higher in Stage IV compared to Stages I & II tumors (p= 0.006 and 0.039 respectively ANOVA and Tukey post hoc analysis). There was no significant difference between mean circulating *miR-195* expression and the stages of disease, however a similar trend of increasing systemic *miR-195* expression with advancing stage of breast cancer was observed (Figure 5). Controlling for age and stage of disease, *miR-195* expression in tumor tissue showed a non-significant positive correlation with circulating *miR-195* levels (Pearson's correlation coefficient 0.126, p=0.321) (Figure 6).

Relationship of circulating miRNAs to clinicopathological parameters

In addition to assessing the relationship of breast tumor and systemic miRNA profiles to the stage of disease, other relevant biopathologic associations of circulating miRNAs were investigated. Lymph node positive patients were found to have significantly lower levels, on average, of circulating *let-7a* compared to those with node negative disease (n = 38 and n = 45 respectively, p=0.002).

Higher circulating levels of *miR-10b* and *miR-21* were observed in patients with ER negative disease (n=15), compared to those with ER positive breast cancer (n=68), (p=0.028 and p=0.004 respectively). A potential relationship between circulating miRNA levels, HER2/*neu* status, and intrinsic subtype was also investigated but no statistically significant difference was identified.

Detection of MiRNA in the circulation of patients with metabolic disorders

Analysis of the miRNA microarray identified a number of miRNAs which were significantly different between obese and non-obese fat. These miRNAs are all potential therapeutic targets for obesity. Successful validation of the miRNA microarray using RQ-PCR and Taqman miRNA primers and probes was achieved, which confirmed the microarray findings to be accurate. This led to the identification of a signature panel of metabolic miRNAs, which have potentially important roles in obesity and development of the metabolic syndrome.

Quantitation of this signature panel of metabolic miRNAs targets in fat tissue and blood from obese and non-obese patients identified *miR-17-5p* as an important miRNA, which is significantly decreased in obesity (in both fat and blood). Additionally *miR-143* is significantly decreased in obese diabetics (in both fat and blood). *MiR-122* expression levels in fat and in blood were shown to correlate positively with total cholesterol and low-density lipoprotein levels. This is the first report of such a finding.

Discussion:

Circulating miRNAs in breast cancer patients and our results demonstrate that cancer-associated miRNAs in blood can potentially serve as novel non-invasive biomarkers for breast cancer. *MiR-195, miR-181c, miR-342* and *let-7a* are significantly increased in the blood of breast cancer patients in comparison to disease-free control subjects. *MiR-195* expression in a similar cohort of breast tumors and TAN specimens shows a similar significant increase in tumor tissue over TAN. When profiling tumor and systemic *miR-195* levels according to the stages of invasive breast cancer, similar profiles are evident in both tissue types. In addition, the use of whole blood in preference to plasma or serum for miRNA detection and quantification was successful. Whole blood samples from patient and control subjects were comparable for white cell counts, hemoglobin and hematocrit levels, thereby eliminating potential bias due to cellular and protein components.

The miRNAs found to be significantly increased in the blood of breast cancer patients, *miR-195, miR-181c, miR-342* and *let-7a,* have previously been described in breast cancer miRNA studies. *MiR-195* was reported by *Mattie et al* to be significantly higher in HER2/*neu* positive compared to HER2/*neu* negative breast cancers [16], a finding which was also true for the cohort of 65 invasive breast tumors analysed for *miR-195* expression in this study (HER2/*neu* positive n=20, HER2/*neu* negative n=45, p=0.002, Figure 7). Furthermore we observed a significant increase in tumor *miR-195* levels in metastatic breast cancers, compared to early stage tumors. This pattern was reflected in the circulation, although to a lesser (non-significant) extent. Interestingly circulating levels of *miR-195* decreased significantly by two weeks following tumor resection (Figure 8) albeit in a small subset of patients. Such observations support the concept of utilising systemic miRNA profiling as a novel and non-invasive biomarker for breast cancer. MiR-181c and miR-342 have both been reported by our group recently to be dysregulated in breast tumours; with levels of miR-342 highest overall in HER2/*neu* over-expressing and Luminal B tumours, and lowest in the triple negative subgroup. MiR-181c was part of a 5 gene signature associated with HER2/*neu* positivity.

The finding that *let-7a* was increased over 5-fold in breast cancer patients was unexpected. *Let-7a* is well described as having a functional role as a tumor suppressor [18] and has been shown to be down-regulated in many solid organ cancers, including lung, colorectal and gastric cancer [19-20]. In relation to breast cancer, *let-7a* in conjunction with miR-16 has been described as a reliable endogenous control for analysis of miRNAs by

RQ-PCR in human breast tissue [13]. As endogenous control genes are tissue and organ specific, it is acceptable that a house keeping gene for one tissue type can be investigated as a target gene in another. The finding that *let-7a* was greatly increased in the blood of breast cancer patients raises an interesting question concerning the origin of circulating miRNAs. Whilst recent blood based miRNA reports, including the present study, clearly show that malignancy alters miRNA levels in the circulation, it is still unknown how tumor associated miRNAs make their way into the bloodstream. Slack *et al* raised two hypotheses in a recent report [21], firstly that tumor miRNAs may be present in circulation as a result of tumor cell death and lyses, or alternatively that tumor cells release miRNAs into the tumor micro- environment, where they enter newly formed blood vessels, and thereby make their way into the circulation. Our findings fit generally with the first hypothesis; however it is clear that further studies are needed to gain greater insight into the origin of circulating miRNAs.

Further evidence from our investigations support these miRNAs as ideal breast cancer biomarkers. Levels of circulating miRNAs were found to decrease postoperatively, two weeks following curative surgical resection of the breast tumour. Systemic miRNA levels also showed significant correlations with stage of disease; for example *miR-195* levels in blood increased as the tumour burden and stage of disease increased, whilst levels of let-7a were highest in patients with non-invasive breast cancer, becoming progressively lower as the stage of invasive disease advanced. Also miR-195 and miR-181c levels were elevated only in breast cancer patients. When miR-195 and miR-181c was measured in the blood of 80 further patients who had just been diagnosed with various cancers other than breast (colon, renal, bladder, prostate, melanoma), levels of miR-195 and miR-181c were elevate only in the breast cancer cohort. Conversely let-7a levels were elevated in almost all cancer patients anlaysed, implicating it as a non-specific general cancer biomarker.

We identified higher circulating levels of *miR-21* and *miR-10b* in patients with ER (oestrogen receptor) negative disease. *MiR-21* has been described as an oncomir, and is up-regulated in many solid and hematological cancers. In relation to breast cancer, higher levels of *miR-21* have been shown to be associated with advanced disease, poorer prognosis, and lymph node metastasis [22-24]. However the relationship of tumor *miR-21* level to ER status has been inconsistently described; Mattie *et al* found higher *miR-21* levels to be associated with ER positive breast cancer in their study of 20 breast tumor

biopsies, 11 of which were ER positive [16]. More recently Qian *et al* showed high *miR-21* levels to be associated with estrogen receptor negative disease in a much larger study of 344 breast tumors, 218 of which were known to be ER positive and 120 ER negative [23]. Our findings in blood correspond to those of Qian *et al* in relation to breast tissue.

Functional studies have shown that *in vitro* manipulation of *miR-21* expression can alter the responsiveness of ER negative cell lines to hormonal therapies. This further highlights the importance of *miR-21* expression in human breast cancer. Although this study did not find circulating *miR-21* to differ significantly between breast cancer patients and controls, its association with clinicopathological parameters such as ER status indicates that circulating *miR-21* may serve as a prognostic molecular marker for breast cancer and disease progression.

The role of *miR-10b* in breast cancer has also been addressed with varying conclusions on its precise function. Early studies collectively found *miR-10b* to be down-regulated in breast tumor compared to normal breast tissue [14 16]. More recently, *Ma et al* contested these findings, and reported that *miR-10b* played a part specifically in the metastatic process but not in primary tumor formation, having found this miRNA to be highly expressed in metastatic breast cancer cells [22]. To our knowledge, this is the first report of a significant association between *miR-10b* and the hormonal status of breast cancers. Given that hormone receptor negative status is considered a poor prognostic factor for breast cancer [25], our observation that circulating *miR-10b* is higher in ER negative disease is in keeping with the findings of *Ma et al*.

Conclusion

The results presented here, showing significantly altered circulating miRNA levels in breast cancer patients compared to healthy individuals, with similar profile for *miR-195* in breast tumor tissues compared to TAN, and the associations of particular circulating miRNAs with commonly used prognostic indicators, highlights the potential of these molecules as novel non-invasive biomarkers for breast cancer. Circulating tumor associated miRNAs have the potential to detect breast cancer even in its earliest stages (DCIS), and can differentiate tumors according to tissue type and histological features such as hormone receptor and lymph node status.

Inherent characteristics of miRNAs such as their lower complexity, tissue specific expression profiles, remarkable stability, and ease with which they are amplified and quantified herald these molecules ideal biomarkers to reflect various physiological and

pathological states. Methods for effective isolation and quantification of miRNA in blood, or its derivatives, provide the opportunity for these potentially useful clinical biomarkers to be measured non-invasively in the circulation. The invention provides an optimal methodological approach to systemic miRNA detection and quantitation. The results presented here indicate that the optimal extraction technique is a modified version of the Trizol copurification protocol. We have identified whole blood as preferable to serum or plasma for circulating miRNA analysis, and that using higher concentrations (100ng per reaction) of systemic miRNA in RQ-PCR studies yields superior results compared to lower concentrations. Finally, we have also observed that miR-16 is stably expressed across all analyzed blood samples, including cancers and healthy controls, and thus appears to be a suitable endogenous control for blood based miRNA investigations.

The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

Example 1

Small RNAs in the circulation were detected as follows:-

1. **LYSIS**: 3ml TRI Reagent + 200 µl BAN + 1ml whole blood

Ratio of reagent volume to sample volume should always be 3:1

In a 5ml clear tube place 3.0ml of TRI Reagent BD supplemented with 200 l of BAN (bromoanisole) and 10 µl of Polyacryl Carrier. Add 1 ml of whole blood.

2. **PHASE SEPARATION**: homogenate

Split the total volume (>4.2ml) across 2 round bottomed 2ml tubes and centrifuge at 14.000 rpm for 15 minutes at 4C.

It is important to separate phases in the cold (4-10 °C). Centrifugation performed at elevated temperatures may sequester DNA into the aqueous phase. The use of bromoanisole for phase separation improves the quality of isolated RNA and eliminates toxic chloroform and bromochloropropane from the isolation protocol.

3. **RNA PRECIPITATION**: 1ml aqueous phase + 1ml isopropanol

Transfer 1ml of each aqueous phase to a fresh 2ml round tube. Precipitate RNA from the aqueous phase by mixing with 1 ml isopropanol. Store samples at room temperature for 5 min and centrifuge at 14,000 rpm for 8 minutes at 18 °C. RNA precipitate forms a

gel-like or white pellet at the bottom of the tube. The 1 ml aliquot of aqueous phases constitutes 80% of the aqueous phase total volume. Leave the remaining 20% undisturbed to prevent accidental collection of DNA from the interphase.

4. **RNA WASH**: 1 ml 75% ethanol

Remove the supernatant and mix the RNA pellet in 1ml 75% ethanol by vortexing. Centrifuge the RNA suspension at 14,000 rpm for 5 minutes at 18 °C. Repeat this step to improve 260/280 ratio of the RNA.

5 **RNA SOLUBILIZATION**: 30 µl water per pellet

Remove the ethanol wash and briefly air-dry the RNA pellet for 5 min. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve the RNA in 30 µl water. Leave the RT for 5 minutes, vortex and spin down 10 sec.

6. RESULTS:

Take a Nanodrop reading at x40 for total RNA and at x33 for miRA. If the duplicate samples are alright, pool the samples, take a fresh NanoDrop reading and reserve 2 µl for Agilent. Store in the appropriately labelled RNA storage tubes (~60 µl vol).

Tri Reagant	750 (µl)	1 ml	3ml
Blood (ml)	250	333.3	1
BAN (µl)	50	66.7	200
Polyacryl Carrier (µl)	2-8	2.7-10.7	8-32
Isopropanol (ml)	500	666.7	2x1
75% Ethanol (ml)	1	1.3	2x1
Water µl	30	30	2x30

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination.

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Claims

1. A diagnostic kit to detect cancers including breast cancer, diabetes, cardiovascular disease, hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, or to stratify patients according to expected prognosis comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising, miR342, miR181c, miR-10b, miR-21, miR145, miR-155, miR-195, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-375, or miR-16 (as endogenous control) biomarkers.

2. A kit as claimed in claim 1 to detect cancers including breast cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c or let-7a biomarkers.

3. A kit as claimed in claim 1 to detect early stage breast cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising comprising miR-16 (as endogenous control), miR-195, miR-342, miR-181c or let-7a.

4. A kit as claimed in claim 1 to detect cancers including breast cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miRNA-195 or let-7a biomarkers.

5. A kit as claimed in claim 1 to detect cancers including breast cancer comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-342 or miR-181c biomarkers.

6. A kit as claimed in claim 1 for detecting or stratifying metabolic diseases comprising at least one oligonucleotide probe capable of binding to at least a portion of a circulating miRNA selected from the group comprising miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-145, miR-375.

7. A kit as claimed in any preceding claim adapted for performance of an assay selected from a real-time PCR assay, a micro-array assay, a histochemical assay or an immunological assay.

8. A method of identifying a therapeutic agent capable of preventing or treating cancers including breast cancer, diabetes, cardiovascular disease, hypercholesterolemia,

obesity and the metabolic syndrome and liver toxicity, comprising testing the ability of the potential therapeutic agent to reduce or enhance the expression of at least one protein selected from the group comprising miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, or miR-375 biomarkers.

9. Use of a circulating miRNA selected from the group comprising miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, or miR-375 biomarkers to detect cancers including breast cancer, diabetes, cardiovascular disease, hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, or to stratify patients according to expected prognosis.

10. Use as claimed in claim 9 wherein the detection is carried out on a blood sample or a sample derived from blood.

11. A method of detecting or screening for early stage breast cancers, comprising analysing a sample of blood taken from a patient for the presence of one or more biomarkers selected from the group comprising miR-16 (as endogenous control), miR-195, miR-342, miR-181c or let-7a, the presence of at least one of these miRNAs in the sample indicating the presence of breast cancer.

12. A method of detecting or screening for metabolic diseases comprising analysing a sample of blood taken from a patient for the presence of one or more biomarkers selected from the group comprising miR-17-5p, miR-29a, miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, miR-145, miR-375, the presence of at least one of these miRNAs in the sample indicating the presence of metabolic disease.

13. A method of detecting small RNAs in the circulation comprising:-

(i) Treating whole blood with TRIzol reagent at a ratio of one part blood to three parts Trizol reagent,

(ii) Adding 1-bromo-4-methoxybenzyene,

(iii) Centrifuging the mixture at at least 12000g for at least 5 min at less than 10°C,

(iv) Precipitating RNA from the aqueous phase,

(v) Quantifying the miRNAs precipatated.

14. A method as claimed in claim 13 wherein the ratio of 1-bromo-4-methoxybenzyene to blood or blood –derived sample is in the range of 0.2 - 0.8ml 1-bromo-4-methoxybenzyene to 1ml blood or blood-derived sample.

15. A method as claimed in claim 14 wherein the ratio the ratio of 1-bromo-4-methoxybenzyene to blood or blood –derived sample is 0.2 ml 1-bromo-4-methoxybenzyene to 1ml blood or blood-derived sample is used.

16. A method as claimed in any of claims 13 to 15 wherein following the addition of 1-bromo-4-methoxybenzyene, polyacryl carrier is added to the blood/trizol/1-bromo-4-methoxybenzyene solution.

17. A method as claimed in any of claims 13 to 16 wherein the RNA is precipitated by use of one part Isopropanol to one part aqueous RNA solution, centrifuged for 8 minutes at 12,000g, at 18 degree Celsius.

18. A method as claimed in any of claims 13 to 17 wherein the precipitated RNA is washed with ethanol, preferably twice.

19. A method as claimed in any of claims 13 to 18 wherein quantification of miRNAs is carried out via NanoDrop® spectrophotometry set at a conversion factor of 33μg/ml or equivalent spectrophotometry.

20. A method as claimed in any of claims 13 to 18 wherein quantification of miRNAs is carried out via the Agilent quantification method

21. A method as claimed in any of claims 13 to 20 wherein cDNA synthesis is performed on 1-1000ng of quantified small RNA.

22. A method as claimed in claim 21 wherein the small RNA is reverse transcribed using stem loop RT primers, specific for each miRNA target and diluted with nuclease – free water to give 50μM concentration per reaction, according to standard terms and conditions

23. A method as claimed in any of claims 13 to 22 whereby miRNA expression levels are quantified (relative quantification)by real-time PCR, using expression level of miR-16 and/or another stably expressed small RNA(s) to normalise the expression level of the target miRNA.

24. A method as claimed in any of claims 13 to 23 for the detection of cancers including breast cancer, as well as a range of benign diseases including diabetes, cardiovascular disease, hypercholesterolemia, obesity and the metabolic syndrome and liver toxicity, or to stratify patients according to expected prognosis wherein the miRNA quantified is at least one miRNA selected from the group comprising miR-16, miR-10b, miR-21, miR145, miR-155, miR-195, miR342, miR181c, let-7a, miR-17-5p, miR-29a,

miR-29b, miR-34a, miR-99a, miR-103, miR-122, miR-132, miR-143, or miR-375 biomarkers .

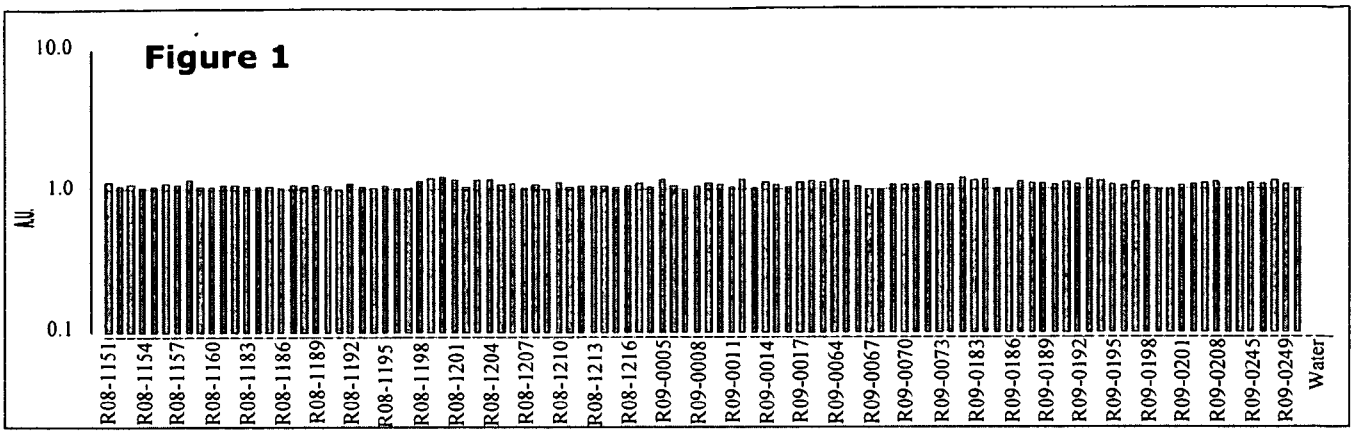


Figure 1
10.0
1.0
0.1
A.U.
R08-1151
R08-1154
R08-1157
R08-1160
R08-1183
R08-1186
R08-1189
R08-1192
R08-1195
R08-1198
R08-1201
R08-1204
R08-1207
R08-1210
R08-1213
R08-1216
R09-0005
R09-0008
R09-0011
R09-0014
R09-0017
R09-0064
R09-0067
R09-0070
R09-0073
R09-0183
R09-0186
R09-0189
R09-0192
R09-0195
R09-0198
R09-0201
R09-0208
R09-0245
R09-0249
Water

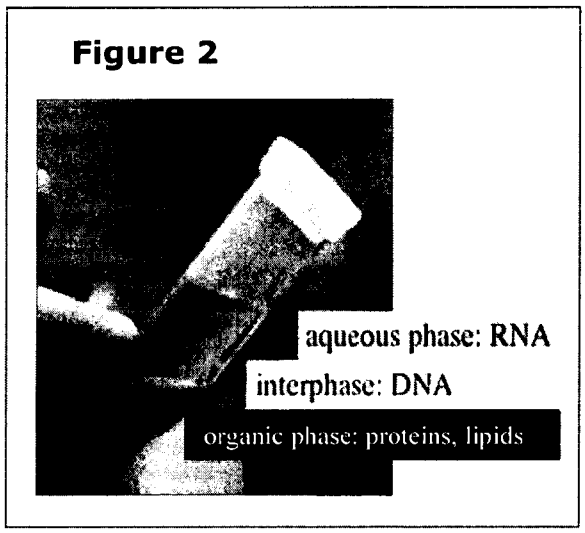


Figure 2
aqueous phase: RNA
interphase: DNA
organic phase: proteins, lipids

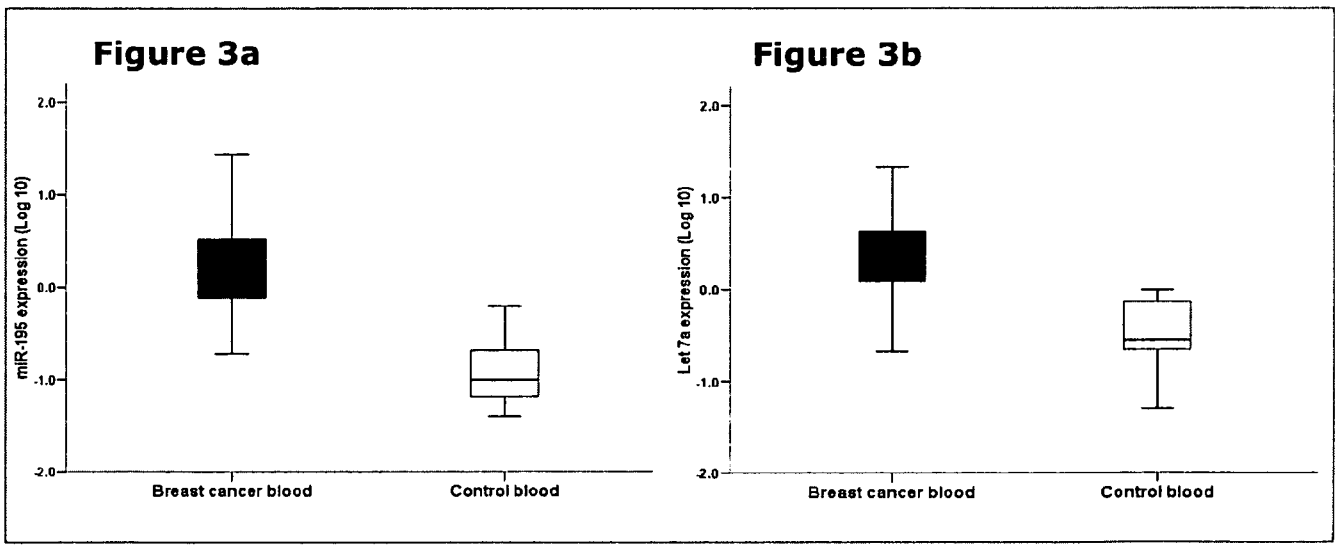


Figure 3a
miR-195 expression (Log 10)
2.0
1.0
0.0
-1.0
-2.0
Breast cancer blood
Control blood
Figure 3b
Let 7a expression (Log 10)
2.0
1.0
0.0
-1.0
-2.0
Breast cancer blood
Control blood

Figure 4

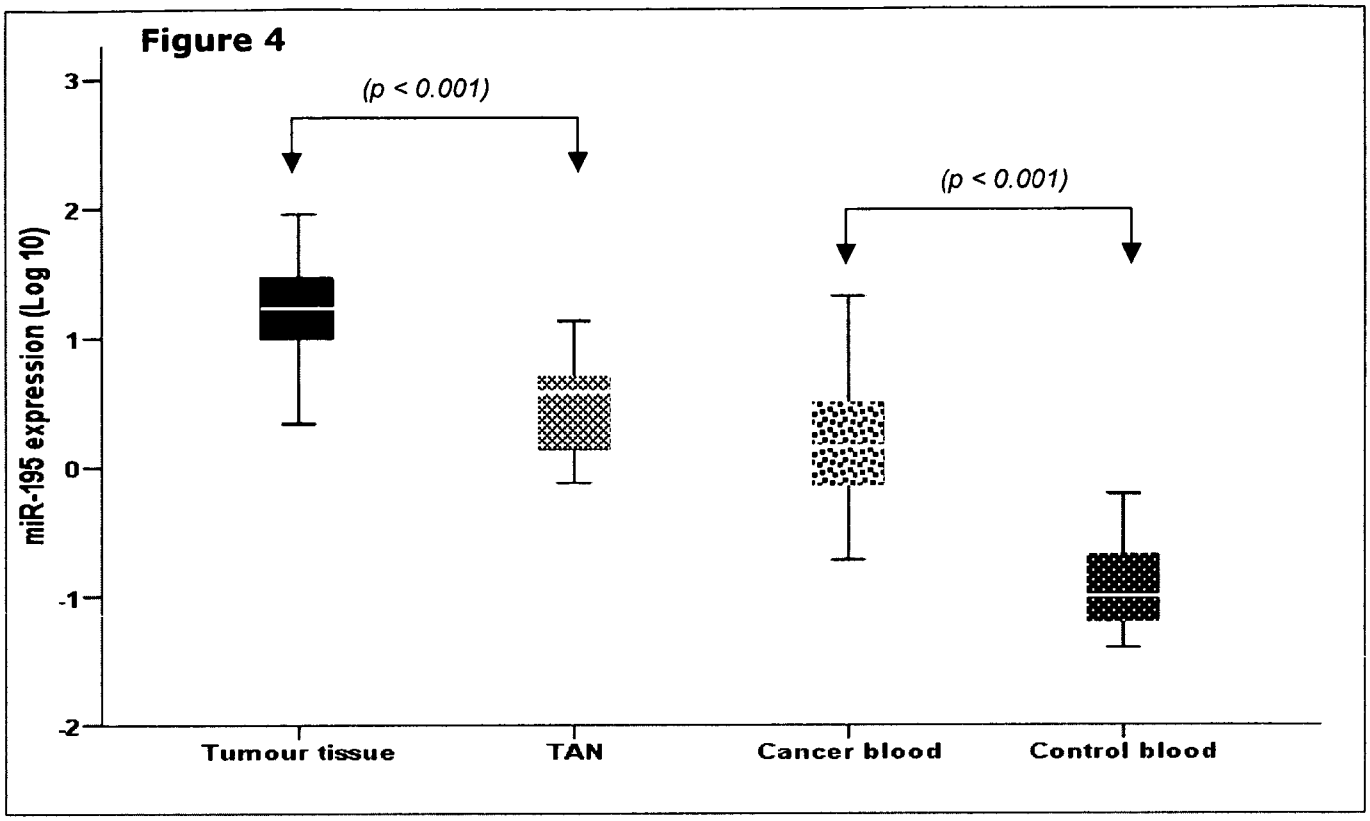


Figure 5

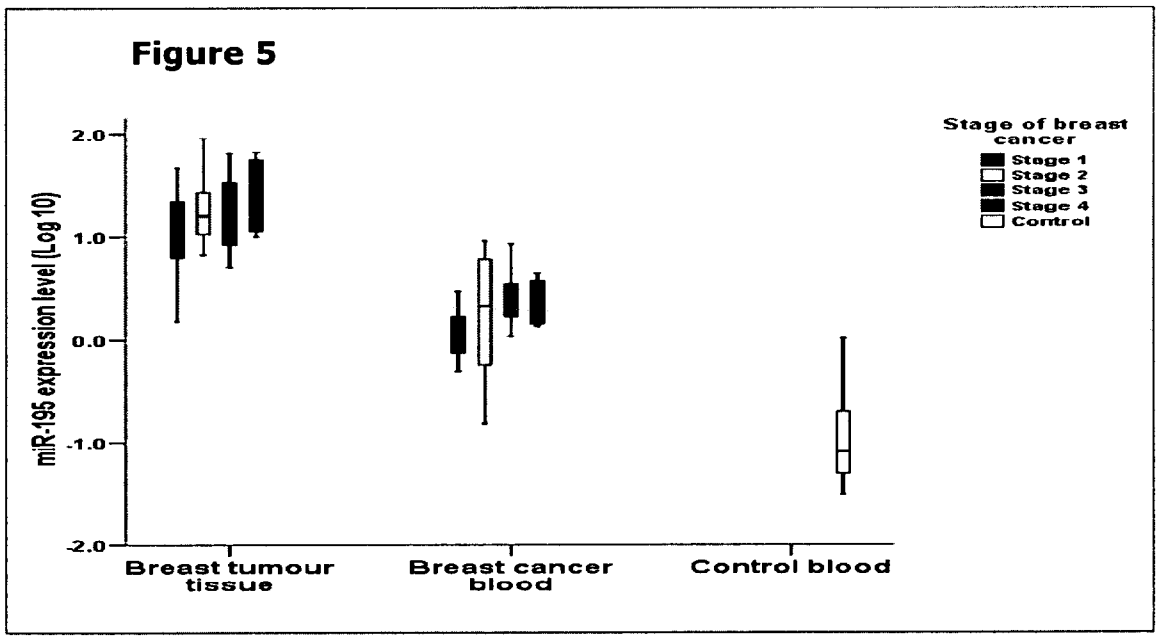


Figure 6

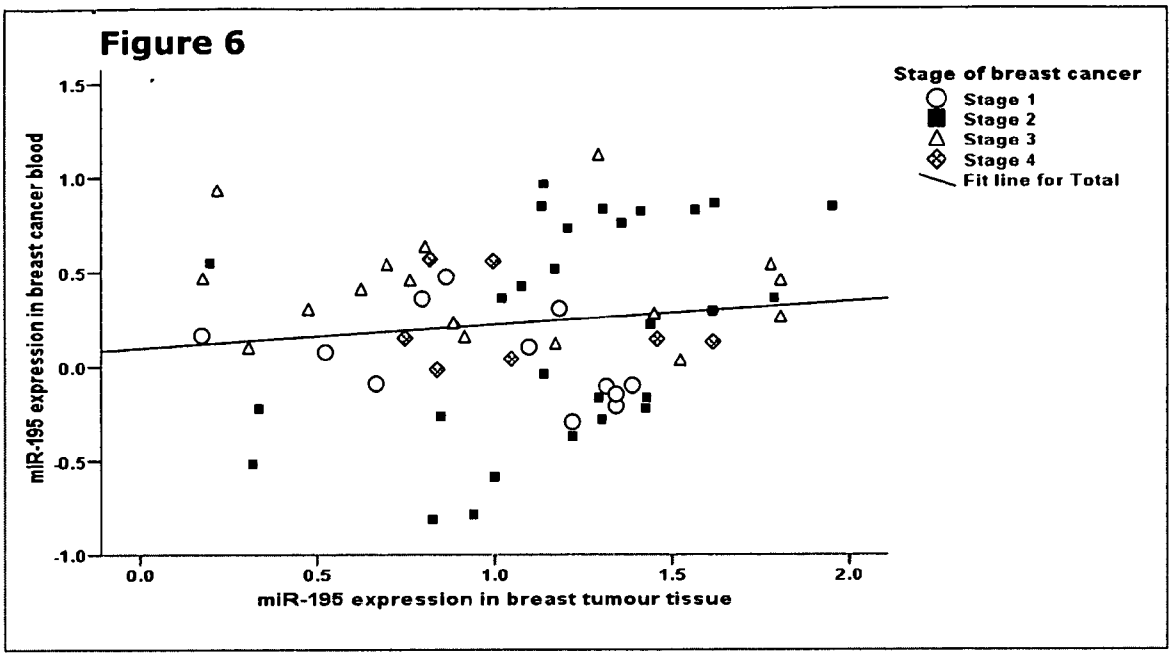


Figure 7

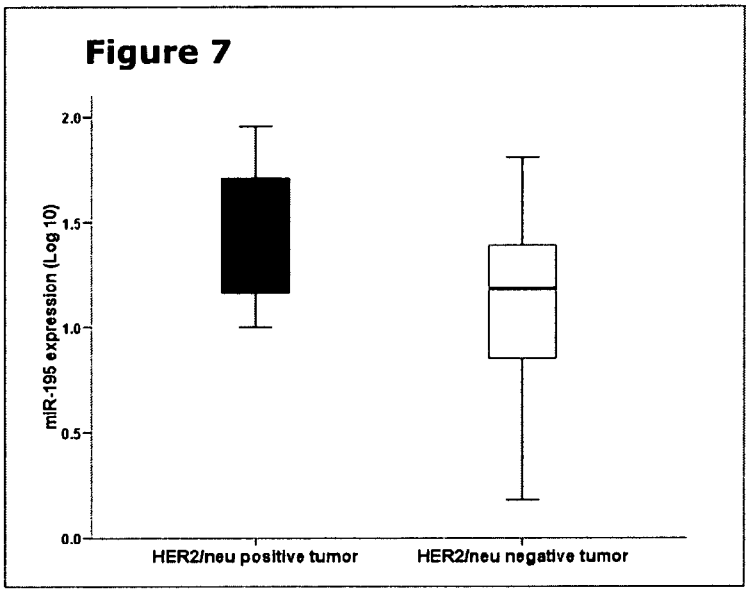


Figure 8

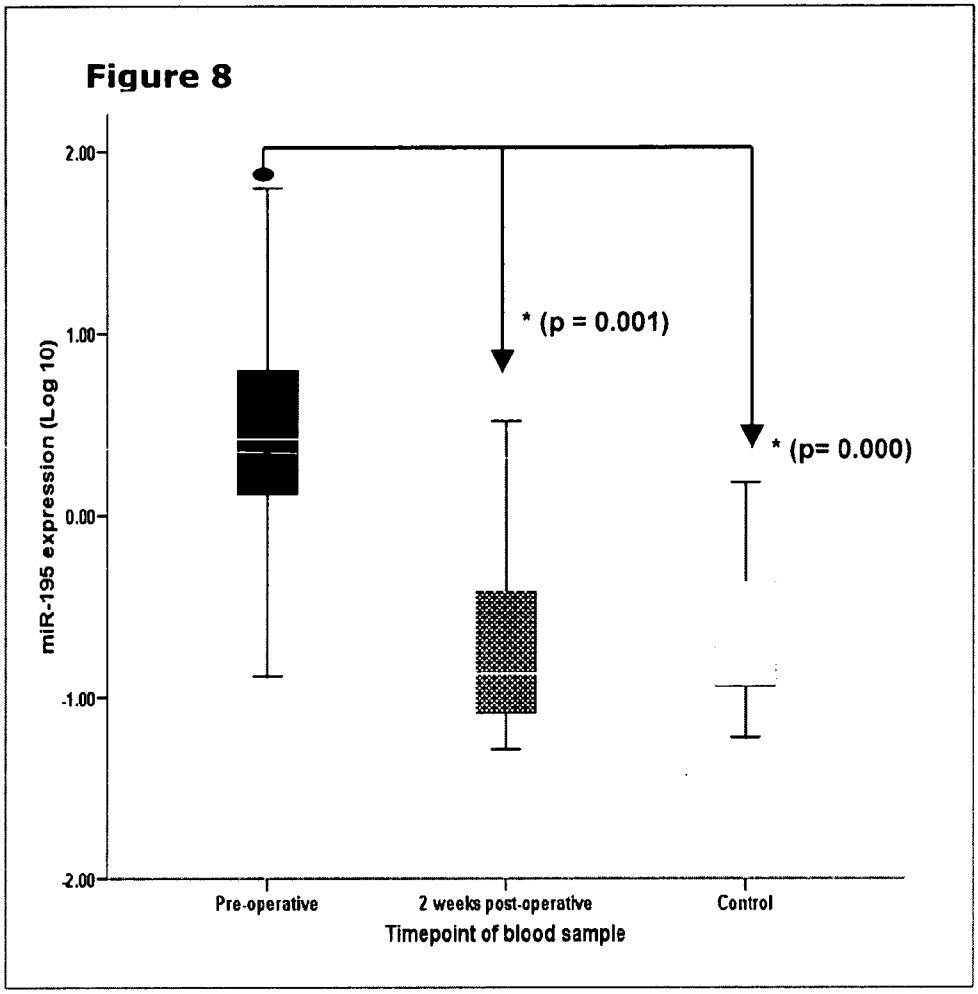
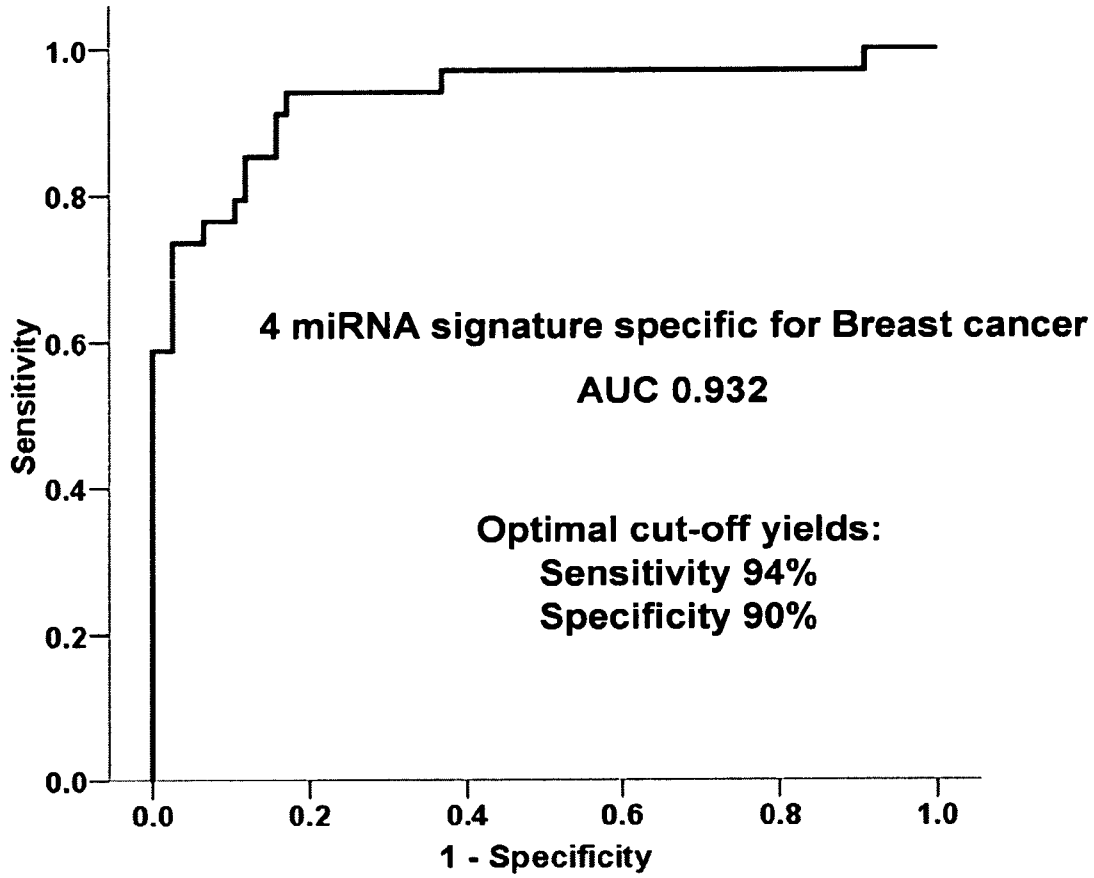

2.00
1.00
0.00
-1.00
-2.00
miR-195 expression (Log 10)
* (p = 0.001)
* (p= 0.000)
Pre-operative
2 weeks post-operative
Control
Timepoint of blood sample

Figure 9


1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity
4 miRNA signature specific for Breast cancer
AUC 0.932
Optimal cut-off yields:
Sensitivity 94%
Specificity 90%

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/053649

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.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/053649

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 practical, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 133 431 A1 (JIANGSU MINGMA BIOTECH CO LTD [CN]) 16 December 2009 (2009-12-16)	1-7,9,10
Y	the whole document para. 13, 25, 36, 42, claims 28-30, Table 1	8,11-24

X	WO 2006/081284 A2 (ROSETTA INPHARMATICS LLC [US]; RAYMOND CHRISTOPHER K [US]) 3 August 2006 (2006-08-03)	1-7
A	the whole document p. 12, l. 20 - p. 13, l. 30, claims 25-40	8-12

X	US 2008/076674 A1 (LITMAN THOMAS [DK] ET AL) 27 March 2008 (2008-03-27)	1-7,9-11
Y	the whole document para. 75, 294, 347, 503, claims 56-58 para. 110, Example 1, Tables 1-3, claims 1 and 18	8,12-24

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[X] Further documents are listed in the continuation of Box C.

[X] 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 document 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	Date of mailing of the international search report
18 July 2011	08/08/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sauer, Tincuta

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2007/016548 A2 (UNIV OHIO STATE RES FOUND [US]; CROCE CARLO M [US]; CALIN GEORGE A [US) 8 February 2007 (2007-02-08) the whole document p. 6, ll. 10-20, claims 33-36 p. 3, ll. 6-14, p. 28, ll. 5-17	8-10 1-7, 11-24

X	CHEN XI ET AL: "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases.", CELL RESEARCH OCT 2008 LNKD-PUBMED:18766170, vol. 18, no. 10, October 2008 (2008-10), pages 997-1006, XP002634302, ISSN: 1748-7838	9,10
Y	the whole document abstract, p. 1000, col. 2 - p. 1003, col. 1; Supplementary Table 3 and 4	1-8, 11-24

X	WANG JIN ET AL: "MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease.", CANCER PREVENTION RESEARCH (PHILADELPHIA, PA.) SEP 2009 LNKD- PUBMED:19723895, vol. 2, no. 9, September 2009 (2009-09), pages 807-813, XP002634303, ISSN: 1940-6215	9,10
Y	the whole document table 2	1-8, 11-24

X	Heneghan HM: "Circulating microRNAs as novel minimally invasive biomarkers for breast cancer", , 3 February 2010 (2010-02-03), pages 1-2, XP002634304, Retrieved from the Internet: URL:http://currentcancer.com/circulating-microrna s-as-novel-minimally-invasive-biomarkers-for-breast-cancer.html [retrieved on 2011-04-27]	9,10
Y	the whole document	1-8, 11-24

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HENEGHAN HELEN M ET AL: "Circulating microRNAs as novel minimally invasive biomarkers for breast cancer", ANNALS OF SURGERY, J.B. LIPPINCOTT COMPANY, PHILADELPHIA, US, vol. 251, no. 3, 1 March 2010 (2010-03-01) , pages 499-505, XP009147535, ISSN: 0003-4932	9,10
Y	the whole document Table 3	1-8, 11-24

X	WO 2009/009457 A1 (UNIV LOUISVILLE RES FOUND [US]; WANG EUGENIA [US]; SCHIPPER HYMAN [CA]) 15 January 2009 (2009-01-15)	1,2,7,9, 10,12
Y	the whole document	13-24
A	p. 4, ll. 18-32, p. 19, ll. 3-31 p. 5, ll. 1-13	3-6,8,11

Y	LOWERY ET AL: "O-7 Micro-RNA expression profiling in primary breast tumours", EUROPEAN JOURNAL OF CANCER. SUPPLEMENT, PERGAMON, OXFORD, GB, vol. 5, no. 3, 1 September 2007 (2007-09-01), page 3, XP022405623, ISSN: 1359-6349, DOI: DOI:10.1016/S1359-6349(07)71697-X the whole document	1-12

Y	HENEGHAN H M ET AL: "Role of microRNAs in obesity and the metabolic syndrome.", OBESITY REVIEWS : AN OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF OBESITY MAY 2010 LNKD-PUBMED:19793375, vol. 11, no. 5, 29 September 2009 (2009-09-29), pages 354-361, XP002634305, ISSN: 1467-789X the whole document p. 359, col. 1, last para.	1-12

Y	Invitrogen: "TRIzol® Reagent and TRIzol® LS Reagent", , 15 December 2007 (2007-12-15), pages 1-33, XP002650899, Retrieved from the Internet: URL:http://tools.invitrogen.com/Content/SFS/ProductNotes/F_071215_Trizol%20and%20Trizol%20LS.pdf [retrieved on 2011-07-18] the whole document p. 4, 26-27	13-24

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ACCERBI MONICA ET AL: "Methods for isolation of total RNA to recover miRNAs and other small RNAs from diverse species", METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.) UNITED STATES 2010,, vol. 592, 1 January 2010 (2010-01-01), pages 31-50, XP009150248, the whole document page 39	13-24
Y	WO 2005/103252 A1 (CHOMCZYNSKI PIOTR [US]) 3 November 2005 (2005-11-03) the whole document p. 6, l. 6 - p. 7, l. 17, claims 12, 28	13-24
Y	Molecular Research Center, Inc: "PRODUCT: TRI REAGENT® RT - RNA / DNA / PROTEIN ISOLATION REAGENT", , 25 January 2008 (2008-01-25), pages 1-11, XP002650900, Retrieved from the Internet: URL:http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/MOR_/RT111.20080125.pdf [retrieved on 2011-07-18] the whole document pages 1-2	13-24
Y	Molecular Research Center, Inc: "TRI REAGENT®BD - RNA / DNA / PROTEIN ISOLATION REAGENT forBLOOD DERIVATIVE", , 20 April 2004 (2004-04-20), pages 1-7, XP002650901, Retrieved from the Internet: URL:http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/MOR_/TB126.20050208.pdf [retrieved on 2011-07-18] the whole document	13-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/053649

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CN 101424640 A	06-05-2009
		US 2010173288 A1	08-07-2010
WO 2006081284 A2	03-08-2006	AT 480644 T	15-09-2010
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		EP 2322613 A1	18-05-2011
		JP 2007532140 A	15-11-2007
		KR 20070042912 A	24-04-2007
		US 2005233333 A1	20-10-2005
		US 2008057560 A1	06-03-2008

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-12

   A diagnostic kit, a method of indentifying a therapeutic agent, use of a circulating miRNA, a method of detecting or screening for early stage breast cancer or metabolic diseases using miRNAs (i.e. products and and methods for detecting diseases associated with miRNA expression);

   ---

2. claims: 13-24

   A method of detecting (i.e. extracting and quantifying) small RNAs in the circulation.
