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# Circulating microRNAs as blood-based biomarkers for breast cancer

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# ABSTRACT

***Background:*** Currently, there is a lack of non-invasive tumour biomarkers with appropriate sensitivity and specificity to be used in routine clinical testing. The use of circulating microRNAs (miRNAs) as cancer biomarkers has been hypothesised based on their presence and stability in the circulation. Promising initial results for these tiny RNAs has been obtained in the field of breast cancer diagnostics. However, the accurate quantification of circulating miRNAs is more challenging than expected. In particular, several pre- and analytical variables have an impact on their final quantification, including the quantification method. Recently, a new droplet digital PCR (ddPCR) system that can be also used for microRNA quantification has been developed and proved to be very promising in liquid biopsy applications.

***Experimental design and findings*:** In order to develop a precise and accurate technique for miRNA quantification, we tested and compared the feasibility of quantifying circulating miRNAs with the new BioRad ddPCR system when used with EvaGreen dye– and TaqMan probe–based assays. In plasma and serum of patients with cancer and healthy controls, two circulating miRNAs and one added exogenous miRNA were quantified with both EvaGreen dye–based miRCURY LNA miRNA assays and TaqMan assays. The EvaGreen-based assay was precise, reproducible and sensitive. In comparison with TaqMan assays, high concordance was obtained for two endogenous miRNAs in serum and plasma. EvaGreen dye–based and TaqMan probe–based assays can be equally used with the ddPCR system to quantify circulating miRNAs. Afterwards, we selected a panel of six miRNAs (miR-10b-5p, miR-145-5p, miR-181a-5p, miR-148b-3p, miR-425-5p, miR-652-3p) derived from microarray experiments or described in literature as potential circulating biomarkers for breast cancer. Then, we assessed their absolute levels in two independent cohorts of breast cancer patients and disease-free controls (Italy; n = 56, and USA; n = 94) using EvaGreen-based ddPCR. MiR-148b-3p, miR-181a-5p and miR-652-3p levels were significantly lower in the serum of breast cancer patients than in controls in both cohorts. For these three miRNAs, the stratification of breast cancer patients versus controls was confirmed by receiver operating characteristic curve analysis. Higher levels of serum miR-10b-5p were associated with clinico-pathological features of poor prognosis. These results confirmed the significant discrimination between breast cancer patients and healthy controls and the direction of down regulation.

***Conclusion*:** This study establishes the basis for using on a ddPCR for quantifying circulating miRNA biomarkers. Both study cohorts revealed very good agreement in terms of comparable absolute miRNA concentrations and consistent trends of dysregulation in breast cancer patients. This study finally powers the use of circulating miRNAs as cancer biomarkers and proposes miR-181a-5p and miR-652-3p as diagnostic biomarker and miR10b-5p as prognostic biomarkers of breast cancer.

# RIASSUNTO

***Stato dell'arte:*** L'assenza di marcatori tumorali non invasivi e con appropriata sensibilità e specificità per un uso clinico, rappresenta un problema fondamentale in ambito oncologico. L'utilizzo di microRNA (miRNA) circolanti come biomarker tumorali è stata ipotizzata sulla base della loro presenza e stabilità nel sangue e in altri fluidi biologici. Promettenti risultati preliminari sono stati ottenuti dall'utilizzo di questi piccoli RNA come biomarcatori del cancro al seno. Tuttavia è risultato fin da subito evidente che la quantificazione accurata dei miRNA circolanti è un processo molto complesso e influenzato da molteplici fattori. In particolare non esiste un accordo su quale sia il metodo di quantificazione migliore. E' stato recentemente sviluppato un nuovo sistema di quantificazione di acidi nucleici chiamato Droplet Digital PCR (ddPCR), ma non era ancora stato testato per la quantificazione di miRNA circolanti.

***Disegno sperimentale e risultati:*** Al fine di sviluppare una tecnica precisa ed accurata per la quantificazione dei miRNA circolanti, abbiamo testato l'affidabilità del nuovo sistema ddPCR di BioRad (QX200) e confrontato i risultati della quantificazione dei miRNA circolanti con due chimiche diverse, una basata sull'intercalante EvaGreen e una basata su sonde TaqMan. Nel plasma e siero dei pazienti con cancro e controlli sani, due miRNA circolanti e un miRNA aggiunto esogenamente sono stati quantificati con saggi per miRNA basati su primer a LNA (Exiqon) combinati con EvaGreen o su sonde TaqMan (Applied Biosystem). Entrambi i saggi si sono dimostrati precisi, riproducibili e sensibili. La concordanza tra i dati di quantificazione ottenuti con le due metodiche è risultata essere molto buona. Abbiamo pertanto concluso che sia saggi basati su EvaGreen che sull'uso di sonde TaqMan possono essere ugualmente utilizzati con il sistema ddPCR per quantificare i miRNA circolanti. In seguito, abbiamo selezionato un gruppo di sei miRNA (miR-10b-5p, miR-145-5p, miR-181a-5p, miR-148b-3p, miR-425-5p, miR-652-3p) derivati da esperimenti microarray o descritti in letteratura come potenziali biomarker circolanti per il cancro al seno. Abbiamo quindi valutato i loro livelli assoluti (copie/µl) nel siero in due coorti indipendenti di pazienti con carcinoma mammario e controlli sani (Italia; $n$ = 56, and USA; $n$ = 94) utilizzando saggi ddPCR basati su primer a LNA e sistema EvaGreen.

MiR-148b-3p, miR-181a-5p e miR-652-3p sono risultati significativamente più bassi nel siero dei pazienti con cancro al seno rispetto ai controlli in entrambe le coorti. Per questi tre miRNA la stratificazione dei pazienti con carcinoma mammario rispetto ai controlli è stata confermata tramite l'analisi di curve ROC. Inoltre, livelli sierici più elevati di miR-10b-5p sono stati associati con alcune caratteristiche clinico-biologiche a prognosi negativa degli stessi campioni.

***Conclusione:*** Questo studio stabilisce la base per l'utilizzo di test basati su ddPCR per la quantificazione di miRNA circolanti quali biomarcatori di tumore al seno. Entrambe le coorti studiate hanno rivelato un ottimo accordo in termini di concentrazioni assolute miRNA e tendenze coerenti di disregolazione in pazienti con cancro al seno rispetto ai controlli. Questo studio suggerisce pertanto l'uso di miRNA come biomarcatori tumorali circolanti e propone miR-181a-5p e miR-652-3p come biomarcatori diagnostici e miR10b-5p come biomarcatore prognostico del tumore al seno.

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# Dedication

I would like to dedicate my dissertation

To

**My Mother**

A strong and gentle soul who taught me to trust in Allah, believe in hard work and that so much could be done with little

**My Father**

For spending his whole life earning an honest living for us and for supporting and encouraging me to believe in myself

**My family**

I hope that this achievement will complete the dream we have always had when we were young

**For all friends, relatives and colleagues**

Who always support, encourage and give me hope in achieving what's not there

**For University of Khartoum-Sudan**

&

**All breast cancer patients**

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# KEY WORDS

**Breast cancer**

**Circulating microRNAs (miRNAs, miRs)**

**Diagnostic biomarkers**

**Prognostic biomarker**

**Droplet digital PCR (ddPCR)**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| < | Less-than sign |
| °C | Celsius degree |
| 3’UTR | 3’untranslated region |
| Ago2 | Argonaute2 |
| AUC | Area Under Curve |
| bp | Base pairs |
| BRCA | Breast cancer patients |
| CA15-3 | Carbohydate antigen 15-3 |
| cDNAs | Complementary DNAs |
| CEA | Carcinoembryonic antigen |
| Ct | Threshold cycle |
| CV | Coefficient of variation |
| ddPCR | Droplet digital PCR |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | Deoxynucleotide Triphosphate |
| dsRNA | Double Strand RNA |
| EDTA | Ethylene diamine tetra acetic acid |
| ER | Estrogen receptor |
| g | Gram |
| H2O | Water |
| HER2/neu | Human epidermal growth factor receptor 2 |
| kb | Kilobase |
| MBC | Metastatic breast cancer |
| mg | Miligram |
| min | Minute(s) |
| miR | MicroRNA(s) |
| miRISC | MiRNA induced silencing complex |
| miRNA | MicroRNAs |
| mM | Milimolar |
| ng | Nanogram |
| nM | Nanomolar |
| nt | Nucleotide |
| NTC | No template control |
| PBC | Primary breast cancer |
| PCR | Polymerase Chain Reaction |
| pg | Picogram |
| PR | progesterone receptor |
| PR | progesterone receptor |
| Pre- miRNAs | Precursor miRNA |

| | |
|---|---|
| Pri- miRNAs | Primary miRNAs |
| PTEN | Phosphoinositide 3-kinase pathway phosphatase and tensin homolog |
| qPCR | Quantitative PCR |
| qRT-PCR | quantitative Real Time PCR |
| RNA | Ribonucleic acid |
| ROC | Receiver Operating Characteristic |
| RT-qPCR | reversetranscription- quantitative polymerase chain reaction |
| s | Second(s) |
| SD | Standard deviation |
| U | Unite |
| X | Times |
| µg | Microgram |
| µL | microliter |
| µl | Microliter |
| µM | Micromolar |

# 1. Introduction

## 1.1. Breast cancer

Breast cancer is the most common type of cancer and the second cause of cancer-related death among women in industrialized countries. Worldwide approximately 1.3 million women develop breast cancer each year (1). Advances in early diagnosis and treatments have contributed to the decrease of mortality rates over the years. The overall 5- year survival is 90 % when breast cancer is diagnosed at an early stage as opposed to 20 % if disease has spread to distant organs (2). Physical examination, mammography, and biopsy are the current approaches to breast cancer diagnosis (3). Carcinoembryonic antigen and carbohydrate antigen 15–3 are circulating tutor markers that are mainly used for patient follow-up (4). However, the sensitivity of these markers is low, thus calling attention to the need for novel and more accurate non-invasive diagnostic biomarkers.

Breast cancer is a complex heterogeneous disease, comprising different histological and biological features, clinical presentations and behaviors that vary in prognosis and response to therapy. Despite improvements in early diagnosis, approximately one out of every three breast cancer patients will suffer metastatic disease. In the last decade, advancements in technologies have helped the researchers to understand breast cancer complexity more thoroughly. Based on gene expression profile and the phenotype, the breast cancer was divided into six major subtypes. Luminal A, luminal B, tumour enriched with human epidermal growth factor receptor 2 (Her2), basal-like, normal-like and claudin-low subtype (5-8). Among them, luminal A is the most common subtype identified by the expression of estrogen receptor (ER), progesterone receptor (PR), Bcl-2 and absence of Her2. It constitutes 50–60% of all breast cancer cases (6, 7). The luminal B subtype is identified by the expression of ER, PR and absence of Her2. High Ki67 staining can differentiate luminal B from luminal A. These two tumour subtypes are associated with good prognosis. Her2 positive subtype accounts for 15–20% of all the breast carcinomas. It is characterized by the high expression of the Her2 gene and high proliferation rate. The overall prognosis still remains poor, despite the improvements in the survival rate in the last decade. Comparing to

other tumour subtypes, basal-like breast cancer expresses none of the three markers (ER, PR and Her2), representing 10–20% of all breast cancer, and is often associated with a poor prognosis. Normal-like subtype is the rarest form of breast cancer subtype and accounts for only 5–10% of all breast cancer. Although poorly characterized this subtype has been demonstrated to express ER, Her2 and PR and has a clinical outcome between basal like and luminal A subtype. Claudin-low breast cancer subtype accounts 12–14% of breast cancer cases with a poor long-term prognosis (9). In nature, is triple negative characterized by low expression of claudin- 3, 4, 7, ocludin and E-cadherin with cancer stem like features.

## 1.2. MicroRNAs: Biogenesis, processing and function

### 1.2.1. MicroRNAs at a glance

It has been well recognised that the majority of non-protein-coding genomic DNA is not “junk” but specifies a range of regulatory RNA molecules which finely regulate gene expression. A particular class of these regulatory RNAs, the microRNAs (miRNAs), represent a highly conserved class of endogenous small non-coding RNAs (ncRNAs) of 20-23 nucleotides in length, which are expressed in a tissue specific manner (10-12). They constitute about 1–2% of the known genes in eukaryotes (13). As post-transcriptional regulators of gene expression, miRNAs regulate the expression of the variety of genes (14-18) and play a central role in virtually all biological pathways in mammals and other multicellular organisms. Accordingly, miRNAs influence a wide array of cancer-relevant processes such as proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism (19).

Since their discovery in 1993 by Wightman et. al and Lee et. al (20, 21), miRNAs have attracted wide attention due to their unique functional significance and mode of action. Lin-4 was the first miRNA, discovered from C. elegans in 1993 (20). Seven years later, the second miRNA let-7 was also discovered from C. elegans (22). This publication was certainly distinct and unique, as it proposed, for the first time, that small dsRNAs could play a role in gene expression. In 2001, this kind of endogenous tiny non-protein coding single-stranded RNAs were first designated as “microRNAs” (23). As a result, characterization of miRNAs in normal physiology

and pathology was emerged in almost all fields of biological and biomedical fields. Consequently, more and more miRNAs were identified in many species ranging from plants to human (24).

Up to date, thousands of miRNAs have been identified in a wide variety of species. A total of 1881 precursors and 2588 mature human miRNAs have been identified according to miRBase Release 21(see link below). With increasing number of identified miRNA, rules of annotation have been suggested to designate individual miRNAs, such as hsa- miR-21 with "hsa" standing for the homo sapiens, "miR" for microRNA, and the number "21" indicating the order of being discovered (25). http://www.mirbase.org/cgibin/mirna_summary.pl?org=hsa

### 1.2.2. Genomic Organization

MiRNA coding sequences can be found in exons or introns of a protein-coding gene or in the intergenic regions. Considerably, several miRNA genes are clustered along the genome (also known as cistrons) sharing the same promoter. Moreover, miRNAs can also be present individually (25). Cistronic miRNAs are typically located within 5 kb of each other in intergenic regions or within the same intron/exon and are co-transcribed and yield similar read counts for each member of a given miRNA precursor cluster (26). MiRNA genes are transcribed into a large, non-coding messenger RNA strand known as primary miRNA transcript (pri-miRNA), with coding capacity for one or more mature miRNAs (25).

### 1.2.3. Biogenesis and processing

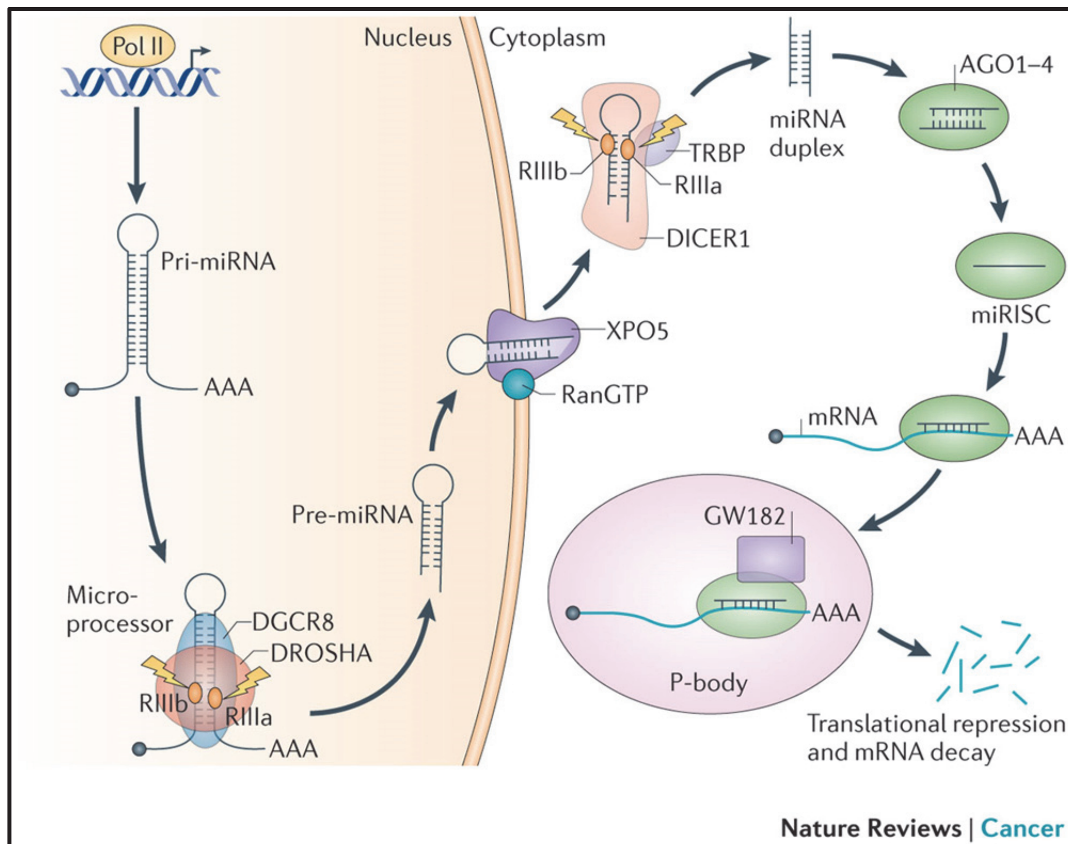
MiRNAs biogenesis is regulated at multiple levels, including at the level of miRNA transcription; it's processing by Drosha and Dicer in the nucleus and cytoplasm, respectively; its modification by RNA editing, RNA methylation, uridylation and adenylation; Argonaute loading; and RNA decay (**Figure. 1**).

In mammal, many aspects of the miRNA biogenesis pathway and repressive mechanisms are still obscure. Principally, because identification of the miRNA promoters is still a challenging task (27). However, the fundamental processes have been described. The biosynthesis of miRNAs is a tightly regulated multistep process that starts in the nucleus of the cell, following transcription, and continues through the cytoplasm where finally the mature miRNA molecule exerts its main

function. For most miRNAs, RNA polymerase II typically transcribed miRNAs (exceptions: a few are manuscript of RNA polymerase III ) as long pri-miRNA molecules from intergenic regions of the genome, but may also be derived from intronic and exonic regions of coding and non-coding genes (28).

In the nucleus, canonical pri-miRNAs are capped with 7-methylguanosine and polyadenylated and cleaved by the RNAse III enzyme Drosha and its cofactor Pasha (or DGCR8) to produce a 60–100 nt precursor miRNA (pre-miRNA) hairpin molecule (29). RAN-GTP and exportin-5 complex, subsequently transport the pre-miRNAs into the cytoplasm.

In the cytoplasm, Dicer, also an RNAse III endonuclease, interacts with TRBP (Tar RNA Binding Protein) to mediate further processing of pre-miRNA to form a mature 20–23 nt miRNA–miRNA* duplex (30). Helicase unwind the duplex and the mature miRNA is incorporated into effectors' complex known as miRISC (miRNA induced silencing complex) (27, 31, 32), while the miRNA* (read as miRNA "star") is degraded. The core components of miRISC are Argonaute proteins (AGO/EIF2C) (33) and GW182/TNRC6 protein families (34, 35). Add to the above, the non-canonical pathways for miRNA biogenesis, including those that are independent of Drosha or Dicer, are also emerging (36).




(Lin & Gregory, Nat Rev Cancer, 2015)

**Figure 1. Overview of miRNA biogenesis pathway.**
The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is degraded.

### 1.2.4. Target recognition and function

As master gene regulators, within the RISC complex, miRNAs bind through imperfect base pairing to the 3'untranslated region (3'UTR) of target miRNAs (37), subsequently cleaving or silencing the target miRNA. Albeit the precise mechanism of such inhibitory effect on translation remains to be validated, there is an evidence suggesting that miRNA interferes with protein factors involved in the elongation process during translation (38). The binding specificity and efficiency is believed to be determined by 6–7 nucleotide sequence near 5′ region of miRNA. This sequence is called the "seed sequence" and is the initial binding site of the miRNA to the 3'UTR of the target miRNA (39). It has been proposed that the degree of complementarity between miRNAs and their targets is essential factor on which the mode of miRNA function is determined. Translational repression of gene targets caused by imperfect complementarily of the miRNAs and the target miRNA, while perfect matching, recruits the CAF1–CCR4 miRNA deadenylation complex to initiate miRNA degradation (31).

Surprisingly, recent studies have suggested that a number of miRNAs are able to activate the expression of certain target genes in a sequence-specific manner instead of silencing them. This novel phenomenon, although largely remaining unknown, is termed "RNA activation" (RNAa). Although still the exact mechanisms of RNAa remain to be elucidated, the Ago2 protein and histone changes linked to gene activation process could be involved in the process (40).

MiRNAs and their targets seem to form a complex regulatory network. It is believed that a single miRNA can regulate multiple mRNAs and a single mRNA can be targeted by a number of different miRNAs. This complexity represents an important characteristic of miRNAs to modulate fundamental biological processes. It has been estimated that regulate more than 60% of all human genes (41) based on computational predictions, with many miRNAs identified as key players in wide variety of fundamental changes at the cellular and organism level. Among them, including development (42), cell proliferation (43, 44), apoptosis (45, 46), aging (47) and stress response (48) by regulation of gene expression through a posttranscriptional mechanism, including mRNA degradation and translational repression (13). Over all, number of genes found to be under the modulation of miRNAs has increased sharply (25) and aberrant miRNA expression may affect a

multitude of transcripts and profoundly influence disease-related signalling pathways (19).

In normal physiology, miRNAs are crucial parts of feedback circuits and through their buffering effects add durability to key biological processes. For instance, miRNA may serve to increase the precision of gene expression by dampening the production of proteins being expressed beyond their physiologically boundaries (49). In mammals, individual miRNAs may serve as switches in many differentiation pathways, as for instance exemplified by the regulation of skin differentiation by miR-203 (50) or smooth muscle cells by miR-143/145 (51). Moreover, the ability of even a single miRNA to influence cell identity was further confirmed early in overexpression studies displaying that transfecting HeLa cells with a single miRNA, miR-124, shifted the expression profile towards that found in the brain, a tissue in which miR-124 is highly expressed (52). A part from their role in differentiation, formation of cellular identity and their noise-dampening effect, it follows that loss of miRNA function may lead to de-differentiation, increased cellular plasticity, and a higher probability for oncogenic transformation. Interestingly, miRNA also play key roles in stem cells and their differentiation (53) and induced pluripotency. Recently, a single miRNA cluster, miR-302, was demonstrate to be able to produce iPSC from both human and mouse fibroblasts (54).

In pathological conditions, miRNAs have been implicated in various diseases, for instance, diabetes (55), cancer (56), hepatitis C (57), neuro-developmental (58) and mental (59) disorders.

Overall, rapidly growing knowledge of miRNAs as potent regulators in health and disease makes miRNAs attractive as targets for therapeutic intervention (60, 61) as well as for diagnostic markers (62, 63).

### 1.2.5. Regulation of microRNA expression

Several mechanisms, including gene amplification, deletion, epigenetic alterations, and single-nucleotide substitution, have been implicated in altered miRNA expression (64). In 2011, Yang et. al reviewed regulation of microRNA expression and function by nuclear receptor signaling. Nuclear receptors (NRs) are ligand-activated transcription factors that regulate the expression of target genes by

binding to cis-acting DNA sequences. Since miRNAs are encoded by genes that are mainly transcribed by RNA polymerase II, their transcription can be regulated by a variety of transcription factors including NRs. For example, ERα binds directly to the promoter region of miR-221/222 and recruits NCoR and SMRT to suppress miR-221/222 expression. Androgen receptor (AR) can also bind to the promoter region of miR-221 to repress miR-221 expression in LNCaP cells. AR is a ligand-dependent transcription factor that regulates the expression of androgen target genes (65).

Post-transcriptional regulation has also been shown to essentially influence miRNA turnover rate or inhibitory activity. The 3′ end of mature miRNA can further be subject to a series of additional modifications such as uridylation that blocks Dicer processing and induces miRNA degradation and adenylation that affects miRNA stabilization. On the other hand, in plants, 2′-O-methylation protects miRNA from uridylation, decay, and miRNA editing that makes an adenosine to inosine conversion resulting in altered mRNA target specificity. MiRNAs expression can be dynamically regulated under certain conditions. For example, under inflammatory conditions, KH-type splicing regulatory protein (KSRP) can promote miR-155 maturation through binding to the terminal loop of pre-miRNA. In addition, expression of Dicer can be suppressed upon activation of various stress pathways, suggesting an implication of stress in alteration of miRNA expression (66).

It has been demonstrated that the infection of Epstein-Barr virus (EBV) could influence human cellular miRNA expression. Godshalk et. al. found that 99.5% of tested miRNAs displayed an average down-regulation of 19.92-fold upon initial EBV infection of primary cultured human B-cells, suggesting an activation of global suppression of miRNA-generating machinery (67).

Taken together, the growing body of literature has indicated that the miRNA biogenesis and its expression regulation are highly complicated, requiring participation of multiple layers of transcriptional and post-transcriptional modifications and subject to tight and dynamic control under distinct physiological and pathological conditions (66).

Thus, expression of this important class of molecules is usually correlated with an array of pathological conditions, among which cancer may represent one of the most relevant diseases related to aberrant expression and/or functions of miRNAs (25).

## 1.3. MicroRNAs and cancer

In 2002, the significance of miRNA in human cancers introduce to be revealed when Croce and colleagues identified that a small genomic region in chromosome 13q14 that is commonly deleted in chronic lymphocytic leukaemia (CLL) contained miR-15a and miR-16-1 genes, suggesting a link of these miRNAs to CLL (68). After this revelation, more and more miRNAs have been revealed to be aberrantly expressed in numerous types of cancer cell lines and clinical tumor specimens. Besides the abnormal levels of specific miRNAs in certain types of human cancers, biological evidence that suggests crucial roles of miRNAs in cancer development and progression was also experimentally validated in animal models (12).

Afterwards, cancer-associated miRNAs classified as either oncogenic microRNAs or tumour-suppressive microRNAs (25). Nevertheless, classification of miRNAs as oncomiRs or tumour suppressor miRNAs can be difficult by cause of it is not clear if altered miRNA patterns are the direct cause of the cancer or rather an indirect effect of changes in cellular phenotype. In addition to, a single miRNA can regulate multiple targets. These issues coupled with miRNAs expression patterns alter for specific tissues and stage of differentiation could implicate a single miRNA as a tumour suppressor in one context and an oncogene in another, which posit difficulties in classification (69).

Up to date, several studies have examines the miRNA expression in cancer patients and found that miRNAs are differentially expressed in normal versus tumour tissues in every type of human cancer (that has been studied so far) including including breast (70, 71), leukemia (72), lymphoma (73), glioblastoma (74, 75), neuroblastoma (76), papillary thyroid carcinoma (77, 78), lung (79), liver (57), pancreas (80), gastric (62), colorectal (46, 81), ovarian(82), prostate (70, 83, 84), kidney and bladder cancer (85). Such researches revealed aberrational expression of miRNAs during carcinogenesis. The first report of miRNA

deregulation in solid tumours was the observation of a consistent down-regulation of miR-143 and miR-145 in colorectal cancer (86). In the case of lung cancer a reduction of >80% of let-7 expression was found by northern blotting in 44% (7 out of 16) of patients compared with healthy controls. Consequently, many researches showed that profiles of miRNA expression are highly informative for tumour classification, prognosis, and response to therapy. Lu et al. at showed that miRNA expression profiles can be used for early cancer detection and classification (87).

In 2005, three reports provided the first mechanistic insight into how miRNAs might contribute to carcinogenesis. Two independent reports illustrated an interaction between miR-17-92 cluster and Myc (88, 89), and the third one uncovered the regulation of Ras by let-7 (90). Lee et. al showed that miRNA precursor processing reduce in cancer cells compared to normal human cell lines and tissues (91). Also, miRNA processing machineries, Drosha and Dicer, have a decreased expression in cancer cells. In ovarian cancer cases, the reduction of Dicer and Drosha correlates with a poor prognosis, suboptimal surgical cytoreduction and an advanced tumor stages (92).

Altogether, these findings support the idea of a cancer progression deeply influenced by a handful of miRNAs with a strong power that confers to these miRNAs a very promising role as cancer biomarker.

## 1.4. MicroRNAs and breast cancer

Conspicuously, it is beyond the scope of this study to show the role of all miRNAs that have been linked to breast cancer in the past 10 years. Indeed, it was hypothesized many years ago, specifically the involvement of miRNAs through an extensive target genes modulation in every step of cancer initiation, development and progression, today it is evidence based knowledge.

Breast cancer was among the first tumours for which miRNAs deregulation was described through a comparison of 76 cancerous and 10 normal breast tissues by miRNA microarray (71). Lorio and his colleagues provided the earliest observation that miRNAs are differentially expressed in breast cancer tumors as compared with normal breast tissue. In their paper, they identified 29 miRNAs differentially expressed in breast cancer tumours as compared with normal breast tissue.

Several functional studies regarding the involvement of various specific miRNAs in breast cancer initiation and development as well as their role in metastatic breast cancer have been issued. Furthermore, miRNAs expression studies in breast cancer also revealed the importance and potential use in tumour classification and better prognosis (71).

MiRNAs profiling studies have led to the identification of miRNAs that are aberrantly expressed in human breast cancer. Among the most commonly deregulated miRNAs, the putative suppressors belonging to the let-7 family, miR-145, miR-125 and miR-200 family and the oncogenic miR-21 and miR-155 whose expression is significantly dysregulated in breast cancer tissues.

Let-7, for instance, is one of the earliest discovered miRNAs and its reduced expression has been observed in many human cancers. In their paper, Yu and colleagues described a role for let-7 in maintaining stem-cell properties of breast tumor-initiating cells (BT-ICs) (93). Increasing let-7 levels in BT-ICs impaired their proliferation rate, self-renewal properties and metastasizing capability. On the contrary, silencing of let-7 known targets, such as RAS or HMGA2, did not obtained that wide effects meaning that the effect of let-7 modulation is not due to its action on a single target gene. In the same line, Volinia et. al have found miRNAs specific for invasiveness capable of regulating transition from ductal carcinoma in situ to invasive ductal carcinoma. In particular, let-7d, miR-210 and miR-221 were downregulated in the ductal carcinoma in situ while upregulated in the invasive transition (94).

Extensive next-generation miRNA sequencing of paired tumour miRNAs and normal tissue from 5 patients with breast cancer, revealed more than 500, including a novel miRNA (miR-4728) encoded within the Her2 gene, which was overexpressed in Her2-amplified tumours (95).

MiR-195 was found to be significantly elevated in breast cancer patients versus disease fee controls. Moreover, significant reduction in miR-195 in post-operative whole blood compared to the pre-operative samples of the same patients was revealed (96).

MiR-125 is another tumor suppressor miRNA, first described as down-regulated in breast cancer compared to normal mammary tissue (71). Its importance in breast

cancer derives from the capability to recognize a complementary site in ERBB2 (HER2) and ERBB3 (HER3) 3'UTRs thereby inducing suppression of HER2/HER3 protein levels and of HER2/HER3 induced proliferation and migration in SKBR3 cells consequently (97). More recently, other important targets of miR-125, involved in cell proliferation, apoptosis, invasion and drug resistance, have been identified strengthening the role of miR-125 as a tumor suppressor miRNA in breast cancer.

An interesting role seems to be exceptionally performed in breast cancer by the "oncogenic" miR-17/92 cluster. Indeed, in breast cancer this cluster is expressed at the same or at reduced levels than in matched normal mammary tissue (98), particularly in lymph node positive tumors. Moreover, miR-17/20 was revealed to target cyclin D1 and IL-8 in breast cancer cell lines thereby inhibiting cell proliferation, migration and invasion (98, 99). The role of tumor suppressor for miR-17/92 cluster was confirmed also by Hossain at al. through the identification of AIB1, an estrogen receptor coactivator, as a target of miR-17-5p (100). Surprisingly, a recent paper by Li at al. presented data supporting an opposite role in breast cancer cell lines for miR-17-5p (101), that is described as an invasion promoter. This discordant information needs to be further clarified.

Since the discovery of the role of miR-10b in breast cancer invasion and metastasis, the functional role of miRNAs in tumour initiation and progression has been the focal point (102). MiR-10b has been shown to be deregulated in several tumour types including, pancreatic adenocarcinoma, glioma,and glioblastoma. Serum miR-10b concentrations were significantly higher in patients with bone metastases than in those without bone metastases, Zhao and colleagues say(103). These findings, together with previous studies that have reported increased miR-10b concentrations in breast cancer metastasis, suggest a role for miR-10b in cancer and metastatic behaviour.

## 1.5. Circulating miRNAs and cancer

The involvement of miRNAs in cancer has been repeated and clearly proven by many studies. The pattern of miRNA expression in tumours can be potentially used as biomarkers for tumour characterization and cancer prognosis (68, 87). Circulating miRNAs, defined as miRNAs present in the cell-free component of

blood and body fluids. As first report, Lawrie and colleagues found increased levels of miR-21 in serum of large B-cell lymphoma patients (104). This was coupled with confirmation by Mitchell and colleagues, when they discovered the association between circulating miR-141 and prostate cancer, spanning the importance of cell-free miRNAs to solid cancers (105).

Recently, several studies have reported the occurrence of circulating miRNAs in serum and plasma samples from cancer patients and healthy controls (106-110). In colorectal cancer, Huang et. al showed plasma miRNAs were highly sensitive for detecting colorectal cancer and advanced adenomas and miR-29a and miR-92a were associated with advanced neoplasia. Further, in the light of origins of these circulating miRNAs, they compared the levels of miR-29a and miR-92a expression in plasma samples of pre-operative and post-operative bloods and revealed that both miRNAs were significantly reduced if compared to the preoperative samples of the same patients.

## 1.6. Circulating miRNAs and breast cancer

In spite of comprehensive research on molecular mechanisms involved in breast cancer has been done over the decades, challenges still dominate in the early diagnosis and management of breast cancer patients, such as unpredictable response and development of resistance to adjuvant therapies. MiRNAs, as mRNA regulators, could serve as novel diagnostic and prognostic candidates and potential therapeutic targets (111). Moreover, alteration of miRNAs expressions in breast cancer may provide better contribution in pathophysiological research (112).

### 1.6.1. Circulating microRNAs as potential diagnostic biomarkers

One of the major challenges in oncology is the early diagnosis of cancer patients, in order to reduce morbidity and mortality rates associated with the disease. Early, Zhu and colleagues provided the first study demonstrating the differential expression of circulating miRNAs in breast cancer patients compared to healthy controls, and found a correlation of miR-155 to progesterone receptor status (113). Another study revealed that tumour-associated circulating miRNAs are increased in the blood of breast cancer patients and associated with tumour progression.

Moreover the relative concentrations of total RNA and miR-155 in serum significantly discriminated primary breast cancer from healthy women (114). In consistence with these findings, significant higher level of miR-155 has been observed in breast cancer patients serum compared to healthy controls (115, 116). In addition, the levels of serum miR-155 may be eligible to discriminate breast cancer patients from healthy subjects (116). On the other hand, significantly elevated circulating miR-195 was found to be breast cancer specific and could differentiate breast cancer from other cancers and from healthy controls with a sensitivity of 88% and specificity of 91%. A combination of circulating levels of miR-195, let-7a, and miR-155 circulating miRNAs, including miR-195, further enhanced the discriminative power of this test for breast cancer to 94% (117).

More recently, further reports confirming significant difference between levels of specific breast cancer circulating miRNAs compared to healthy individuals were published. Ng et. al performed TaqMan-based miRNA profiling and identified a significant increase of levels of miR-16, miR-21, and miR-451 and significant reduction of level of miR-145 in plasma of breast cancer patients. Intriguingly, they showed that the combination of plasma miR-145 and miR-451 levels provided the best markers for discriminating breast cancer from healthy controls and all other types of cancers recruited in the study (colorectal cancer, esophagus cancer, gastric cancer, hepatocellular carcinoma and lung cancer). Interestingly, the increase of plasma miRNAs was detectable also in in early stages (pre-invasive stages) of breast cancer suggested that this marker might be very useful for early diagnosis (118).

Add to the above, seven miRNAs (miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-191 and miR-382) had differentially expressed in serum of breast cancer patients compared to healthy controls. Furthermore, combined receiver operating characteristic (ROC) curve analyses of a panel of three miRNAs (miR-145, miR-155 and miR-382) revealed that, those miRNAs are valuable biomarkers for distinguishing breast cancer from normal controls and the combination of the three miRNAs have better sensitivity (97.6%) and specificity (100%) than any other miRNA analysed separately (115).

In consistency with previous report, the level of miR-92a was found to be significantly lower, while miR-21 was higher, in tissue and serum samples of

breast cancer than that of healthy controls. Contrary, the statistical assessments between the concentrations of miRNAs and the other clinical and histopathological data as well as the number of circulating tumour cells (CTC) did not reach any statistical significance (114).

An effort to identify the specific miRNAs that are co-expressed in serum and tissue of breast cancer patients, a genome-wide miRNA analysis by next-generation sequencing was performed. MiR-103, miR-23a, miR-29a, miR-222, miR-23b, miR-24 and miR-25 were found to be co-upregulated. Among them, miR-222 was significantly increased in serum of breast cancer patients which may serve as valuable biomarker for differentiating breast cancer patients from normal individuals (119).

The number of papers reporting that circulating miRNAs could serve as non-invasive biomarkers for breast cancer detection is increasing. A genome-wide approach investigating circulating mRNA in primary breast cancer, showed that miR-148b, miR-376c, miR-409-3p, and miR-801 were identified as potential early stage breast cancer biomarkers in plasma (120). Chan et. al studied 20 circulating microRNAs signatures using a cohort of Asian Chinese breast cancer patients, and compare miRNA profiles between tumour and serum samples. MiR-1, miR-92a, miR-133a, and miR-133b were identified as the most important diagnostic markers, ROC combination of these miRNAs exhibited areas under the curves of 0.90 to 0.91 (121).

Taken together, in the recent years, circulating microRNAs have garnered a lot of attention and interest with differential levels in the patients with breast cancer compared to disease free controls. Certainly, they could be emerging as revolutionary sources of biomarker for breast cancer diagnosis.

### 1.6.2. Circulating microRNAs as potential prognostic biomarkers

Metastatic breast cancer (MBC) is a leading cause of morbidity and mortality among females. There is an urgent need for predictive as well as prognostic biomarkers that can improve the quality of life for these patients (122).

The identification of circulating miRNAs as potential non-invasive biomarkers for breast cancer and other diseases was followed by studies attempt to identify

miRNAs able to detect MBC. If the detection of invasive metastasizing cancer cells and their miRNAs migration to the lymph node is feasible, their potential use as minimally invasive biomarker would be an incredible breakthrough in disease monitoring.

The use of circulating tumour cells (CTC) as a prognostic marker in metastatic breast cancer (MBC) has been well described. Nevertheless, their efficacy and accuracy are still under scrutiny mainly because of methods of their enrichment and identification. Further, eight miRNAs, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801, were found to be significantly higher in plasma of CTC-positive metastatic breast cancer compared to CTC-negative patients and healthy controls. Combinations of these miRNAs or even miR-200b alone could represent valuable promising prognostic marker for progression-free survival and overall survival (122).

Deregulation of circulating miR-215, miR-299-5p and miR-41 were detected in patients with metastatic breast cancer, suggesting their value markers for metastases detection (123). Investigations in breast cancer cell line MCF-7 showed that miR-21 is responsible for migration and invasion by activation of the epithelial mesenchymal transition (EMT) (124). Furthermore, association of serum miR-21 with tumour size and a positive lymph node status have also been reported (112). Circulating miR-214, which targets the tumour suppressor gene phosphatase and tensin homolog (PTEN), which represent an indicator of metastatic spread to regional lymph nodes, was described in breast cancer (125).

Level of circulating miR-155, which mediates transforming growth factor-beta (TGF-β)-induced EMT and cell invasion, was differentially expressed in the serum of patients with hormone-sensitive breast carcinoma from patients with hormone-insensitive breast carcinoma (113) and further, correlated with p53 status, clinical stage and proliferation marker Ki-67 (126).

Additionally, changes in levels of circulating miRNAs; miR10b, miR34a and miR155 were linked to the presence of metastases. MiR-10b and miR-373 have been suggested to be a useful biomarker to differentiate between breast ductal carcinoma patients with lymph node metastasis and patients without lymph node metastasis with sensitivity of 71 % and of 68 % respectively and specificity of 72 %

and 89 % respectively. A combination of the two circulating miRNAs further enhanced the sensitivity to 72 % and the specificity to 94.3 % (127). Higher serum levels of miR-34a were detected in patients with advanced-stage disease compared to patients at early tumour stages.

Although promising, these results need to be further strengthen by independent confirmations before miRNAs can be successfully applied as biomarkers in clinical settings. In addition, several pre-analytical and analytical issues should be concern.

### 1.6.3. Circulating microRNAs as potential predictive biomarkers

In addition to the diagnostic and prognostic values, changes in levels of circulating miRNAs were hypothesised to play a role in the treatment of patients with breast cancer. Serum and in vitro measurements showed that miR-125b, which is down - regulated in HER2-amplified and -overexpressing breast cancers (70), may predict chemo-resistance response. In breast cancer cells, ectopic expression of miR-125b was correlated with increased resistance to anti-cancer drug administration. Moreover, when the level of miR-125b decreased breast cancer cells became sensitized to chemotherapy (128).

Due to treatment with the anti-HER2 monoclonal antibody trastuzumab has significantly improved the survival of patients with HER2-positive breast cancer, trastuzumab belongs to the standard treatment for these patients. However, not all patients respond to the treatment of trastuzumab. Interestingly, circulating miR-210 could additionally be beneficial because plasma miR-210, which was associated with trastuzumab sensitivity, may predict and monitor therapeutic response to trastuzumab (129). Furthermore, miR-210 seems to be a marker for hypoxia levels in tumours and is induced in hypoxic cancer cells (130). Previously, tumour hypoxia has been associated with a poor prognosis and resistance to chemotherapy and radio therapy (131), suggesting the use of miR-210 as therapeutic indicator for breast cancer.

## 1.7. Pros and cons of current methodologies for breast cancer management

Whereas most studies focus on improving prognosis with better therapies, an equally promising approach is to detect cancer at an early stage. Mammography is the current gold standard tool using ionizing radiation for breast cancer detection, but can have false negative rates of up to 20% (National Cancer Institute (Bethesda, MD) data; http://www.cancer.gov). The diagnosis of breast cancer relies on the histologic examination of tissue biopsies or cytology of fine-needle aspirates, which are both invasive procedures (132). Then again, as serum based biomarkers, carbohydrate antigen 15-3 (CA 15-3) is the most widely applied for breast cancer. However, the lack of sensitivity hinders its clinical use in early stage cancer. Other serum markers such as carcinoembryonic antigen (CEA) and tissue polypeptide specific antigen (TPS) are even less sensitive than CA 15-3 and so they are not useful for the early detection of breast cancer. Currently, CA 15-3 and CEA are mainly used to monitor therapy in metastatic breast cancer in combination with history, physical examination and imaging (116). Furthermore, prognostic markers of primary breast cancer such as plasminogen activator inhibitor 1, urokinase type plasminogen activator and *ERBB2*, have a limited ability to predict eventual metastatic progression (103).

Given these points, the development of novel, minimally invasive, diagnostic, clinically validated approaches to supplement breast imaging and improve detection rates and breast cancer screening compliance, remains an unmet challenge for breast cancer. Also, urgent improvements in the detection of metastasis from primary breast tumours are required in order to reduce mortality and also to reduce treatment costs.

## 1.8. Breast cancer needs new biomarkers

As challenges in management of breast cancer, not only treatment but also discoveries of sensitive and specific minimally invasive biomarkers that can be exploited to detect early neoplastic changes are important. Consequently, facilitate the detection of breast cancer at an early stage and of course greatly reduce the worldwide health burden of cancer. The ideal biomarker should be easily accessible for example, it can be relatively noninvasively sampled, sensitive

enough to detect early presence of tumours in almost all patients, and absent or minimally present in healthy tumour free individuals (133). On one hand, for those patients whose tumour tissue are not available, assays that allow the repetitive monitoring of patients by using blood samples may be efficient in evaluating cancer progression. On the whole, all things considered none of the existing diagnostic tools and biomarkers for breast cancer meets the above criteria.

In order to move beyond the modest benefits achieved to date, truly innovative approaches those can complement and improve on current strategies for cancer detection are urgently desirable. Obviously, microRNA expression studies have been performed on tissue specimens. Rather, tumour-derived microRNAs can be present in blood and appear to be stable and protected from endogenous ribonuclease activity in circulation. Cell-free microRNAs (miRNAs) represent one of the novel strategies for cancer screening (116). With this in mind, some studies have shown diagnostic and prognostic potential for circulating microRNAs (134).

## 1.9. Characteristics of circulating miRNAs as biomarkers

Since discovery, circulating miRNAs have attracted a great deal of attention as promising non-invasive biomarkers for breast cancer (102). The promise of circulating miRNAs as an early detection/prognostic/predictive marker has been evaluated in different solid carcinomas, including early breast cancer as well as in metastatic breast cancer (107).

As gorgeous revelation, Mitchell et. al found that, circulating miRNAs represent suitable biomarkers for an early cancer detection because of their presence and inherent stability human serum or plasma (105), ease of sampling by minimally invasive methods and the proven role of miRNAs in cancer development and progression. Furthermore, circulating populations of miRNAs are, in part, tumour derived (135). Subsequently, they appear to be differentially expressed in cancer patients compare to healthy controls. Henegan et al. described a consistent and specific up-regulation of miR-195 levels in blood of breast cancer patients (107), neither detected in blood of healthy controls nor in patients with other examined cancer types (prostatic, renal, colorectal and melanoma) (96). Add to the above, circulating miRNAs exhibit altered expression levels in association with clinicopathologic features of breast cancer.

It should be noted, all these characteristics open up the possibility of using circulating miRNAs as non-invasive, specific and sensitive biomarkers for breast cancer.

Notwithstanding with the promising findings, a comprehensive review of genome-wide circulating miRNA data showed that, data was almost completely lacking concordance. Hence, the utility of quantification of circulating miRNAs for breast cancer detection is still questionable (136).These discrepancies might be due mainly to the lack of a standardized method and an established endogenous miRNA control to normalize miRNA amounts (125).

## 1.10. Challenges of adopting circulating miRNAs as biomarkers

Literature on circulating miRNAs and breast cancer is enormous (>100 PubMed hits as of December, 2015) and very diverse in experimental approaches taken. To date, development of a reliable, reproducible, and non-invasive clinical test using circulating miRNAs is still in its infancy and there is a long way to go before using circulating miRNAs as clinical biomarkers for breast cancer. The accurate measurement of these miRNAs has been associated with many challenges, including those related to pre-analytic and analytical variations. Most importantly, detailed validation of the pre-analytical variables affecting miRNA detection and quantification is critical when considering the use of individual miRNAs as clinical biomarkers (137). In the same way, many studies have been conducted in circulating miRNA analysis cannot be replicated because data are missing or procedure are inadequately described.

*What is the suitable sample for circulating miRNA assessment?*

Is it blood, serum or plasma? While Mitchell et al. found no significant differences when comparing serum and plasma levels of miRNAs (105, 138), this was limited to only four miRNAs and might not represent the global situation. Recently, researchers found that serum samples yielded lower miRNA concentrations (107) (107). Further study, linked that difference to the presence of cellular contamination, in particular, platelets. Serum samples should be more suitable to minimize the variation introduced by variable levels of platelet contamination. Moreover, type of anticoagulant used, Ethylene diamine tetra acetic acid (EDTA)

and citrate are acceptable, but heparin impedes the reverse transcription quantitative PCR (RT-qPCR). Also, centrifugation protocols used to separate serum or plasma require normalization before results can be compared (139).

*What is the appropriate normalization for circulating miRNA quantification?*

Obviously, crucial analytical issue is the use of appropriate normalization controls. So far, several normalization strategies have been used for the analysis of circulating miRNAs, however, there is no consensus. Some genes such as RNU6B or 18S rRNA have been used to normalize data (107, 115), however in other studies they were found to be highly variable or sensitive to degradation. MiR-16 has been used in many studies as an internal normalization control (114, 133), but was later found to be susceptible to hemolysis and was related to some diseases that would make it unstable in circulation (139). Synthetic C. elegans miRNAs, such as Cel-miR-39 been used as spike-in control during RNA isolation (120, 140). Nevertheless, they were later found to be degraded by RNase in the circulation. For the above reasons, some researchers chose to perform normalization without the use of a reference gene. For instance, Hu et al. (141) used a healthy donor sample, which was processed together with the test samples.

Taken together, it is clear why circulating miRNAs analysis is yet to be implemented in the clinic: certain variables remain to explored and normalized.

## 1.11. Absolute quantification of nucleic acids by droplet digital PCR

Digital PCR, as the less familiar cousin of widely used quantitative PCR moves mainstream, researchers have more options to choose from.

The concept of digital PCR was first described in 1992 to describe a PCR carried out in limiting dilution conditions and able to detect very rare targets (142, 143). For many years, although the theory was simple, its implementation was no. But the recent development of several commercial platforms has brought the technology into the research and diagnostic settings (144). In particular, the droplet digital PCR (ddPCR) system (145) is based on the partitioning of a reaction mixture into thousands of oil-dispersed, nanoliter-sized microdroplets. The ddPCR

methodology has been successfully applied in the detection of copy-number variations (146, 147) and in the diagnosis of viral and bacterial infections (148, 149), providing equivalent results to standard procedures using RT-qPCR. In general, digital PCR uses the same primers and probes as qPCR but is capable of higher sensitivity and precision. In standard implementations, qPCR cannot distinguish gene expression differences or copy number variants smaller than about twofold. Additionally, ddPCR proved to be more tolerant than qPCR to the presence of inhibitors of the amplification reaction (150).

Recently, ddPCR has also been applied to the quantification of miRNAs circulating in the blood. In particular, Hindson and colleagues (10) found that, for quantifying circulating miRNAs, a prototype of the QX100 Droplet Digital PCR system (Bio-Rad Laboratories) was superior to qPCR carried out with TaqMan miRNA assays (Applied Biosystems and Life Technologies). Now, a second-generation instrument (named QX200) has been developed that is able to detect both TaqMan probe and DNA-binding dye chemistries with comparable precision and accuracy (151). In that work, ddPCR using the recently commercialized EvaGreen DNA-binding dye was shown to be comparable with TaqMan assays for quantification of mRNA; however, miRNA was not investigated.

## 1.12. Rationale and objectives

Currently, there is a lack of non-invasive tumour biomarkers with appropriate sensitivity and specificity to be used in routine clinical testing. The use of circulating microRNAs (miRNAs) as cancer biomarkers has been hypothesised based on their presence and stability in the circulation. Promising initial results for these tiny RNAs has been obtained in the field of breast cancer diagnostics. However, the accurate quantification of circulating miRNAs is more challenging than expected. In particular, several pre- and analytical variables have an impact on their final quantification, including the quantification method.

A comparison of miRNA quantification systems and platforms using body-fluids derived miRNAs has been performed, in the context of a more comprehensive study, by Mestdagh and colleagues (152). This study highlighted the lower sensitivity of hybridization and sequencing technologies when quantifying serum miRNAs if compared to qPCR platforms.

The ddPCR technology has been applied to the quantification of microRNAs (miRNAs) circulating in the blood. Droplet digital PCR (Bio-Rad Laboratories) has been successfully used with TaqMan assays to assess gene expression through the quantification of mRNA and miRNA. The system was superior to conventional qPCR, allowing also an absolute quantification, Hindson and colleagues say (151).

Recently, a second-generation instrument has been developed that is able to detect both the TaqMan-probe as well as DNA-binding dye chemistries with comparable precision and accuracy and proved to be very promising in liquid biopsy applications (153), but it has not yet been tested for miRNA.

In the first part of the study, in order to develop a precise and accurate technique for miRNA quantification, we took the advantage of dd-PCR technology to test and compare the feasibility of quantifying circulating miRNAs with the new BioRad ddPCR system (named QX200) when used with EvaGreen dye– and TaqMan probe–based assays.

While sensitivity and specificity of currently used breast cancer biomarkers include carcinoembryonic antigen and carbohydrate antigen 15–3 are low, other

biomarkers might be actively involved and thus present novel and more useful biomarkers for breast cancer. Among the type of currently under investigation as potential biomarkers are circulating microRNAs. MiRNAs can be detected in serum or plasma, and their levels may be specifically altered in pathological conditions. Because of their remarkable stability in plasma and serum and the possibility of measuring their levels using non-invasive methods, various studies have suggested a role for circulating miRNAs as novel cancer biomarkers (104, 105, 114, 133, 154).

In second and third part of the study, we aimed to investigate our selected panel of six circulating miRNAs in breast cancer patients and disease free controls, and further evaluate their diagnostic and prognostic potential as biomarkers for breast cancer using our newly developed EvaGreen-based dd-PCR miRNA protocol.

# 2. Materials and Methods

## 2.1. Study description

This study was carried out in department of Experimental and Diagnostic Medicine, University of Ferrara during the period from 2013 to 2015.

## 2.2. Ethics Statement

Ethical approval was granted as a part of ongoing cancer researches by local Ethics committee of Ferrara University Hospital (Italy) and of institutional review board of Mercy Women's Center in Oklahoma-USA. Before the sample was taken, written informed consent from all participants involved in the study was obtained for use of their samples for research purposes.

## 2.3. Study population

### 2.3.1. Cancer Patients and disease free Controls

At Ferrara University Hospital (Ferrara, Italy) peripheral venous blood was collected from 164 persons. Of whom, 46 with breast cancer, 18 with colorectal cancer, 18 with lung cancer, and 8 with melanoma tumours, and from age-matched 47 healthy donors. At Mercy Hospital (OK, USA), blood was collected from 90 patients, 60 of whom had their sample drawn pre-biopsy for what proved to be primary breast carcinoma. The 30 controls were drawn from subjects in a high-risk surveillance program, where women were followed with a combination of mammography and breast MRI. Subjects were designated as controls only when breast imaging was completely negative.

No patients had received any treatment prior to surgery, neither neo-adjuvant chemotherapy nor radiation therapy. Controls blood samples were collected concurrently, using the same collection and sampling procedures.

### 2.3.2. Clinical and pathological data of the study population

A questionnaire containing essential patient's identification data (personal, life style and clinical data) was obtained from each patient. For all patients analysed, histopathological diagnosis was confirmed after surgical resection of the tumours

and histopathological records of tumours sample were retrieved from the surgery units of the two diagnostic centres.

## 2.4. Study design

This study consisted of three parts (**Figure 2**); at first, development of Droplet Digital PCR (ddPCR) protocol for quantification of circulating microRNA and comparison of EvaGreen and TaqMan-based chemistries in quantification of circulating miRNAs by ddPCR. Second, selection of circulating miRNAs candidate for breast cancer from qualitative microRNA microarray screening and from published literature. Finally, validation of a panel of selected six miRNAs candidates in tow independent cohorts of sera of breast cancer patients and healthy controls and further, assessment of their diagnostic and prognostic value in breast cancer.

### 2.4.1. Part 1: Development of methodology for circulating miRNAs quantification

Plasma and serum collected from each of 28 individuals, of whom, 18 persons with any type of cancer and from 10 healthy persons at Ferrara University Hospital. RNA was reverse-transcribed and quantified for two circulating miRNAs and one added exogenous miRNA, with both EvaGreen dye–based miRCURY LNA miRNA assays and TaqMan assays. Amplification and detection of target miRNAs were performed on the QX200 ddPCR system. Conditions required to run miRCURY LNA miRNA assays were optimized.

### 2.4.2. Part 2: Selection of candidate circulating miRNAs for breast cancer

We selected candidates circulating miRNAs in breast cancer based on results of microarray screening of cell-free miRNAs revealed by facilities in our laboratory and by search in recently published scientific literature about potential circulating microRNAs biomarkers in breast cancer.

#### 2.4.2.1. MicroRNA Microarray

Plasma samples were evaluated for global miRNA expression profile using the Agilent miRNA microarrays (G4870A) at the Microarray Facility of Ferrara University. This array is capable to assess the expression of 1200 human

miRNAs. Eighty samples from 18 breast cancer (BRCA), 18 colorectal cancer (CRC), 18 lung cancer (LC), 8 melanoma (M) and 18 healthy control (C) samples were evaluated. A fixed input volume (15 µl) of RNA was used for the hybridization procedures. Experiments were performed as previously described [20]. Microarray raw data were imported and analyzed using GeneSpring GX 12 software (Agilent Technologies). Since a signal was detectable for at least 150 probes for each sample, a quantile was applied for normalization before statistical analyses. Global correlation coefficients were calculated using GeneSpring software.

#### 2.4.2.2. Selection of candidate circulating miRNAs for breast cancer

In this part of the study, we have searched in PubMed for the recent-findings in breast cancer related circulating miRNAs with keywords of: circulating, plasma, serum, blood, microRNAs, miRNAs and breast cancer. Then we have presented the data in a tabular form containing a summary of published scientific literature for circulating miRNAs as being potential diagnostic and/or prognostic biomarkers of both primary and metastatic breast cancers.

### 2.4.3. Part 3: Validation of circulating microRNAs in the serum of breast cancer patients by Eva Green-ddPCR

Six candidates of circulating miRNAs were investigated in serum samples of two cohorts obtained from two diagnostic centers. Italy cohort (n=56) was collected at the General Surgery Unit of the University Hospital of Ferrara, Italy during 2012 to 2014, of whom, 28 breast cancer patients pre-surgery and 27 age-matched disease-free controls. USA cohort (n=94) was collected at Mercy Women's Center in Oklahoma City, OK, USA, during 2005 to 2013, of whom, 59 breast cancer patients pre- surgery and 35 age-matched controls.

## 2.5. Blood samples and RNA isolation

Blood samples were obtained from cancer patients prior to surgery. Serum and plasma were prepared within 1 h of sample collection and immediately stored at −80 °C.

### 2.5.1 Plasma and Serum collection

For plasma preparation, 5 mL blood was collected in EDTA (Ethylene diamine tetra acetic acid) tubes (Vacuette); samples were centrifuged at 1,000 g for 10 minutes to remove blood cells, and the supernatant plasma was dispensed in aliquots and stored at -80°C.

For serum preparation, 5mL blood samples from Italy and USA cohorts were collected in red stopper clot tubes (BD Vacutainer) and kept at room temperature to clot for at least 1 hour; they were centrifuged at 1000g for 10 minutes at room temperature then, serum was removed and dispensed in aliquots and stored at -80°C.

### 2.5.2. RNA Isolation procedure:

Total RNA including miRNA was extracted from a fixed volume of 200 µl of plasma or serum using the miRNeasy Mini Kit (cat. no. 217004; Qiagen) according to the manufacturer's supplementary protocol (155) with two minor modifications. To be mentioned, addition of synthetic miRNA cel-miR-39-3p from C. elegans and RNA elution was performed in 35 µL nuclease-free water. The procedure was performed as follows: Frozen serum or plasma was thawed, 200 µl of the sample dispensed into labelled 2 ml tube and homogenized in 1 ml of QIAzol Lysis Reagent. The homogenized sample was mixed by vortexing and incubated for 5 min at room temperature (15–25°C) to permit complete dissociation of the nucleoprotein complex. As Spike-In control, 3 µl of a 4.16 nmol/L solution of the synthetic miRNA cel-miR-39-3p from C. elegans (custom synthesized by Integrated DNA Technologies) was added and mixed thoroughly. Then, 200 µl of chloroform per 1 mL of QIAzol was added and the tube was shaked vigorously by hand for 15 seconds (s). After incubation for 2–3 minutes (min) at room temperature, the tube was centrifuged for 15 min at 12,000 x *g* at 4°C. Only the colourless upper aqueous phase of the samples was transferred to a new collection tube. Then, 700 µl of 100% ethanol was added and mixed thoroughly by pipetting up and down several times. Without delay, 700 µl of the sample, including any precipitate that may have formed was transferred into an RNeasy MinElute spin column in a 2 ml collection tube. After centrifugation at 8000 x *g* for 15 s at room temperature, the flow-through was discarded. The last step was

repeated using the remainder of the sample. Also the flow-through was discarded each time. To wash the column membrane, three buffer washes were performed by adding 700 µl of buffer RWT into the spin column followed by two washes of 500 µl of buffer RPE. In each wash, the buffer was added, the tube was centrifuged for 15 s at 8000 x *g* and the flow-through was discarded. Afterward, 500 µl of 80% ethanol was added into the spin column and then centrifugation was performed for 2 min at 8000 x *g*. To avoid carryover of ethanol, carefully the spin column was removed from the collection tube. Then, the collection tube was discarded with the flow-through. The spin column was placed into a new 2 ml collection tube. To dry the membrane, the column was centrifuged at full speed for 5 min and the collection tube was discarded with the flow-through. Then, the spin column was placed in a new 1.5 ml collection tube. Finally, to elute the RNA, 35 µl RNase-free water was added directly to the centre of the spin column membrane, the lid gently was closed, and the column was centrifuged for 1 min at full speed. The RNA samples were immediately stored at -80°C.

## 2.6. Reverse Transcription and Droplet Digital PCR

### 2.6.1. Universal Reverse transcription for EvaGreen assays

Synthesis of cDNA was performed in a 20 µL reaction using the Universal cDNA synthesis kit II (Cat. No. 203301; Exiqon) starting from 3 µL of RNA according to the manufacturer’s guidelines for miRNA profiling in serum and plasma samples (156) as follows; Firstly, the 5x Reaction buffer and nuclease-free water were thawed, immediately placed on ice and mixed by vortexing. Afterward, the RNA spike-ins control was re-suspended according to the appropriate RNA Spike-ins protocol and then leaved on ice for 15-20 min. Immediately before use, the Enzyme mix was removed from the freezer, mixed by flicking and placed on ice. All reagents were spun down. The required amount of reverse transcription (RT) working solution for one reaction was prepared from; 4 µL of 5x Reaction buffer, 9 µL water, 1 µL Synthetic RNA spike ins (Uni Sp6), 2 µL Enzyme mix, and 4 µL RNA and then placed on ice. RT working solution was dispensed into nuclease free tubes and the template RNA was added in each tube. To ensure that all reagents are thoroughly mixed, the reaction was mixed very gently by vortexing and spun down. After that, the reaction was incubated for 60 min at 42℃, followed by heat-inactivation of the reverse transcriptase for 5 min at 95℃. Finally, the

reaction was immediately cooled to 4°C and cDNA samples were stored at at -20°C.

In nuclease free water, the synthesized cDNA was diluted 50-folds before the amplification for all investigated miRNAs except for the cel-miR-39-3p and miR-21-5p assays were diluted 500-folds. To optimize the sensitivity and accuracy of the assays, over a range of target cDNA concentrations and different volume of primers solution were tested.

### 2.6.2. Droplet digital PCR-EvaGreen assays

PCR was performed in a 20 µL reaction volume using EvaGreen supermix (Bio-Rad; cat, 1864034). Nuclease free water and cDNA were thawed for 15-20 min at room temperature. EvaGreen Supermix vial was always protected from light. Immediately before use, all reagents were mixed by vortexing and spined down. The optimum condition of the PCR primers and PCR EvaGreen Supermix was prepared in the proportion as follows; 10 µL of 2X EvaGreen supermix (Bio- Rad), and 0.5, 1 or 2 µL of one of the following miRCURY LNA PCR primer sets (Exiqon): hsamiR-320a (ID, 204154), cel-miR-39-3p (ID, 203952), hsamiR- 21-5p (ID, 204230), hsamiR-10b-5p (ID, 205637), hsamiR-145-5p (ID, 204483), hsamiR-148b-3p (ID, 204047), miR-181a-5p (ID, 204566), hsamiR-425-5p (ID, 204337) and hsamiR-652-3p (ID, 204387). Afterwards, 12 µl of the master mix was dispensed into each well and 8 µL of diluted cDNA was added to the corresponding well.

### 2.6.3. Reverse transcription for TaqMan assays

cDNA was synthesized in 15 µL reaction using the standard protocol TaqMan miRNA Reverse Transcription kits (Cat. No. 4366596; Life Technologies) from human plasma and serum samples. The process for one reaction involves the following procedures. To prepare RT master mix, all the kit components were allowed to thaw on ice and then spun down. In a polypropylene tube, 7ul of the RT master mix working solution for one reaction was prepared as follows; The 10 µL-reverse transcription reaction contained 0.15 µL of 100 mM dNTPs, 1 µL MultiScribe Reverse Transcriptase (50 U/µL), 1.5 µL of 10× Reverse Transcription Buffer, 0.19 µL of RNase Inhibitor (20 U/µL), nuclease-free water. Afterwards, the reaction was mixed gently and shortly centrifuged to bring the solution to the

bottom of the tube. Then, 5 µL RNA for each sample and 3 µL of primers specific for two circulating human miRNAs; miR-320a (assay ID, 002277), and miR-21-5p (assay ID, 000397), and one added control miRNA; cel-miR-39-3p (assay ID, 000200) was transferred into the corresponding RT reaction tube. Then the tube was closed, mixed gently and centrifuged to bring solution to the bottom of the tube. After incubation of the tube on ice for 5 min, on Thermal Cycler (name) the reaction was carried out at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes.

The resulting cDNA (undiluted for miR-320a or diluted 1:100 for miR-21-5p and cel-miR-39-3p) was prepared for amplification.

### 2.6.2. Droplet digital-TaqMan assays

PCR was performed in a 20 µL reaction volume according to manufacturer's protocol for ddPCR™ Supermix for Probes (Cat. No.1863010; Bio-Rad). Nuclease free water and cDNA were thawed for 15-20 min at room temperature. 2X ddPCR Supermix for probes vial was protected from light. Immediately before use, all reagents were mixed by vortexing and spinned down. Master mix working solution of the PCR primers and ddPCR Supermix for Probes was prepared in the following proportion as follows; 10 µL of 2X ddPCR Supermix for Probes (Bio-Rad) and 1 µL of 20X TaqMan miRNA PCR primer probe set of one of the following primer sets miR-320a, miR-21-5p or cel-miR-39-3p. Afterwards, 12 µl of the master mix was dispensed into each well and 8 µL of diluted cDNA was added to the corresponding well.

## 2.7. Droplet digital PCR workflow

### 2.1.2. Droplet Generation

Each sample was loaded into individual well of a disposable eight wells droplet generator cartridge (Bio-Rad). Then, 70 µL of droplet generator oil for EvaGreen or TaqMan probes (Bio-Rad) (according to the assay) was loaded into each of the eight oil wells. Samples then were placed into the QX200 Droplet Generator, which utilizes proprietary reagents and microfluidics to partition the samples into 20,000 nanoliter-sized droplets. The droplets created by the QX200 Droplet Generator are uniform in size and volume.

### 2.7.3. PCR Amplification of Droplets

Each sample was then transferred into a 96-PCR plate (Eppendorf) using a Rainin multichannel pipet then the plate was heat-sealed with foil and PCR performed to end-point according manufacture's protocol (Bio-Rad). Thermal cycling conditions for EvaGreen assays were as follows: 95° C for 5 minutes, then 40 cycles of 95°C for 30 seconds and (56-58°C according to specific miRNA) for 1 min (ramping rate reduced to 2%), and three final steps at 4° C for 5 minutes, 90°C for 5 minutes, and a 4°C indefinite hold to enhance dye stabilization (153). Thermal cycling conditions for TaqMan assays were as follows: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1min (ramping rate reduced to 2%), and a final inactivation step at 98°C for 10 min. A no template control (NTC) and a negative control for each reverse transcription reaction (RT-neg) were included in every assay.

### 2.7.4. Droplet reading and results analysis

After the PCR amplification of the nucleic acid target, the QX200 droplet reader (Bio-Rad) and its bundled QuantaSoft™ software were used to count PCR-positive and PCR-negative droplets: the "singulator" unpacks the emulsified droplets and streams them in a single line past a two-color optical detection system. Positive droplets, which contain at least one copy of the target miRNA, exhibit increased fluorescence compared to negative droplets. The fraction of PCR-positive droplets enables the target to be quantified according to Poisson distribution to determine the absolute initial copy number of the target miRNA molecule in the input reaction mixture in units of copies/μl. we calculated the absolute copies in 1 μl of plasma or serum multiplying the obtained concentration in ddPCR reaction value for a dilution factor (145.83).

## 2.8. Reverse transcription quantitative PCR (RT-qPCR).

Reverse transcription quantitative PCR was performed in 20 μl reaction for miR-10b- 5p and miR-652-3p using the Precsicion Melt Supermix (Cat No.172-5110; Bio-Rad). In brief ,10 μL of Precsicion Melt Supermix (Bio-Rad), and 0.5 or 1μL of one of miRCURY LNA PCR primer sets (Exiqon): hsamiR-10b-5p (ID, 205637), and hsamiR-652-3p (ID, 204387). Afterwards, 12 μl of the master mix was dispensed into each well and 8 μL of diluted cDNA (reversed transcribed using Universal cDNA synthesis kit II as mentioned before). Thermal cycling conditions

for were as follows: 95° C for 5 minutes, then 40 cycles of 95°C for 30 seconds and (56-58°C according to specific miRNA) for 1 min (ramping rate reduced to 2%), and three final steps at 4° C for 5 minutes, 90°C for 5 minutes, and a 4°C indefinite hold to enhance dye stabilization. The expression levels of miRNAs were normalized to Cel-miR-39 (ID, 203952) with the 2^-ΔΔCt method.

## 2.9. Statistical analysis of validation of data

Statistical analysis was performed using Prism software version 5.0 (GraphPad, La Jolla, CA). Data were $log_2$ transformed for graphical representation. The unpaired T-test with or without Welch's correction was performed to assess significance of differences between data distribution. P-value less than 0.05 were deemed to be significant. Binary logistic regression analysis was performed and receiver operating characteristic (ROC) curves were generated to assess the ability of chosen miRNAs to distinguish cancer cases from controls. The area under the ROC curve (AUC) was then estimated. P values less than 0.05 were considered to be statistically significant.

# 3. Results

This study consisted of three parts (**Figure 2**); at first, development of Droplet Digital PCR (ddPCR) protocol for quantification of circulating microRNA and comparison of EvaGreen and TaqMan-based chemistries in quantification of circulating miRNAs by ddPCR. Second, selection of circulating miRNAs candidate for breast cancer from qualitative microRNA microarray screening and from published literature. Finally, validation of a panel of selected six miRNAs candidates in tow independent cohorts of sera of breast cancer patients and healthy controls and further, assessment of their diagnostic and prognostic value in breast cancer.

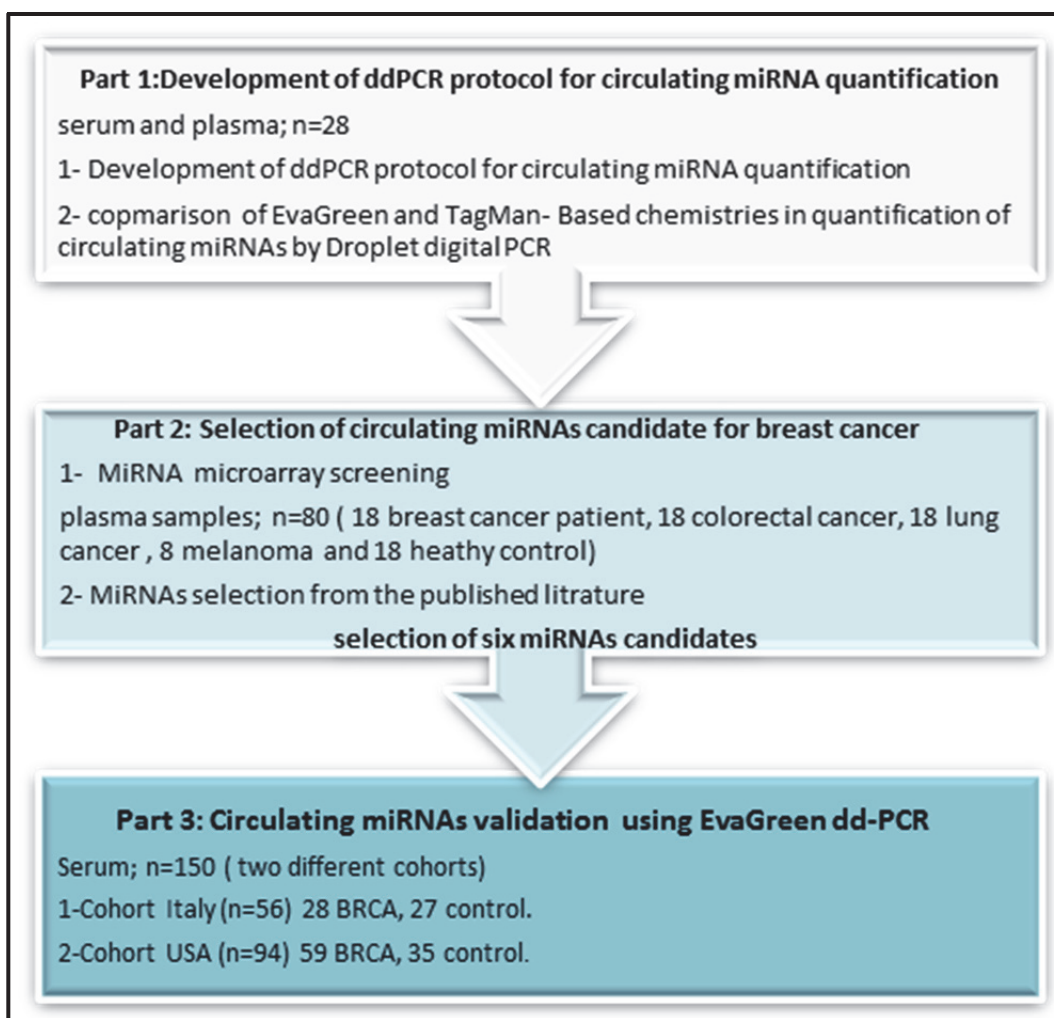



**Figure 2. Schematic diagram of the workflow of this study**

# 3.1. Part 1: Development of ddPCR protocol for circulating miRNA quantification

## 3.1.1. Optimization of EvaGreen-based ddPCR for miRNA quantification

At first, EvaGreen assays have not been tested for miRNA quantification on a ddPCR instrument. Therefore, we had to identify the optimal concentrations of miRCURY LNA primers (Exiqon) for the QX200 Droplet Digital system (Bio-Rad). Moreover, because plasma presents a complex background for PCR, we tested the assay using 10 different RNA preparations from plasma of patients with cancer and healthy individuals. We quantified both a synthetic miRNA (cel-miR-39-3p), the spike-in that was added to plasma at the beginning of RNA extraction and a human miRNA (miR-320a) which was previously figured out by microarray experiments to be present in the samples.

### 3.1.1.1. Concentration of the starting materials

To avoid positive droplet saturation, target miRNAs were quantified starting from a cDNA dilution of 50 folds for the miR-320a assay and 500 folds for the cel-miR-39-3p.

### 3.1.1.2. Optimum primer concentration

To optimize the primer concentration, both the recommended volume (2 µL) and half of that volume (1 µL) were tested, to examine which volume gives a better separation between positive and negative droplets; this step was motivated by the fact that EvaGreen can bind with low affinity to single-stranded DNA **(157).** The results of the ddPCR for cel-miR-39-3p in two plasma samples are shown in **Figure. 3A and B**. Reducing the volume of primers from 2 µL to 1 µL gave better performance in terms of droplet separation and reduced the spread of negative droplets, seen for two samples in the cel-miR-39-3p assay; no positive droplets were obtained for the NTC (non template control).

Comparable results were revealed among quantification of cel-miR-39-3p in two samples with reduction of primer volume from 2 µL to 1 µL. Quantification in terms of number of copies per microliter in the two samples did not change as indicated in **Figure (3C)**. Furthermore, the miR-320a assay was also performed using both 2 µL and 1 µL of LNA primers and cDNA from 6 samples **(Figure 3D).** Reduction of

the amount of primer did not change the quantification results. Almost identical results were obtained and 1μL of primer solution was decided to be used in subsequent experiments.

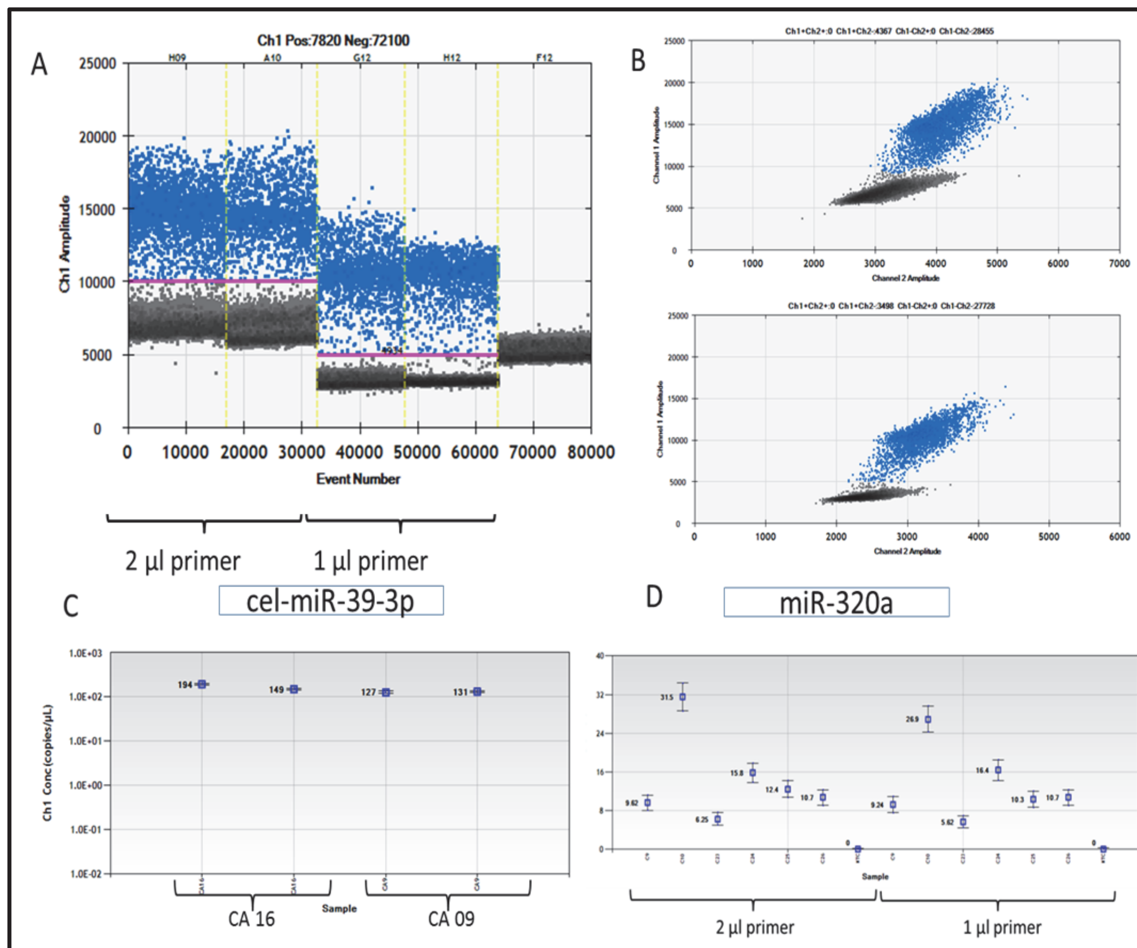



**Figure 3. Primers optimization.**

Effect of LNA primer concentration on the amplitude of positive (blue) and negative (black) droplets in EvaGreen-based ddPCR. Results are presented as copies per microliter in the amplification reaction. **A**, reducing the volume of primers from 2 μL to 1 μL gives a better performance in terms of droplet separation and reduces the spread of negative droplets, seen for two samples, CA16 and CA9, in the cel miR-39-3p assay; no positive droplets were obtained for the NTC. **B**, bi-dimensional droplet plots for the cel-miR-39-3p assay with 2 μL (top) or 1 μL (bottom) of LNA primers. **C,** reducing the volume of primers for cel-miR-39-3p did not change the quantification results; from the left, normalized copies/mL of CA16 sample with 2 or 1 μL of primer solution and of CA9 sample with 2 μL or 1 μL of primers. **D**, EvaGreen quantification of miR-320a in 6 samples using 2 μL LNA primer (left) or 1 μL LNA primer (right). Reducing the amount of primer did not change the quantification results.

### 3.1.2. Precision and sensitivity of EvaGreen-based ddPCR assays

#### 3.1.2.1. Within-run and overall precision

To calculate within-run and overall precision of EvaGreen-based ddPCR assays, cel-miR-39- 3p assay was run on 10 RNA samples from plasma in triplicate. In addition, analysis was repeated over 3 days to determine overall precision. The mean within-run coefficient of variation (CV) was 5.1% (range, 0.7%–10.7%). Furthermore, the overall mean of CV was 13.4% (range, 7.3%–20.4%) as shown in **Figure 4A** and **Table 1**.

#### 3.1.2.2. Concordance of the EvaGreen-based ddPCR assay in reverse-transcription duplicate

Working in duplicate, RNA from 8 plasma samples were reverse-transcribed and cel-miR-39-3p was amplified (which had been added before RNA extraction) using the EvaGreen-based assay **(Figure 4B).** Quantification of this miRNA in the two independent series displayed high concordance (Pearson r = 0.96), although we observed a high variability in cel-miR-39-3p recovery.

#### 3.1.2.3 Sensitivity and accuracy of EvaGreen-ddPCR

To assess the sensitivity and accuracy of EvaGreen ddPCR at different target cDNA concentrations, we ran the assay in duplicate on serial dilutions of cel-miR-39-3p (10–10,000 copies/µL) in water (**Figure 4C**). We found an extremely high concordance between the two series, and the responses were highly linear over four orders of magnitude. Moreover, the assay was able to detect cel-miR-39-3p down to the lower limit of 1 copy/ µL.

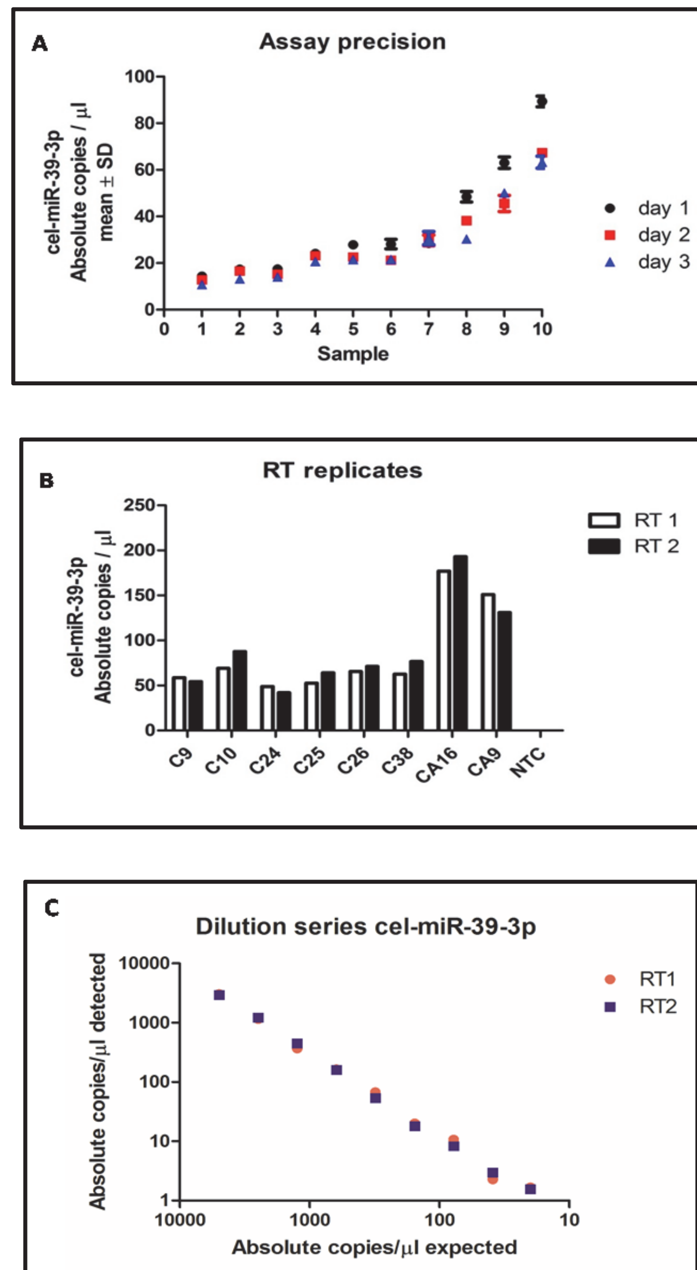



**Figure 4. Precision and performance of EvaGreen ddPCR assay.**
**A**; cel miR- 39-3p was quantified in triplicate on 3 days in a panel of 10 RNA preparations from plasma to which cel-miR-39-3p had been added. Each dot represent; average and SD of one day. Day replicates are represented by different colours. 1. **B**; replicate analyses of cel-miR-39-3p assay over two independent RT reactions from eight RNA samples and NTC. Pearson correlation of RT replicates was 0.96. **C**; quantification of cel-miR-39-3p in a water matrix by EvaGreen ddPCR shows linearity across the dynamic range of miRNA concentrations. Two independent RT reactions and dilution series were performed starting from a known amount of cel-miR- 39-3p. Linearity was maintained across four orders of magnitude. The lower limit of detection was 1 copy/ µL. Results are presented as copies per microliter of the amplification reaction mixture.

**Table 1. Within-run and overall precision variability of EvaGreen dye-based ddPCR assay for 10 samples.**

| | Day 1 | | | Day 2 | | | Day 3 | | | Overall precision | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (copies/ µl) | SD | CV | Mean copies/ µl | SD | CV | Mean copies/ µl | SD | CV | Average of 3 days copies/µl | SD | CV |
| **S1** | 14.367 | 0.777 | 0.054 | 12.900 | 0.608 | 0.047 | 10.767 | 0.751 | 0.070 | 12.678 | 1.686 | 0.133 |
| **S2** | 17.400 | 0.458 | 0.026 | 16.600 | 1.609 | 0.097 | 13.167 | 1.405 | 0.107 | 15.722 | 2.233 | 0.142 |
| **S3** | 17.467 | 1.250 | 0.072 | 15.300 | 0.755 | 0.049 | 14.067 | 0.907 | 0.065 | 15.611 | 1.721 | 0.110 |
| **S4** | 24.133 | 1.007 | 0.042 | 23.200 | 0.600 | 0.026 | 20.733 | 0.681 | 0.033 | 22.689 | 1.665 | 0.073 |
| **S5** | 27.900 | 0.265 | 0.009 | 22.600 | 0.436 | 0.019 | 21.533 | 1.644 | 0.076 | 24.011 | 3.076 | 0.128 |
| **S6** | 28.167 | 2.011 | 0.071 | 21.300 | 1.353 | 0.064 | 21.667 | 1.550 | 0.072 | 23.711 | 3.642 | 0.154 |
| **S7** | 28.467 | 1.234 | 0.043 | 29.767 | 2.268 | 0.076 | 30.733 | 2.822 | 0.092 | 29.656 | 2.151 | 0.073 |
| **S8** | 48.467 | 2.250 | 0.046 | 38.167 | 0.833 | 0.022 | 30.400 | 0.436 | 0.014 | 39.011 | 7.943 | 0.204 |
| **S9** | 63.067 | 2.454 | 0.039 | 45.600 | 3.477 | 0.076 | 50.133 | 1.762 | 0.035 | 52.933 | 8.180 | 0.155 |
| **S10** | 89.333 | 2.309 | 0.026 | 67.333 | 0.473 | 0.007 | 63.300 | 2.553 | 0.040 | 73.322 | 12.258 | 0.167 |

Results are presented as copies of cel-miR-39 per microliter of the amplification reaction. SD: standard deviation. CV: coefficient of variation calculated over 3 replicates or over 3 days (overall precision.

### 3.1.3. Comparison of EvaGreen- and TaqMan-ddPCR for miRNA quantification

#### 3.1.3.1. Comparison of EvaGreen- and TaqMan-ddPCR for miRNA quantification in plasma

To determine if the EvaGreen-based assay gives comparable results to the TaqMan assay on the QX200 ddPCR system, the synthetic cel-miR-39-3p (added as internal control) and the naturally occurring human circulating miR-320a were quantified in 10 RNA preparations from plasma. For TaqMan assays, the two miRNAs were reverse-transcribed using miRNA-specific primers and the resulting cDNA was used for amplification diluted 100-folds for cel-miR-39-3p and undiluted for miR-320a. To run the EvaGreen-based assay, the RNA samples were reverse-transcribed using the Exiqon Universal cDNA Synthesis Kit and the resulting cDNA was diluted 500-folds for cel-miR-39-3p and 50-folds for miR-320a. The quantification, expressed as absolute copies per microliter plasma, was highly concordant between the two assays for both miRNAs (**Figures 5A and B**). Pearson correlation coefficients between TaqMan and EvaGreen based assays were $r$ = 0.917 for cel-miR-39-3p, and $r$ = 0.986 for miR-320a. The concentration of the endogenous miR-320a ranged from 1,500 to 23,000 copies/µL of plasma **(Figure 5B**).

#### 3.1.3.2. Comparison of EvaGreen- and TaqMan-ddPCR for low abundance miRNA quantification in serum

The two assays were compared for their ability to quantify another naturally occurring miRNA in serum (miR-21-5p), where concentrations of endogenous miRNAs are lower than in plasma. This work showed that, in 16 RNA preparations from serum, miR 21-5p was indeed present at low concentrations (**Figure 5C**). In this case, the EvaGreen-based assay gave approximately 2-folds higher concentrations in the amplification reaction solution (range, 0.96–19.6 copies/µL) than the TaqMan assay (range, 1.3–8.2 copies/µL). Nevertheless, there was a high concordance between the two methodologies (Pearson $r$ = 0.92).

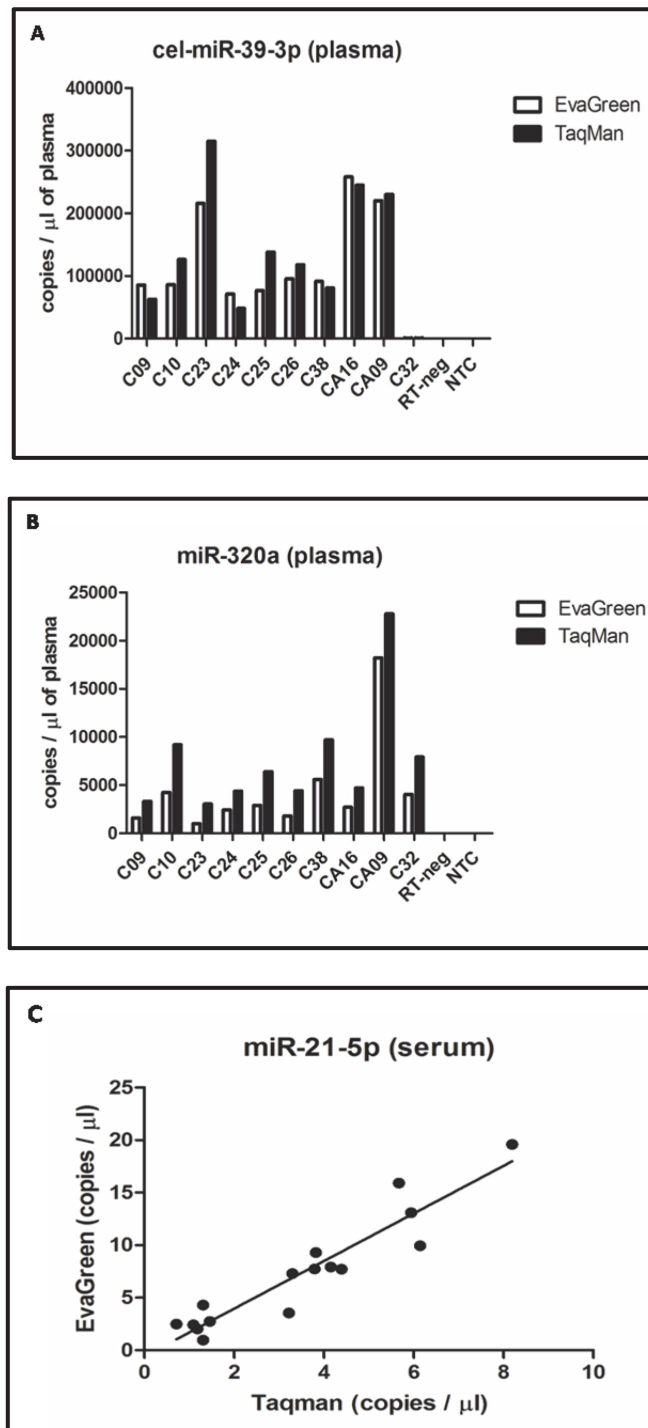



**Figure 5. Concordance of miRNA quantification by TaqMan and EvaGreen ddPCR.**
**A;** concentrations of exogenous cel-miR-39-3p added to plasma obtained with TaqMan- (black) and EvaGreen–based (white) assays. Results, presented as absolute copies per microliter plasma, were highly concordant for 10 RNA preparations (Pearson r = 0.917). **B**; concentrations of endogenous human miR-320a in plasma using the same assays show high concordance (Pearson r = 0.986). **C;** quantification of miR-21-5p in 16 serum samples using the same assays gives values that are highly concordant (Pearson r = 0.92). Results are presented as absolute copies per microliter of amplification reaction.

## 3.2. Part 2: Selection of circulating miRNA candidate

We selected six miRNAs (miR-10b-5p, miR-145-5p, miR-148b-3p, miR-181a-5p, miR-425-5p and miR-652-3p) derived from microarray experiments (158) or described in recently published scientific literature as being potential circulating biomarkers **(Table 2 and 3).**

### 3.2.1. MiRNA Microarray screening

Qualitative analysis was performed for miRNAs species circulating in 80 plasma samples of four different cancer types and healthy controls. Of whom, 18 breast cancer (BC), 18 colorectal cancer (CRC), 18 lung cancer (LC), 8 melanoma (M) and 18 healthy control (C) using microarray. The microRNA expression profile was generated using Agilent miRNA microarrays. Fixed volumes of total RNA were hybridized, derived from fixed plasma volumes.

Bioinformatics analyses revealed that 255 miRNAs were expressed in at least one sample (**Table 2**). Microarray experiments from plasma samples revealed a reduced consistency between samples from the same tumour type, if compared to other experiments performed with low-abundance RNA samples (159). Indeed, a poor correlation was found between patients with the same tumour. We used microarray data to obtain the global miRNA expression profile of all individual cancer types and control group.

### 3.2.2. MiRNA selection from published literature

In this part of the study, we have searched in PubMed for the recent findings in breast cancer related circulating miRNAs with keywords of: circulating, plasma, serum, blood, microRNAs, miRNAs and breast cancer. Then we have presented the data in a tabular form containing a summary of published scientific literature for five circulating miRNAs as being potential diagnostic and/or prognostic biomarkers of both primary and metastatic breast cancers (**Table 3**).

**Table 2. Normalized, log2-transformed levels of 255 miRNAs detected in the plasma of cancer patients and healthy subjects by microarray technology.**

| systematic_name | [Healthy](normalized) | [Breast](normalized) | [Colorectal](normalized) | [Lung](normalized) |
|---|---|---|---|---|
| hsa-let-7a-5p | -0.46704933 | -0.36127505 | 0.82199574 | -0.4092247 |
| hsa-let-7b-5p | 0.4864247 | -0.813748 | 0.6458966 | 0.27849776 |
| hsa-let-7c | -1.2824045 | -0.8480108 | -0.7759244 | -0.7053463 |
| hsa-let-7d-3p | -1.2824045 | -0.8518935 | -0.12207147 | -1.0784427 |
| hsa-let-7d-5p | -1.2824045 | -0.51310694 | 0.012715671 | -0.74021876 |
| hsa-let-7f-5p | -1.0067921 | -0.807136 | -0.22314477 | -1.020123 |
| hsa-let-7g-5p | 0.4296368 | -0.17533451 | 0.5791697 | -0.32400787 |
| hsa-let-7i-5p | 1.383868 | 0.503433 | 1.959558 | 0.8532918 |
| hsa-miR-101-3p | -0.7358751 | -1.1657774 | -1.3805063 | -0.88966686 |
| hsa-miR-103a-3p | 0.99691653 | 0.16487579 | 1.4226149 | 0.24456549 |
| hsa-miR-106b-5p | 0.759486 | 0.1802597 | 1.1785517 | -0.5977091 |
| hsa-miR-107 | 1.7537442 | 0.36129352 | 1.4995334 | 1.4695745 |
| hsa-miR-1180 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-1181 | 0.49245378 | -0.5704251 | -1.3805063 | -0.087510556 |
| hsa-miR-1182 | -0.94234985 | -1.4159147 | -1.3805063 | -0.8914354 |
| hsa-miR-1183 | -0.8681443 | -0.79406005 | -1.3805063 | -0.8277643 |
| hsa-miR-1202 | -1.054873 | -0.95691174 | -1.1482188 | -0.8541108 |
| hsa-miR-1207-5p | -0.7323167 | -0.8807661 | -1.0707039 | -0.9087234 |
| hsa-miR-122-5p | -0.35368782 | -0.5575304 | -0.84237415 | -0.6323043 |
| hsa-miR-1224-5p | 1.7828242 | 1.9568975 | 0.6317603 | 0.95204777 |
| hsa-miR-1225-3p | -0.9211494 | -1.0935578 | -1.3524427 | -0.8283473 |
| hsa-miR-1225-5p | -0.86395156 | -0.8786032 | -1.0410699 | -0.6565665 |
| hsa-miR-1226-5p | -0.6461885 | -0.6403432 | -1.3805063 | -0.87915665 |
| hsa-miR-1228-3p | -0.9262348 | -1.0737879 | -1.0455978 | -0.91683304 |
| hsa-miR-1228-5p | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-1229-3p | -0.16833009 | -0.38046205 | -0.36984742 | -0.24986045 |
| hsa-miR-1234-3p | -0.98061097 | -1.0795331 | -1.021668 | -0.8561147 |
| hsa-miR-1237-3p | 0.8166915 | 0.42202032 | 0.93275857 | 2.1721902 |
| hsa-miR-1238-3p | -0.94945186 | -1.0807948 | -1.0227942 | -0.9108155 |
| hsa-miR-1246 | 1.6018798 | 0.48315683 | 0.52122927 | 1.9537425 |
| hsa-miR-1249 | 1.226533 | 1.3916422 | 0.71214825 | 0.93025017 |
| hsa-miR-125a-3p | -1.2824045 | -1.4159147 | -1.1169324 | -1.2976347 |
| hsa-miR-125a-5p | -1.2824045 | -1.1319501 | -1.0929533 | -1.2976347 |
| hsa-miR-126-3p | -0.42701986 | -0.16380572 | 0.44486272 | -0.708022 |
| hsa-miR-1260a | 1.1891894 | 0.9676977 | 1.32424 | 1.88968 |
| hsa-miR-1260b | -1.2824045 | -1.1443322 | -1.3805063 | -0.77529854 |
| hsa-miR-1268a | -0.94030595 | -0.9526885 | -1.0896443 | -0.81269145 |
| hsa-miR-1273e | -1.2824045 | -1.2050394 | -1.3805063 | -1.2976347 |
| hsa-miR-1274a_v16.0 | -1.2824045 | -1.4159147 | -1.3805063 | -1.0114006 |
| hsa-miR-1274b_v16.0 | -1.4447086 | -0.29142728 | -0.5580933 | -0.8771031 |
| hsa-miR-1275 | -0.27414972 | -0.67388195 | -0.9049241 | -0.80179584 |

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| hsa-miR-1280_v18.0 | -0.8856801 | -1.0311278 | -1.0391788 | -0.70090044 |
| hsa-miR-1281 | -1.0701377 | -1.2069749 | -0.9213273 | -0.97610545 |
| hsa-miR-1287 | -1.2824045 | -1.4159147 | -1.3805063 | -0.6582118 |
| hsa-miR-1290 | -0.25438643 | -0.31705916 | -0.28947112 | 0.7238816 |
| hsa-miR-1295a | -0.92358047 | -1.1010746 | -1.1369905 | -0.39078897 |
| hsa-miR-1299 | -1.9229808 | -2.3636198 | -2.4734354 | -0.14900336 |
| hsa-miR-1306-3p | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
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| hsa-miR-130a-3p | -0.24556462 | -0.3351302 | 0.59211314 | 0.22887462 |
| hsa-miR-130b-3p | -0.99936223 | -0.73083884 | 0.2517574 | -0.70389533 |
| hsa-miR-134 | 0.9474284 | 0.38374504 | -1.0100888 | -0.042937968 |
| hsa-miR-135a-3p | 1.9614084 | 0.9895225 | -0.003809823 | 1.1009402 |
| hsa-miR-139-3p | -1.2824045 | -1.4159147 | -1.3805063 | -0.9653562 |
| hsa-miR-140-3p | 1.7215008 | 0.44962645 | 0.6608922 | 1.3532578 |
| hsa-miR-142-3p | -0.71415657 | -0.4137491 | 0.32476658 | -1.0311519 |
| hsa-miR-142-5p | -1.2824045 | -1.4159147 | -1.062109 | -1.0156173 |
| hsa-miR-144-3p | 1.2764777 | -0.03419309 | -0.42224374 | 0.16432701 |
| hsa-miR-146a-5p | -1.2824045 | -0.8079372 | -0.13303731 | -0.9920905 |
| hsa-miR-1471 | -0.5932255 | -1.0918913 | -1.3805063 | -0.846276 |
| hsa-miR-149-3p | 0.61673707 | 0.24185586 | -0.35264704 | -0.99766856 |
| hsa-miR-150-3p | -0.44433674 | -0.7385017 | -1.394449 | -1.1231768 |
| hsa-miR-150-5p | 0.8796475 | 0.056251638 | 0.40132985 | -0.25907645 |
| hsa-miR-151a-3p | -0.7331017 | -0.6607383 | 0.29737166 | -0.71247554 |
| hsa-miR-151a-5p | -1.2824045 | -0.77020574 | -0.04729814 | -1.2976347 |
| hsa-miR-1539 | -0.8050931 | -0.94102 | -0.7749078 | -1.0573063 |
| hsa-miR-15a-5p | 0.67961854 | -0.074502125 | 1.0104252 | -0.061566755 |
| hsa-miR-15b-5p | 0.87150097 | -0.023401193 | 1.1364322 | -0.67173374 |
| hsa-miR-16-5p | -0.8601122 | -1.7077851 | -1.4463747 | -2.14139 |
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| hsa-miR-185-5p | 0.95543236 | 0.29582706 | 0.9915462 | -0.15334673 |
| hsa-miR-187-5p | -0.71315914 | -1.2141304 | -1.3805063 | -0.89820564 |
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| hsa-miR-18b-5p | -1.2824045 | -1.4159147 | -1.3805063 | -1.0872508 |
| hsa-miR-191-3p | -0.99408066 | -1.1243131 | -1.069276 | -0.9358293 |
| hsa-miR-1914-3p | -0.6347608 | -0.8717506 | -1.0685077 | -0.9304413 |
| hsa-miR-1915-3p | -0.643559 | -0.8522072 | -0.9891398 | -0.8002764 |
| hsa-miR-193a-5p | -0.7100808 | 0.31532225 | -0.5885722 | -0.25352997 |
| hsa-miR-195-3p | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-197-3p | 0.43841603 | -0.029791275 | 1.125133 | 1.1375453 |
| hsa-miR-1973 | -0.99811107 | -1.4159147 | -1.1266166 | -0.33394426 |
| hsa-miR-198 | -0.89944774 | -1.4159147 | -1.3805063 | -0.88843465 |
| hsa-miR-199a-3p | -1.2824045 | -1.1275543 | 0.29526293 | -1.2976347 |
| hsa-miR-199a-5p | -1.2824045 | -0.85698265 | -0.20529811 | -1.2976347 |
| hsa-miR-19a-3p | 0.77988243 | 0.2733549 | 1.2784106 | -0.53456426 |
| hsa-miR-19b-3p | -0.062179856 | 0.4441168 | 0.29948503 | -2.3588905 |

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| hsa-miR-206 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-20a-5p | 1.3365445 | 0.48953423 | 1.7313402 | 0.057803974 |
| hsa-miR-20b-5p | 0.06739637 | -0.85531634 | -0.5334021 | -0.92488456 |
| hsa-miR-21-3p | -1.2824045 | -1.4159147 | -1.1184103 | -1.2976347 |
| hsa-miR-21-5p | -0.9853747 | -1.0716273 | 0.7776575 | -1.8300662 |
| hsa-miR-210 | -1.2824045 | -1.4159147 | -1.1003889 | -0.9453227 |
| hsa-miR-22-3p | -0.82073075 | -0.66697687 | -0.37593836 | -1.3026967 |
| hsa-miR-221-3p | -1.2824045 | -0.83346516 | -0.4402536 | -1.2976347 |
| hsa-miR-222-3p | -1.2824045 | -1.4159147 | -1.3805063 | -1.0259224 |
| hsa-miR-223-3p | 1.1135944 | 0.14411464 | 0.9380189 | 0.97938293 |
| hsa-miR-2276 | 2.3437424 | 1.991018 | 0.61229146 | 1.0167577 |
| hsa-miR-2278 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-23a-3p | 0.45115757 | -0.10199363 | 0.8521263 | 0.23649801 |
| hsa-miR-23b-3p | -1.2824045 | -1.4159147 | -0.8236495 | -1.2976347 |
| hsa-miR-24-3p | 1.0764958 | 0.34173816 | 1.7244741 | 0.4999459 |
| hsa-miR-25-3p | 1.2948729 | 0.86878014 | 0.8427911 | 0.2676149 |
| hsa-miR-26a-5p | -0.12748045 | -0.27897093 | 0.89641094 | -0.27826115 |
| hsa-miR-26b-5p | -0.7392442 | -1.1341896 | -0.7789519 | -0.9697827 |
| hsa-miR-27a-3p | -0.9897925 | -0.45076397 | 0.39539686 | -0.73671657 |
| hsa-miR-27b-3p | -1.2824045 | -1.4159147 | -1.1055894 | -1.2976347 |
| hsa-miR-2861 | -0.77032554 | -0.90144324 | -1.0852052 | -1.0435876 |
| hsa-miR-296-5p | -0.5297259 | -0.93851495 | -0.66682404 | -0.7783758 |
| hsa-miR-297 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-29a-3p | -1.0123423 | -0.8931518 | -0.8377231 | -0.45378944 |
| hsa-miR-29c-3p | -0.9580187 | -0.5608343 | -1.054233 | -0.89676124 |
| hsa-miR-301a-3p | -1.2824045 | -1.4159147 | -1.1176043 | -1.0304793 |
| hsa-miR-30a-5p | -1.2824045 | -1.4159147 | -1.3805063 | -1.0073717 |
| hsa-miR-30b-5p | -1.2824045 | -1.4159147 | -0.84618574 | -1.2976347 |
| hsa-miR-30c-1-3p | -1.050714 | -1.4159147 | -1.3805063 | -1.050386 |
| hsa-miR-30c-5p | -1.2824045 | -1.4159147 | -1.0820664 | -1.0301033 |
| hsa-miR-30d-5p | -1.4436047 | -0.46225834 | -0.4295826 | -1.5295652 |
| hsa-miR-30e-5p | 0.6327492 | 0.41475874 | 1.3434991 | 1.6107601 |
| hsa-miR-3124-5p | 1.4766182 | 1.137107 | 1.1298865 | -0.18080732 |
| hsa-miR-3125 | -1.2824045 | -1.0918295 | -1.3805063 | -1.2976347 |
| hsa-miR-3131 | -0.9609439 | -0.5379383 | -1.3805063 | -0.80739856 |
| hsa-miR-3137 | -0.9793327 | -1.4159147 | -1.3805063 | -0.89876676 |
| hsa-miR-3138 | 0.32645643 | 0.15508491 | 0.43199995 | 0.33178425 |
| hsa-miR-3141 | -1.1246516 | -1.0073414 | -1.1943402 | -0.8364318 |
| hsa-miR-3147 | -0.63234025 | -0.10598026 | -0.38234118 | 0.15215611 |
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| hsa-miR-3152-3p | -1.0230801 | -1.4159147 | -1.3805063 | -0.9171237 |
| hsa-miR-3156-5p | -2.1905925 | -1.5737908 | -1.9442245 | -0.032362938 |
| hsa-miR-3162-5p | -0.8514054 | -0.7870216 | -1.0060592 | -0.8827437 |
| hsa-miR-3180-5p | -0.1584693 | -0.3335616 | 0.20124139 | 0.37018543 |
| hsa-miR-3181 | -1.2824045 | -1.4159147 | -1.3805063 | -1.0366035 |
| hsa-miR-3188 | 0.6055193 | 0.83823806 | 0.1763433 | 2.0418813 |

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| hsa-miR-3190-3p | -1.2824045 | -1.4159147 | -1.3805063 | -0.9968485 |
| hsa-miR-3194-5p | -0.05173232 | 0.46820408 | -0.46743473 | 0.40953356 |
| hsa-miR-3195 | -0.9650228 | -0.8850899 | -0.79540867 | -0.72061 |
| hsa-miR-3196 | -0.7057202 | -0.85446644 | -1.172418 | -0.56136537 |
| hsa-miR-3198 | -0.3780787 | -0.5972729 | -1.3805063 | -0.7296024 |
| hsa-miR-32-3p | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
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| hsa-miR-320a | -0.6732969 | -0.55153424 | -0.65681064 | -1.7801293 |
| hsa-miR-320b | -0.82363343 | -0.5325343 | -0.56153584 | -1.1885821 |
| hsa-miR-320c | -0.6545146 | -0.65136296 | -0.8098142 | -1.2085649 |
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| hsa-miR-320e | -0.970612 | -0.3518678 | -0.31348264 | -0.7505515 |
| hsa-miR-324-3p | 1.1843921 | 0.9783967 | 0.85692656 | 1.49122 |
| hsa-miR-326 | -1.2824045 | -1.4159147 | -1.3805063 | -1.0288165 |
| hsa-miR-328 | -1.2824045 | -0.81573707 | -0.5243356 | -1.036643 |
| hsa-miR-331-3p | -1.2824045 | -1.115144 | -0.46077886 | -0.7715779 |
| hsa-miR-33b-3p | 1.1403414 | 0.4841101 | 1.3206019 | 1.394331 |
| hsa-miR-342-3p | -0.23418532 | -0.14056163 | 0.12758893 | -0.18462494 |
| hsa-miR-34a-5p | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-361-5p | -1.2824045 | -0.7516826 | -0.2442817 | -1.2976347 |
| hsa-miR-3610 | 1.3188692 | 0.9389995 | -0.7800763 | 2.222537 |
| hsa-miR-3620-3p | -1.0429865 | -1.1767205 | -1.3805063 | -0.9517532 |
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| hsa-miR-3647-3p_v17.0 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-3648 | -0.9062747 | -1.1463903 | -1.3805063 | -0.87009645 |
| hsa-miR-3652 | -1.8857015 | -2.0804074 | -2.5199041 | -0.43238366 |
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| hsa-miR-3663-3p | -0.7131659 | -0.99861497 | -1.0145475 | -1.0998541 |
| hsa-miR-3665 | -0.7664656 | -0.8785503 | -1.0318463 | -1.0775844 |
| hsa-miR-3667-5p | -0.65192056 | -1.4159147 | -1.3805063 | -0.8883472 |
| hsa-miR-3675-3p | 0.011002647 | -0.18288027 | -0.10175557 | -0.041016895 |
| hsa-miR-3676-3p | -1.1036983 | -1.422292 | -1.3588338 | -0.79219306 |
| hsa-miR-3679-3p | -0.24705166 | -0.38133585 | -0.3523852 | -0.2688445 |
| hsa-miR-3679-5p | -0.9015239 | -0.973078 | -1.1483203 | -0.7659708 |
| hsa-miR-3680-3p | -0.95553875 | -0.9243427 | -1.3805063 | -0.5918631 |
| hsa-miR-3682-3p | -1.2824045 | -0.24432176 | -1.3805063 | -0.9841306 |
| hsa-miR-371a-5p | -0.9713618 | -0.045603964 | -0.22660129 | -0.56515384 |
| hsa-miR-373-5p | -1.0116 | -1.1840001 | -1.3805063 | -1.0071826 |
| hsa-miR-3907 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-3911 | 0.1801362 | 0.67139137 | -0.5141347 | 0.62100065 |
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| hsa-miR-3937 | 0.60888124 | 0.5063042 | 0.4366118 | 0.93924874 |
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| hsa-miR-423-5p | 0.18543267 | 0.34035376 | 0.46165833 | -0.5777306 |
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| hsa-miR-4253 | -0.025746431 | 0.8442053 | -0.17539848 | -0.29454157 |
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| hsa-miR-4323 | 0.37502483 | 0.9943149 | 1.088018 | 2.1997397 |
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| hsa-miR-451a | -0.7705647 | -0.36890426 | -0.62560856 | -1.5828967 |
| hsa-miR-483-3p | -0.9988425 | -1.1417968 | -0.80437577 | -0.7574113 |
| hsa-miR-483-5p | -0.7872411 | -0.7069071 | -0.9561704 | -1.2968979 |
| hsa-miR-484 | -1.0157616 | -0.6229678 | -0.764648 | -0.63736993 |
| hsa-miR-486-3p | -1.0565323 | -0.9922183 | -0.66163796 | -0.8433492 |
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| hsa-miR-494 | -1.0225571 | -0.884897 | -1.1200913 | -0.059509873 |
| hsa-miR-498 | 1.8895959 | 1.3282971 | 1.1418163 | 1.5533813 |
| hsa-miR-501-5p | -1.2824045 | -1.4159147 | -1.3805063 | -1.1037683 |
| hsa-miR-513a-5p | -1.0034939 | -0.6315177 | -1.0994525 | -0.97560966 |
| hsa-miR-514b-5p | -0.68151283 | -0.17919374 | -1.129342 | -0.9568886 |
| hsa-miR-548q | -0.9417756 | -1.4159147 | -1.3805063 | -0.9132606 |
| hsa-miR-550a-5p | -0.72874993 | -1.4159147 | -0.94065124 | -0.7307631 |
| hsa-miR-557 | -0.8784557 | -1.1759375 | -1.3805063 | -0.86950755 |
| hsa-miR-564 | -0.92531383 | -1.4159147 | -1.3805063 | -1.0602051 |
| hsa-miR-572 | -0.76726407 | -0.9182044 | -1.0530065 | -1.0632902 |
| hsa-miR-574-3p | -0.40788606 | -0.77511096 | -0.017488837 | 1.5479448 |
| hsa-miR-574-5p | -1.4109797 | -1.0810039 | -0.45733774 | -0.6276206 |
| hsa-miR-575 | 0.72574663 | 0.47801626 | -0.655823 | 0.6033237 |
| hsa-miR-584-5p | 0.5567631 | 1.5902121 | 0.6711106 | 0.49057186 |
| hsa-miR-595 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
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| hsa-miR-622 | -1.2824045 | -1.1007124 | -1.3805063 | -1.0250392 |
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| hsa-miR-630 | -0.45398754 | -0.42360127 | -0.74500775 | -1.5794584 |
| hsa-miR-634 | -0.72874993 | -0.6296336 | -1.004376 | -0.92797494 |
| hsa-miR-636 | -0.109644145 | -0.31499282 | -0.16542456 | -0.12185354 |
| hsa-miR-638 | -0.71219647 | -0.8631332 | -1.0767816 | -0.9554833 |

| | | | | |
|---|---|---|---|---|
| hsa-miR-642b-3p | -0.6155474 | -0.81425285 | -1.0532161 | -0.6894966 |
| hsa-miR-663a | -0.74150157 | -1.4159147 | -1.3805063 | -0.91657233 |
| hsa-miR-670 | -1.2824045 | -1.4159147 | -1.3805063 | -1.2976347 |
| hsa-miR-671-5p | -0.65881306 | -0.59520304 | -0.86483276 | -1.0589856 |
| hsa-miR-711 | -1.2824045 | -1.4159147 | -1.3805063 | -1.0486478 |
| hsa-miR-718 | -1.324395 | -0.2698376 | -1.6775774 | -1.5229166 |
| hsa-miR-720_v18.0 | -1.0238743 | -0.5087119 | -0.55823964 | -0.9173896 |
| hsa-miR-760 | -1.1373667 | -0.9815091 | -0.83380395 | 0.7364784 |
| hsa-miR-762 | -0.51064324 | -0.73016983 | -1.1104631 | -0.6326083 |
| hsa-miR-765 | 1.1219465 | 1.6292841 | 0.96297497 | 0.7863143 |
| hsa-miR-766-3p | 0.07826439 | 0.3169012 | 1.2050716 | 1.6834062 |
| hsa-miR-874 | -0.41802198 | -0.30130842 | -0.5950329 | -0.9299363 |
| hsa-miR-877-3p | 0.3086067 | 0.1224385 | 1.6268284 | 0.7885615 |
| hsa-miR-877-5p | -0.91677177 | -1.4159147 | -1.3805063 | -0.9469963 |
| hsa-miR-92a-3p | -0.98229516 | -0.18429378 | -0.40052593 | -1.5700414 |
| hsa-miR-93-5p | 1.6754165 | 1.2199533 | 1.006522 | 0.19259581 |
| hsa-miR-933 | -1.182854 | -1.5546248 | -1.4580795 | -0.76235414 |
| hsa-miR-936 | -1.020303 | -1.4159147 | -1.3805063 | -0.9269178 |
| hsa-miR-939-5p | -0.62375873 | -0.81005245 | -1.0150306 | -1.0179119 |
| hsa-miR-940 | -0.9530716 | -1.1749135 | -1.0868282 | -0.8988908 |

**Table 3. Published data on circulating microRNAs in human breast cancer patients**

| miRNA | Sample type | Experimental design | Results | References |
|---|---|---|---|---|
| miR-10b | Serum | SYBR Green qRT-PCR Normalizer: miR-16 | Higher in BRCA with bone metastases than in BRCA without bone metastases or healthy controls | (103) |
| | Serum | TaqMan qRT-PCR Normalizer: 18S RNA | Higher in BRCA vs healthy controls | (115) |
| | Serum | TaqMan qRT-PCR Normalizer: miR-16 | Higher in pM1 BRCA vs pM0 BRCA | (114) |
| | **Blood** | TaqMan qRT-PCR Normalizer: miR-16 | No significant difference between BRCA and healthy controls | (107) |
| | Serum | LNA-based qRT-PCR (miRNA panel) Normalizer: miR-103, and miR-191 | Higher in BRCA vs healthy controls | (121) |
| | Plasma | TaqMan qRT-PCR Normalizer: miR-16 | Higher in pN+ BRCA vs pN0 BRCA | (127) |
| | Serum | LNA-based qRT-PCR QX200 digital PCR system | Higher in BRCA vs healthy controls<br>Higher in BRCA stage II-IV vs I<br>Higher in pN+ BRCA vs pN0 BRCA<br>Higher in Grade III BRCA vs Grade I-II BRCA | This paper |
| miR-145 | Serum | TaqMan qRT-PCR Normalizer: 18S RNA | Higher in BRCA vs healthy controls | (115) |
| | Plasma | SYBR Green qRT-PCR Normalizer: RNU6B | Lower in BRCA vs healthy controls | (118) |
| | Serum | LNA-based qRT-PCR Normalizer: based on the mean of the assays detected in all samples | Lower in ER-positive early-stage BRCA vs healthy controls | (160) |
| | Serum | LNA-based qRT-PCR (miRNA panel) Normalizer: miR-103 and miR-191 | Higher in BRCA vs healthy controls | (121) |
| | Serum | LNA-based qRT-PCR QX200 digital PCR system | Lower in BRCA vs healthy controls | This paper |

**Continue Table 3. Published data on circulating microRNAs in human breast cancer patients.**

| miRNA | Sample type | Experimental design | Results | References |
|---|---|---|---|---|
| **miR-148b** | Plasma | TaqMan qRT-PCR Normalizer: Spiked-in cel-miR-39 | Higher in early BRCA vs healthy controls | (120) |
| | Plasma | TaqMan qRT-PCR Normalizer: Spike-in cel-miR-39 | Higher in BRCA vs healthy controls Higher in benign breast tumours vs healthy controls | (140) |
| | Plasma | TaqMan qRT-PCR Normalizer: miR-93 | Higher in early stage BRCA vs healthy controls | (161) |
| | Serum | LNA-based qRT-PCR QX200 digital PCR system | Lower in BRCA vs healthy controls | This paper |
| **miR-425** | Serum | LNA-based qRT-PCR Normalizer: based on the mean of the assays detected in all samples | Higher in ER-positive early-stage BRCA vs healthy controls | (160) |
| | Serum | LNA-based qRT-PCR (miRNA panel) Normalizer: miR-103 and miR-191 | Higher in BRCA vs healthy controls | (121) |
| | Plasma | TaqMan qRT-PCR Normalizer: miR-16 | Higher in BRCA vs healthy controls | (133) |
| | Serum | LNA-based qRT-PCR QX200 digital PCR system | Lower in BRCA vs healthy controls | This paper |
| **miR-652** | Blood | TaqMan qRT-PCR Normalizer: miR-16 | Lower in Luminal A-like BRCA vs healthy controls | (162) |
| | Plasma | TaqMan qRT-PCR Normalizer: Spike-in cel-miR-39 | Higher in BRCA vs healthy controls Higher in benign breast tumors vs healthy controls | (140) |
| | Serum | LNA-based qRT-PCR QX200 digital PCR system | Lower in BRCA vs healthy controls Lower in Luminal A-like BRCA vs healthy controls | This paper |

BRCA, Breast Cancer patients; ER, Estrogen Receptor; Grade, Tumor grade; LNA, Locked Nucleic Acid; miRNA, microRNA; pM0,Patients with localized breast cancer; pM1, patients with metastatic breast cancer; pN0, no regional lymph node metastases histologically; pN +, regional lymph nodes metastases histologically; qRT-PCR, quantitative Real Time PCR; vs, versus.

## 3.3. Part3: Validation of circulating miRNAs using EvaGreen-ddPCR

### 3.2.1. Demographic characteristics of the study population

Two independent sets of serum samples from breast cancer patients and disease-free controls were analysed. Fifty-six samples were collected at the University Hospital of Ferrara, Italy, from 2012 to 2014. Of 56, 28 were breast cancer patients and 27 disease-free controls (**Italy cohort**). Ninety-four samples were collected at the Mercy's Woman Center in Oklahoma City, OK, USA, from 2005 to 2013. Of 94, 59 were breast cancer patients and 35 disease-free controls (**USA cohort**).

Blood samples from both cohorts were drawn prior to surgery and processed according to the same protocol, and the levels of circulating miRNAs were assessed in serum samples by ddPCR.

Age wise, in Italy cohort, patients and controls were well-matched on age, mean age: 65.3 (±14.4) vs 54.2 (±14.8) respectively. By the same token, in USA cohort, mean age: 56.73 (±10.4) vs 56.73 (±10.4) respectively as shown in **table (4).**

**Table 4. Demographic characteristics of the study population (validation cohorts).**

| | Italy Cohort | | USA Cohort | |
|---|---|---|---|---|
| | Cancer patients | Controls | Cancer patients | Controls |
| **Total** | 28 | 27 | 59 | 35 |
| **Mean age, years (SD)** | 65.3 (±14.4) | 54.2 (±14.8) | 56.73 (±10.4) | 53.2 (±11.5) |
| **Range** | 33-91 | 28-78 | 34-81 | 27-94 |
| **< 50** | 4 (14.3%) | 8 (29.6%) | 15 (25.4%) | 12 (34.3%) |
| **50-60** | 8 (28.6%) | 8 (29.6%) | 22 (37.3%) | 14 (40.0%) |
| **60-70** | 6 (21.4%) | 3 (11.1%) | 15 (25.4%) | 8 (22.9%) |
| **> 70** | 10 (35.7%) | 5 (18.5%) | 7 (11.9%) | 1 (2.9%) |
| **Un-known** | 0 | 3 (11%) | 0 | 0 |

### 3.2.2. Clinico-pathological features of breast cancer patients

Data of clinicopathological features for cases of both cohorts were retrieved from the two diagnostic centers as shown in **table (5)**. All cases were histologically confirmed according to the WHO classification of tumours of the breast, different histological types were revealed among cases of cohort Italy and USA; invasive ductal carcinoma in 21 (75%) and 48 (81%) respectively, invasive lobular carcinoma in 4 (14%) and 4 (7%) respectively, tubular carcinoma in 1 (4%) and 1(2%) respectively and other types in 2 (7%) and 6 (10%) respectively.

Based on the tumor size (pT) patients of Italy and USA cohorts were classified into three groups; pT1 in 21 (75%) and 28 (47%) respectively, pT2 in 7 (25%) and 27 (46%) respectively, notably, pT3 class was not observed among patients of Italy cohort, while only 4 (7%) cases were observed in USA cohort.

Also Lymph node involvement (pN) (NX, N0, N1, N2 or N3) depend on the number, size and location of breast cancer cell deposits in various regional lymph nodes was assessed in both cohort Italy and USA; pN0 in 21 (75%) and 35 (59%) respectively, pN1 6 (21%)16 (27%) respectively, pN2 in 0 and 6 (10%) respectively, pN3 in 1 (4%) and 1 (2%) respectively, one (2%) patient in USA cohort cannot be assessed.

Notably, metastasis was clinically assessed; M0 (no metastasis) were revealed among 28 (100%) and 57 (97%) respectively and M1 (metastasis detected) was not revealed in Italy cohort. However, it was detected in 2 (3%) cases of USA cohort. In addition, stages of breast cancer tumours in our cohorts Italy and USA were assessed; stage-I in 16 (57%) and 24 (41%) respectively, stage-II in 11 (39%) and 24 (41%) respectively, stage-III in 1 (4%) and 9 (15%) respectively while, stage-IV was not detected in Italy cohort and was detected only in 2 (3%) cases of USA cohort.

Grading data of breast tumours were collected for both cohort Italy and USA patients; well differentiated low-grade-I in 5 (18%) and 11 (19%) respectively, moderately differentiated intermediate-grade-II in 18 (64%) and 14 (24%) respectively, and poorly differentiated high-grade-III in 5 (18%) 34 (58%) respectively.

The receptor status of breast cancers from Italy and USA cohorts was identified by immunohistochemistry; ER was positive in 26 (93%) and 41 (69%) respectively, negative in 2 (7%) and 16 (27%) respectively, and 2 (3%) were missing in USA cohort. PR was positive in 19 (68%) and 35 (59%) respectively, negative 9 (21%) and 22 (37%) respectively, 2 (3%) were missing in USA cohort. HER2/neu receptor was positive in 3 (11%) and 11 (19%) respectively, negative in 25 (89%) and 44 (75%) respectively, 1 (2%) case was uncertain and 3 (5%) cases were missing in USA cohort. Additionally, Triple negative ER-/PR-/HER- BC were identified in 21 (4%) and 9 (15%) respectively.

**Table 5. Clinico-pathological features of breast cancer patients**

| Characteristic | | Italy Cohort (n=28) | USA Cohort (n=59) |
|---|---|---|---|
| **Menopausal status** | Pre | 3 (11%) | 14 (24%) |
| | Peri | 7 (25%) | 1 (2%) |
| | Post | 18 (64%) | 44 (75%) |
| **Histological subtype** | Ductal | 21 (75%) | 48 (81%) |
| | Lobular | 4 (14%) | 4 (7%) |
| | Tubular | 1 (4%) | 1(2%) |
| | other | 2 (7%) | 6 (10%) |
| **Tumor (pT)** | pT1 | 21 (75%) | 28 (47%) |
| | pT2 | 7 (25%) | 27 (46%) |
| | pT3 | 0 | 4 (7%) |
| **Lymph node involvement (pN)** | pN0 | 21 (75%) | 35 (59%) |
| | pN1 | 6 (21%) | 16 (27%) |
| | pN2 | 0 | 6 (10%) |
| | pN3 | 1 (4%) | 1 (2%) |
| | pNx | 0 | 1 (2%) |
| **Metastasis (cM)[1]** | M0 | 28 (100%) | 57 (97%) |
| | M1 | 0 | 2 (3%) |
| **Stage** | I | 16 (57%) | 24 (41%) |
| | II | 11 (39%) | 24 (41%) |
| | III | 1 (4%) | 9 (15%) |
| | IV | 0 | 2 (3%) |
| **Grade** | I | 5 (18%) | 11 (19%) |
| | II | 18 (64%) | 14 (24%) |
| | III | 5 (18%) | 34 (58%) |
| **Estrogen receptor** | positive | 26 (93%) | 41 (69%) |
| | negative | 2 (7%) | 16 (27%) |
| | missing | 0 | 2 (3%) |
| **Progesterone receptor** | positive | 19 (68%) | 35 (59%) |
| | negative | 9 (21%) | 22 (37%) |
| | missing | 0 | 2 (3%) |
| **HER2/neu receptor** | positive | 3 (11%) | 11 (19%) |
| | negative | 25 (89%) | 44 (75%) |
| | uncertain | 0 | 1 (2%) |
| | missing | 0 | 3 (5%) |
| **Triple negative** | ER-/PR-/HER2- | 1 (4%) | 9 (15%) |

[1]cM Clinical evidence of metastasis

HER2/neu human epidermal growth factor receptor 2, ER Estrogen receptor, PR Progesterone receptor

### 3.2.3. Circulating miRNAs in sera of breast cancer patients controls

#### 3.2.3.1. Circulating miRNAs significantly down-regulated in breast cancer patients

Quantification of our candidates in serum samples of breast cancer patients and healthy controls revealed that miR-148b-3p, miR-181a-5p and miR-652-3p levels were significantly down-regulated in breast cancer patients in both cohorts ($p = 0.0042$, $p < 0.05$; and $p < 0.0001$, respectively, in Italy cohort; $p = 0.0115$, $p < 0.005$ and $p = 0.0043$, respectively, in USA cohort) (**Figure 6**). In addition, miR-145-5p and miR-425-5p were also down-regulated in breast cancer patients compared with controls in both cohorts. The differences were statistically significant in USA cohort ($p = 0.0257$ and $p = 0.0226$ respectively). On the contrary, there was no significant difference in Italy cohort ($p = 0.261$ and $p = 0.077$) as shown in **Figure 6.**

#### 3.2.3.2. Circulating miRNA significantly up-regulated in breast cancer patients

Among the tested microRNAs, miR-10b-5p exhibited a weak increase in cancer patients compared with controls. This trend was statistically significant in USA cohort ($p = 0.016$). However, there was no significant difference in the level of miR-10b-5p in cancer patients compared with controls in Italy cohort ($P = 0.554$) as indicated in **Figure 6.**

### 3.2.4. Combined analysis of two different cohorts (Italy and USA cohorts)

Results of the two cohorts (Italy and USA) shown in **Figure. 6** were combined. The unpaired t-test with or without Welch's correction was performed to assess significance of differences between breast cancer patients and control groups. Significant discrimination between breast cancer patients and disease-free controls as well as trends of dysregulation were all confirmed. When combined, the two cohorts produced a highly significant discrimination between cancer patients and controls for all investigated miRNAs, with the strongest discrimination achieved by miR-652-3p and miR-148b-3p ($p = 0.008$ and $p < 0.0001$ respectively) (**Figure 7**).

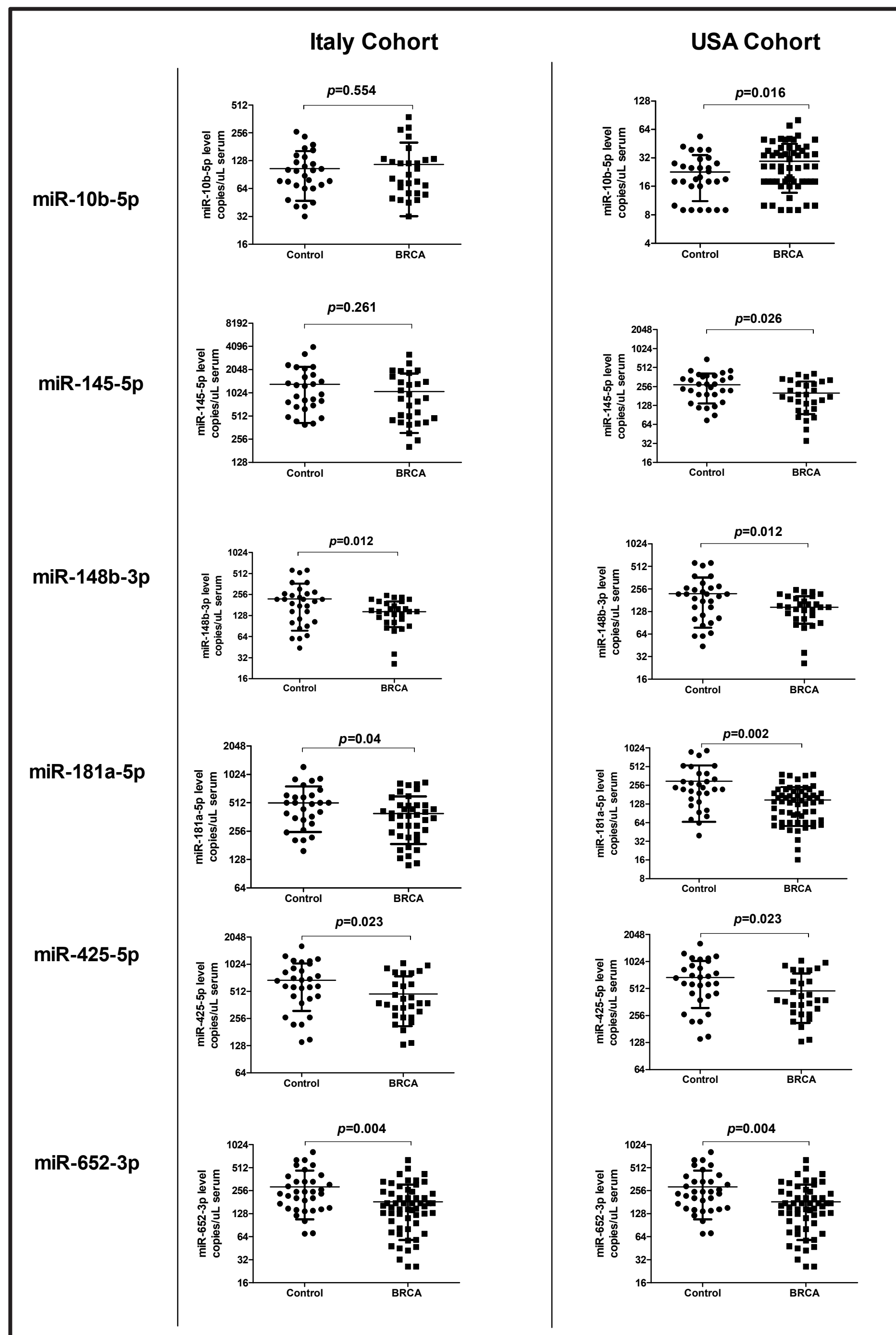



**Figure 6. Levels of miRNAs in sera of two independent cohorts of breast cancer and disease-free patients.**

The level of each miRNA was measured by the ddPCR technique and expressed in copies per microliter in each sample. Each miRNA displays comparable levels and consistent dysregulation in both cohorts. MiR-652-3p, miR-181a-5p, miR-148b-3p exhibited statistically significant reduction in breast cancer patients in both cohorts. The unpaired t-test with or without Welch's correction was performed to assess significance of differences between patient and control groups. P-values of less than 0.05 were deemed to be significant. BRCA; breast cancer patients.

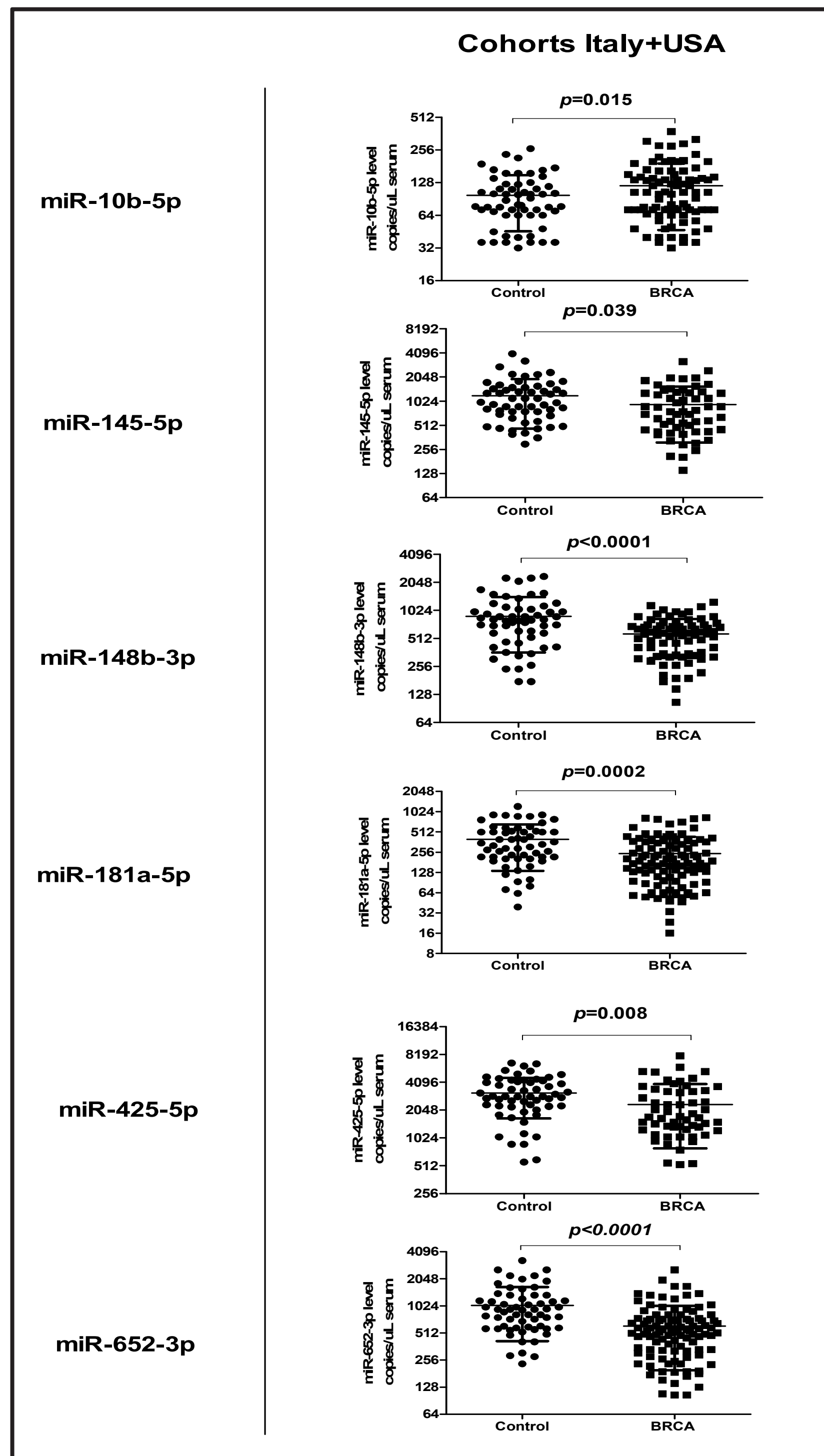



**Figure 7. Levels of miRNAs in sera of the combined two cohorts of breast cancer and disease-free patients.**

Results of the two cohorts shown in figure 6 were combined. The unpaired t-test with or without Welch's correction was performed to assess significance of differences between breast cancer patients and control groups. P-values of less than 0.05 were deemed to be significant. Significant discrimination between breast cancer patients and disease-free controls as well as trends of dysregulation were all confirmed. BRCA; breast cancer patients.

### 3.2.5. Confirmation of the ddPCR results using RT-qPCR

We further verified our candidate miRNAs with the most commonly used method based on reverse transcription quantitative PCR (RT-qPCR). We investigated serum levels of miR-10b-5p and miR-652-3p. As RT-PCR normalizer, we employed the Cel-miR-39, which we routinely added at a defined concentration to any serum sample. Our results confirmed the significant discrimination between samples from breast cancer patients versus controls and the direction of down regulation ($p = 0.0076$ and $p = 0.0092$ respectively) (**Figure 8**).

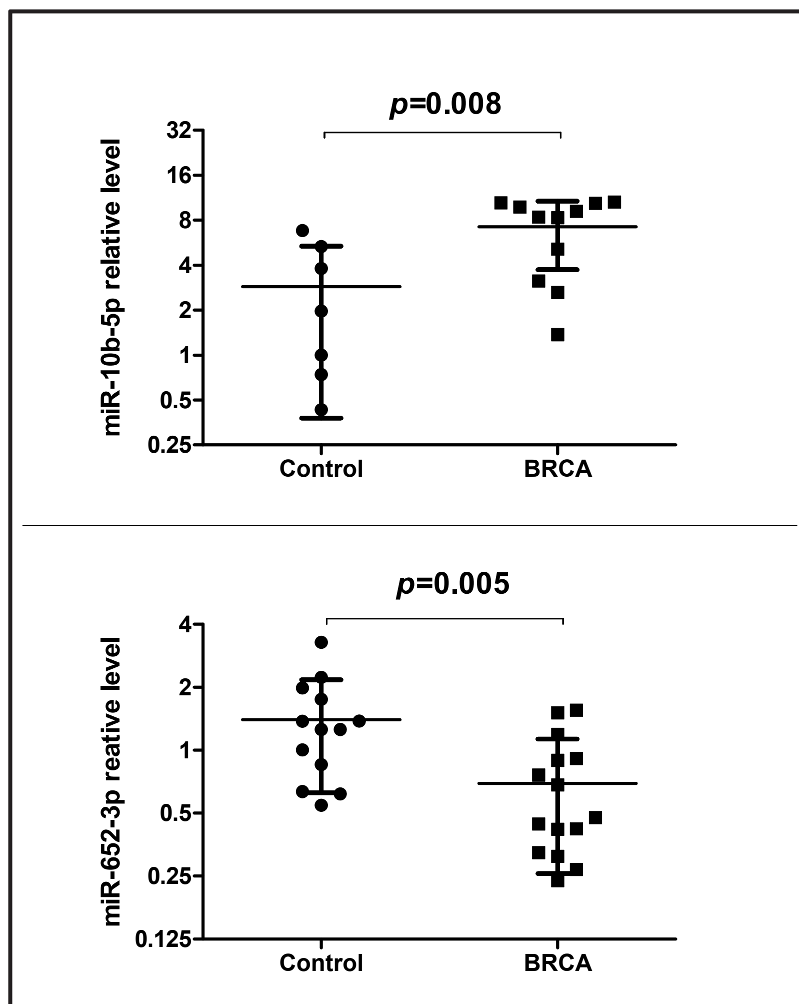
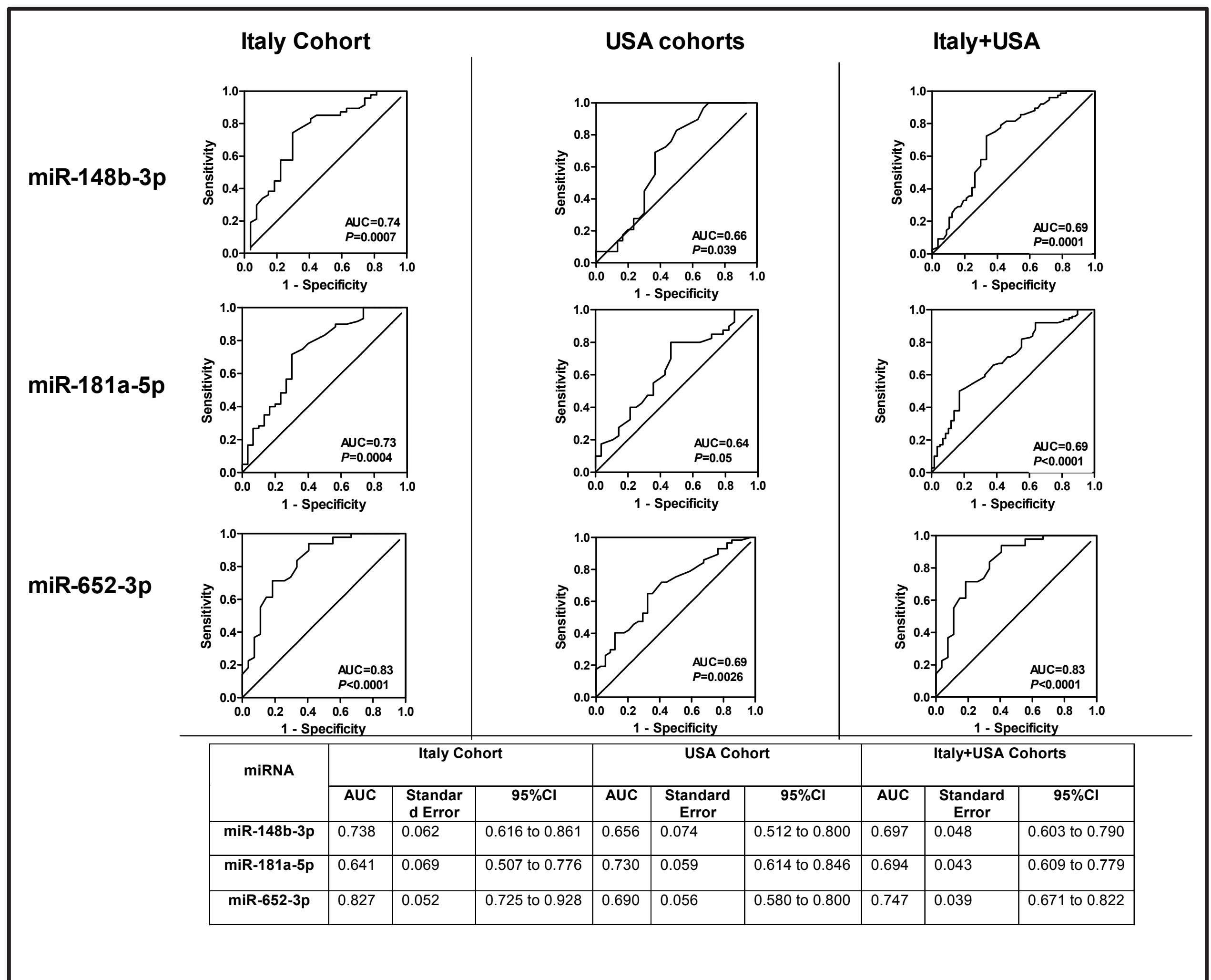



**Figure 8. Confirmation of ddPCR results using RT-qPCR.**
The serum levels of miR-10b-5p (up) and miR-652-3p (down) were investigated in a subset of samples from USA cohort using Real-Time PCR instead of ddPCR method. The same trend of dysregulation found with ddPCR was also confirmed with this method. Cel-miR-39 was used as standard reference to normalize results.

### 3.2.6. Diagnostic value of circulating miRNAs in breast cancer

Receiver operating characteristic (ROC) curve analysis and calculation of the area under each ROC curve (AUC) were performed to evaluate the diagnostic ability of the chosen six miRNAs to distinguish cancer patients from controls in Italy and USA cohorts separately and combined Italy and USA cohorts. As a result**,** miR-148b, miR-181a-5p and miR-652-3p appeared to represent valuable diagnostic biomarkers. MiR-148b showed a ROC curve area of 0.74 ($p = 0.0007$) in Italy cohort, 0.66 ($p = 0.039$) in USA cohort and 0.70 ($p = 0.0001$) in combined analysis of cohort Italy and USA. In the same way miR-181a-5p yielded a ROC curve area of 0.665 ($p = 0.01$) (95% confidence interval, 0.535– 0.795) for the Italy cohort and a ROC curve area of 0.73 ($p < 0.001$) (95% confidence interval, 0.614–0.845) for USA cohort and 0.694 ($p <0.0001$) in combined cohort analysis. MiR-652 was of particular interest because of the highly significant ROC curves in both cohorts (AUC = 0.83, $P = <0.0001$ in Italy cohort; AUC = 0.69, $P=0.0026$ in USA cohort; AUC = 0.75, $P = <0.0001$ in combined Italy and USA) **(Figure 9)**. On the contrary, miR-10b-5p, miR-145-5p and miR-425-5p showed lower sensitivity and specificity.




| miRNA | Italy Cohort | | | USA Cohort | | | Italy+USA Cohorts | | |
|---|---|---|---|---|---|---|---|---|---|
| | AUC | Standard Error | 95%CI | AUC | Standard Error | 95%CI | AUC | Standard Error | 95%CI |
| miR-148b-3p | 0.738 | 0.062 | 0.616 to 0.861 | 0.656 | 0.074 | 0.512 to 0.800 | 0.697 | 0.048 | 0.603 to 0.790 |
| miR-181a-5p | 0.641 | 0.069 | 0.507 to 0.776 | 0.730 | 0.059 | 0.614 to 0.846 | 0.694 | 0.043 | 0.609 to 0.779 |
| miR-652-3p | 0.827 | 0.052 | 0.725 to 0.928 | 0.690 | 0.056 | 0.580 to 0.800 | 0.747 | 0.039 | 0.671 to 0.822 |

**Figure 9. Diagnostic potential of miR-148b-3p, miR-181a-5p and miR-652-3p.** Receiver operating characteristic (ROC) curve analyses shows that miR-148b-3p and miR-652-3p exhibit a significant ability to predict breast cancer in both cohorts. AUC; area under the curve, CI; confidence interval.

### 3.2.7. Association of circulating miRNAs with clinico-pathological features in breast cancer patients

The levels of miRNAs were investigated in association with clinico-pathological features in patients of USA cohort. Italy cohort included only patients with stage I or II tumors, consequently the results of this cohort were not used to perform these analyses. The statistical correlation between the serum levels of miR-145-5p, miR-148b-3p, miR-425-5p, miR-652-3p and clinical and histopathological data did not reach a statistical significance. However, miR-10b-5p showed a significant correlation with various clinico-pathological features (**Table 6**)**.**

#### 3.2.7.1. Association of miR-10b-5p with prognostic parameters

In USA cohort, the level of serum miR-10b-5p revealed a significant increase with tumour stage. Patients with stage II to IV tumor exhibited a significant higher levels of miR-10b in comparison with patients with stage I tumour ($p = 0.005$) or controls ($p = 0.003$). Conversely, no significant difference was revealed between stage I cases and controls. Besides the significant difference of serum level miR-10b-5p with cancer stage in USA cohort, its diagnostic performance was assessed for the discrimination of patients with breast cancer stage I from breast cancer stage II to IV, the AUC = 0.69 ($p = 0.017$) as indicated in **Figure 10**.

#### 3.2.7.2. Association of miR-10b-5p expression with other clinico-pathological features

Notably, miR-10b-5p was also significantly up-regulated in association with various clinico-pathological features, including tumor grading**.** Patients with grade III tumor showed a significant higher levels of miR-10b-5p in comparison with patients with stage I-II tumour ($p = 0.026$). In addition, patients with lymph node metastases (pN1-2-3) exhibited significant increase in the level of circulating miR-10b-5p compared to cancer patients without lymph node metastasis (pN0) ($p = 0.014$) (**Figure 10**).

Albeit statistically not significant, the level of miR-10b-5p was also higher in patients carrying HER2 positive versus HER2 negative cancer patients ($p = 0.203$) or ER-/PR- cancers versus ER+/PR+ cancer patients. Because of their paucity, we

could not assess the significance of miR-10b-5p in patients with metastatic disease. However, the level of this miRNA was very high in the two available cases. In the same way, trends toward increased level of miR-10b-5p were also detected in cases characterized by increased tumour size ($p = 0.123$) or in post-menopausal compared to pre-menopausal ($p = 0.893$) (**Figure 10**).

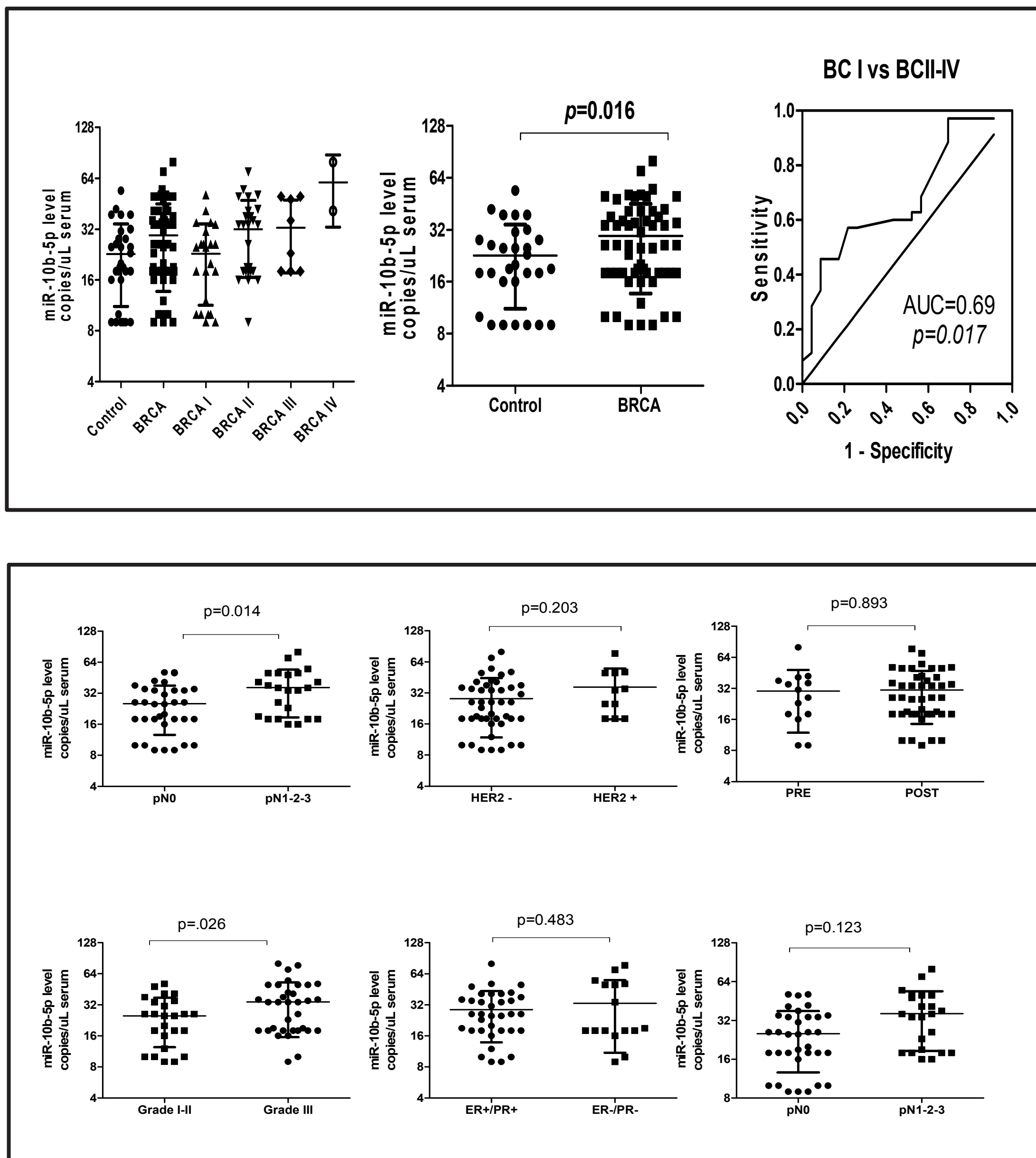



**Figure 10. Serum miR-10b-5p increases in patients presenting poor prognostic parameters.**

In USA Cohort. Upper figure; shows Levels of miR-10b increased in line with worsening clinical stage. Levels from patients with stages II-IV cancer exhibit a significant difference in comparison to those from patients with stage I cancer (p = 0.005) or controls (p = 0.003). The diagnostic value of miR-10b-5p was assessed by receiver operating characteristic curve analysis. No significant difference was found between stage I and controls. BRCA breast cancer patients, AUC area under the curve. bottom figure shows, higher levels of miR-10b-5p were associated with various clinicopathological parameters: lymph node metastasis (pN) (p = 0.014) and tumour grade (p = 0.026). Albeit not statistically significant, a trend toward increased levels of miR-10b was also detected in cases characterized by increased tumor size (pT) or as being human epidermal growth factor receptor 2 positive (HER2+) or estrogen receptor/progesterone receptor negative (ER-/PR-). “PRE” or “POST” refer to the menopausal status.

**Table 6. Association of circulating miR-10b-5p with clinicopathological features in breast cancer patient.**

| Feature | | Cohort (Italy) Average Copies/uL ± SD (n= number of patients) | Cohort (USA) Average Copies/uL ± SD (n= number of patients) |
|---|---|---|---|
| **Tumor size (pT)** | pT1 | 108.7±78.0 (n=19) | 98.6±47.3 (n=28) |
| | pT2 | 97.3±55.9 (n=6) | 143.4±70.3 (n=27) |
| | pT3 | / | 75.0±11.9 (n=4) |
| **Lymph node involvement (pN)** | pN0 | 88.7±37.2 (n=18) | 101.2±54.6 (n=35) |
| | pN1-2-3 | 113.3±89.4 (n=7) | 145.2±70.8 (n=23) |
| **Stage** | I | 82.9±28.5 (n=14) | 91.3±45.9 (n=23) |
| | II | 107.7±79.9 (n=10) | 128.0±61.8 (n=25) |
| | III | 134.0 (n=1) | 130.5±60.2 (n=8) |
| | IV | / | 242.2±110.3 (n=2) |
| **Grade** | I | 80.8±36.5 (n=5) | 114.9±43.2 (n=11) |
| | II | 112.7±88.6 (n=18) | 91.2±58.8 (n=13) |
| | III | 105.0±54.3 (n=5) | 136.0±74.81 (n=34) |
| **ER/PR Status** | ER+/PR+ | 142.1±98.44 (n=17) | 115.0±60.1 (n=35) |
| | ER-/PR- | 74.0 (n=1) | 131.9±89.4 (n=16) |
| **HER2/neu receptor** | positive | 103.7±80.8 (n=3) | 112.9±65.5 (n=11) |
| | negative | 115.8±88.4 (n=25) | 145.8±75.0 (n=44) |
| **Triple Negative** | | 74.0 (n=1) | 117.8±90.2 (n=9) |
| **Others** | | 118.2±85.61 (n=27) | 121.5±63.36 (n=49) |

Results represented in copies per µL of serum according to clinico-pathological features.

#### 3.2.7.3. Association of miR-652-3p with the molecular subtypes of breast cancer

To assess whether the circulating miRNAs were specific to breast cancer molecular subtypes. Patients stratified by molecular subtypes were assessed by comparing the level of each miRNA in Luminal A, Non-Luminal A and Triple negative/basal-like breast cancers. Notably, miR-10b-5p, miR-145-5p, miR-148b-3p, miR-425-5p and miR-652-3p levels did not vary significantly across Triple negative breast cancer (p = 0.908, p = 0.609, p = 0.125, p = 0.410, p = 0.883 respectively) (**Figure 11**).

Here, we confirmed a significant lower level of miR-652-3p in Luminal A cancer patients ER, PR positive and HER2 negative versus controls (p = 0.021 ). Interestingly, the level of miR-652-3p was significantly lower in non-Luminal A cancer patients, versus controls (p = 0.004) **(Figure 12A)**. In addition, miR-652-3p was found to distinguish Luminal A cancer patients, from controls (AUC = 0.669, p = 0.021) and non-Luminal A cancer patients, from controls AUC = 0.7106,p = 0.005) **(Figure 12B)**.

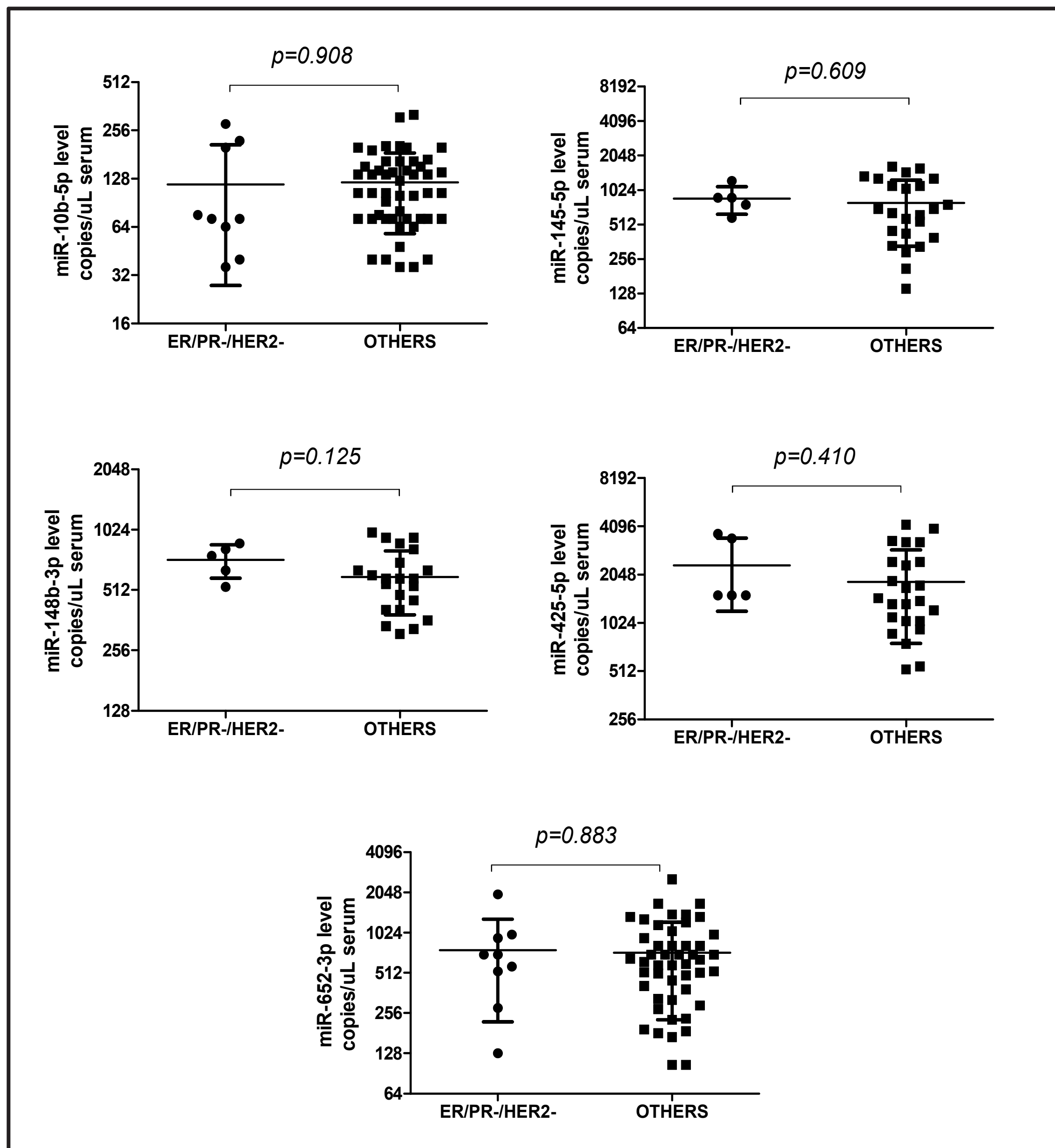



**Figure 11. Association of circulating miRNAs with triple negative breast cancer.** Triple negative breast cancer did not shown different in levels of miR-10b-5p, miR-145-5p, miR-148b-3p, miR-425-5p and miR-652-3p compared with others ER/PR/HER2 positive breast cancers. P; p value.

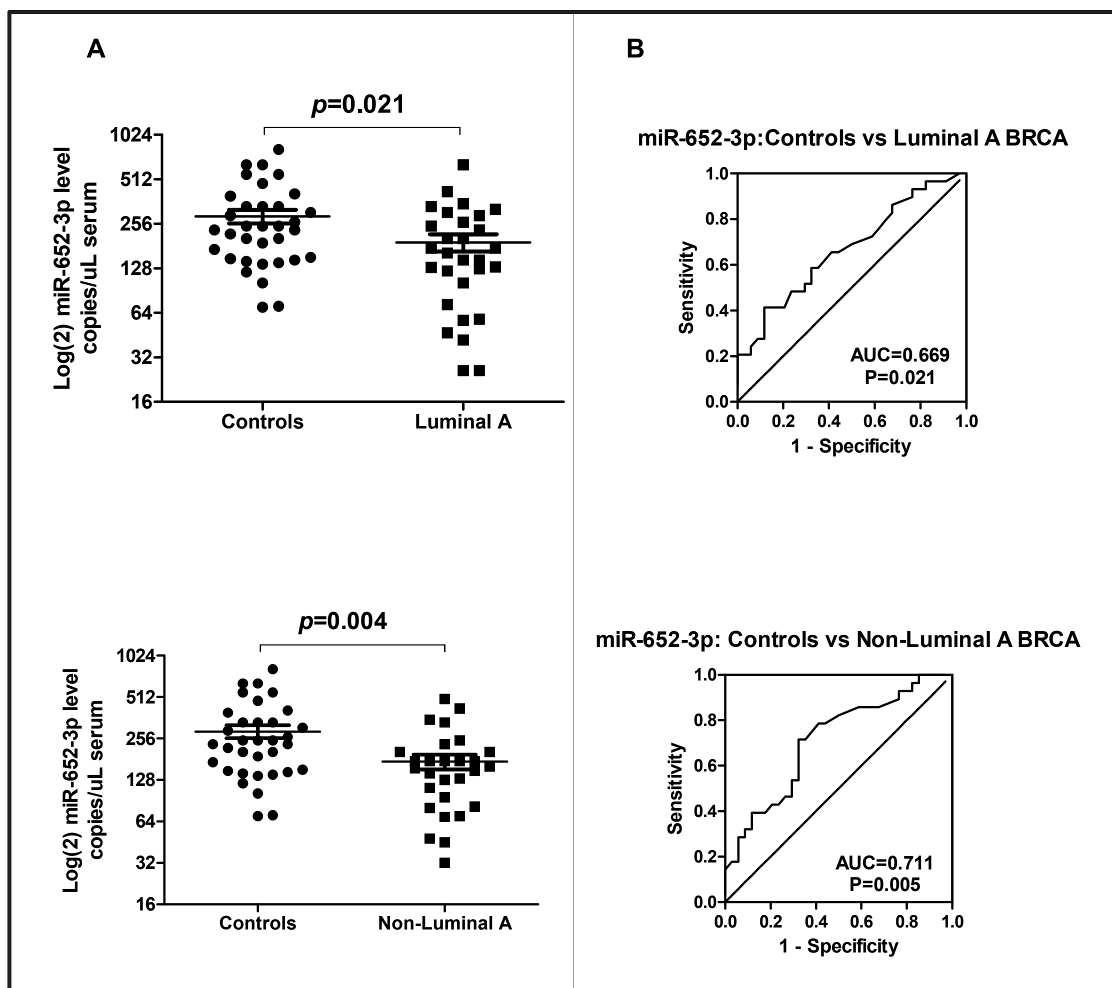



**Figure 12. Association of miR-652-3p with molecular subtypes of breast cancer**
**A**; Serum miR-652-3p is significantly reduced in patients of either Luminal A or non-Luminal A breast cancers compared to controls. A, Analysis of USA cohort indicated significantly lower levels of serum miR-652-3p in Luminal A (ER+/PR+/HER2-) and other non-Luminal A cases in comparison with disease-free controls. The same results were obtained by analysis of Italy cohort. **B;** miR-652-3p was found to distinguish Luminal A cancer patients, from controls and non-Luminal A from cancer patients, from controls. AUC; area under the curve. P; p value.

# 4. Discussion

Since their first discovery, circulating microRNAs became an active and rapidly evolving area of research with the potential to transform cancer management in terms of diagnosis and prognosis. Since they are extremely stable in plasma and serum, circulating miRNAs emerged as potential non-invasive biomarkers in cancer patients. In particular, miRNAs could provide novel biomarkers for breast cancer, the most frequent cancer among women worldwide.

In the last few years, many authors have demonstrated that a number of circulating miRNAs could discriminate breast cancer patients from healthy individuals (115, 120) or could be linked to breast cancer subtypes (120, 160, 162). Unfortunately, miRNA quantification has been performed in a variety of manners and the combined effect of several variables generated poorly overlapping and hardly reproducible results and their translation into clinically useful applications is not feasible (152, 163-166).

Obviously, the differences in sample type (whole blood, plasma or serum) or sample processing protocols (blood collection tube, tissue centrifugation and conservation) could have been responsible for the apparent discrepancies among the various studies, as confirmed by published studies (167, 168). Thereby, we cannot compare the miRNA profiles across studies in which tissues have been collected using different protocols. Moreover, in several studies this information is missing in the published methods. The RNA extraction procedures and the quantification assay have a relevant impact as well. In our study, we give a detailed description of all pre-analytical steps, henceforth guarantying the possibility to reproduce the presented results. This information is relevant to avoid biased results when performing meta-analyses of miRNA biomarkers data. The use of whole blood will lead to isolation of miRNAs not only circulating miRNA but also from blood cell (169) Earlier, Plasma and serum were considered to be equivalent (170). Obviously, now there allot of discrepancies between published data on circulating miRNAs on serum and plasma.

It has been hypothesized that the main differences between serum and plasma could be linked to the permanence of microvescicles and exosomes that contains

microRNAs (171). Indeed, stronger centrifugation steps (3200xg minimum) are necessary to remove most platelets (164) and some part of microvescicles. According to this hypothesis, circulating miRNAs linked to Argonaute protein or HDL will display similar levels in plasma and serum, while exosome and microvescicle encapsulated miRNAs will display different levels, since microvescicles are trapped inside serum clot and discharged. Further study, linked that difference to the presence of cellular contamination, in particular, platelets. Serum samples should be more suitable to minimize the variation introduced by variable levels of platelet contamination. In our validation step we analysed serum, instead of plasma, as it contains minimal cellular contamination, most commonly available patient material, the procedure used to collect serum is homogeneous at different institutions. Thus, helping to reduce uncertainties in pre-analytical procedures.

Another issue that is frequently underestimated in miRNA biomarkers studies concerns with a variety of methods were used to normalize data, thereby producing non comparable results (165). Here we took advantage of droplet digital PCR technology to develop an analytical approach for absolute quantification of specific circulating miRNAs, working in every step with constant volumes. As mentioned before, digital PCR can be considered superior to RT-qPCR in circulating miRNA quantification, because of its higher sensitivity and reduced variability in low abundance miRNA detection (151). By using this technology, we have been able to perform an absolute quantification of miRNA copies, without the need of additional normalization steps. Therefore, results obtained using droplet digital PCR can be easily used to calculate the number of each miRNA molecule that are present in one ml of plasma or serum.

In the first part of the study, we tested and compared the feasibility of quantifying miRNA with the new QX200 Droplet Digital PCR system when used with EvaGreen dye– and TaqMan probe–based assays. As a result, we demonstrated that two commercial miRNA assays, TaqMan (Life Technologies) and miRCURY LNA (Exiqon), can be successfully used to quantify specific miRNAs in human biofluids. Indeed, although based on different detection chemistries (e.g., TaqMan probes and DNA-binding EvaGreen dye), the assays provided comparable results on the QX200 ddPCR system. When miRNA assays are performed for diagnostic

purposes, TaqMan and miRCURY LNA assays are considered highly reliable because of their sensitivity and precision. It was therefore relevant to understand whether both assays could be run on a ddPCR system. Our results demonstrate that both miRNA quantification methodologies whether based on probes or DNA-binding dye detection chemistries can be performed using a digital PCR approach.

Circulating miRNAs are present in blood at extremely low concentrations (14), so they are difficult to quantify with other techniques such as microarray and RNA sequencing. Moreover, the amount of RNA that can be extracted from plasma and serum samples is low. In this context, it is of great importance to be able to quantify any desired miRNA using individual assays and ddPCR technology, without having to do miRNA-specific reverse transcription (as for TaqMan assays). Therefore, a universal cDNA system, like that developed by Exiqon, paired with a specific PCR assay makes the Eva-Green-based ddPCR assay attractive. With this assay, we can combine the advantages provided by digital PCR technologies with the specificities inherent to the use of LNA primers, thus expanding the spectrum of applications of ddPCR technology in the biomarker field

In the second part of this study, we selected a panel of candidate biomarkers for breast cancer. Among the six selected miRNAs, miR181a-5p and miR-425-5p have been identified to be abundant miRNA species in plasma of breast cancer patients using a microarray screening. By searching in the published literature, we selected six circulating miRNAs (miR-10b, miR-145-, miR-148b-, and miR-652-) and presented a tabular summary for their associated with diagnosis and /or prognosis of both primary and metastatic breast cancer. Hence, they are very attractive candidates for clinical application as biomarkers for breast cancer.

In the third part of the study (our validation step), using EvaGreen-based ddPCR, we analysed the levels of six miRNAs in the serum of two independent cohorts of breast cancer patients and disease-free controls. Blood samples were collected in two different countries (Italy and USA) in different years and processed separately, with no differences in procedures for obtaining serum samples.

Despite all the technical issues, our data clearly indicate that miRNA molecules are present in the circulation at levels that change according to the cancer status. Notably, all of the analysed miRNAs (miR-145-5p, miR-148b-3p, miR181a-5p,

miR-425-5p and miR-652-3p) showed comparable absolute levels in the sera of the two cohorts. Interestingly, both cohorts exhibited consistent trends of dysregulation in breast cancer patients versus controls (see figure 6). Most important, we observed that among the six examined miRNAs in the two independent cohorts (Italy and USA) 5 miRNAs were decreased, while one was increased in sera of breast cancer patients compared to controls. The differences between breast cancer cases and controls in USA cohort were statistically significant for all six miRNAs, whereas only miR-148b-3p, miR181a-5p and miR-652-3p reached statistical significance in Italy cohort, possibly because of differences in clinico-pathological characteristics.

In this study, we further performed ROC curve analyses, by which we evaluated the possible diagnostic potential of the six circulating miRNAs. Areas under the curves and p-values were significant for miR-148b-3p, miR181a-5p and miR-652-3p in both cohorts MiR-148b showed a ROC curve area of 0.74 (p = 0.0007) in Italy cohort, 0.66 (p = 0.039) in USA cohort and 0.70 (p = 0.0001) in combined analysis of cohort Italy and USA

MiR-148b showed a ROC curve area of 0.74 (p = 0.0007) in Italy cohort, 0.66 (p = 0.039) in USA cohort and 0.70 (p = 0.0001) in combined analysis of cohort Italy and USA. In the same way miR-181a-5p yielded a ROC curve area of 0.665 (p = 0.01) (95% confidence interval, 0.535– 0.795) for the Italy cohort and a ROC curve area of 0.73 (p < 0.001) (95% confidence interval, 0.614–0.845) for USA cohort and 0.694 (p <0.0001) in combined cohort analysis. MiR-652 was of particular interest because of the highly significant ROC curves in both cohorts (AUC = 0.83, P = <0.0001 in Italy cohort; AUC = 0.69, P=0.0026 in USA cohort; AUC = 0.75, P = <0.0001 in combined Italy and USA) **(see Figure 9)**. Taken together, these findings suggest the potential value of these three miRNAs as breast cancer biomarkers.

Our data on circulating miRNAs show both similarities and discrepancies to previous reports. This is not surprising, considering the wide variation in experimental settings and technical approaches. Similar to our findings, higher level of miR-10b-5p in advanced stages breast cancer patients is in agreement with several published reports (103, 115, 121) but in contrast with those reported by Heneghan et al. showing similar level of miR-10b in breast cancer patients

compared to healthy controls (96). Our Italy group consisted of patients carrying stage I or II cancers. This is the reason why we did not find significant differences in miR-10b-5p levels in this cohort. Nevertheless, this finding does not contradict the positive correlation between miR-10b-5p and advanced stage disease that we observed in our USA group; it is also consistent with the results described in a report by Roth et al., who found a higher level of miR-10b in patients with metastatic disease (114).

To what concern miR-145-5p, three previous reports found a decreased level of miR-145-5p in breast cancer patients (118, 121, 160). On the contrary, Mar-Aguilar et. al found that miR-145 was significantly higher in breast cancer serum than in healthy controls (115). Circulating miRNA-145 has been found to be significantly elevated in malignant myoepithelioma but not in basal cell-like breast cancer (172). As a known tumour suppressor in numerous human cancers, miR-145 exhibits an inhibitory role in tumoural angiogenesis, cell growth and invasion and tumour growth through the post-transcriptional regulation of the novel targets N-RAS and VEGF-A, and may plays important inhibitory role in breast cancer malignancy by targeting these genes (173), early manifestation of altered miR-145 expression in atypical hyperplasia and carcinoma *in situ* lesions in breast cancer suggests that this miRNA may have a potential clinical application as a novel biomarker for early detection (174).

With reference to miR-181a-5p, McDermott and colleagues assessed miR -181a in a cohort of 44 patients and 46 controls by RT-qPCR starting from whole blood RNA, using miR-16 for normalization. They found that, the level of circulating miR-181a-5p levels was reduced in the blood of Luminal A breast cancer (162). Similar to our findings of declined serum miR-181a-5p levels in breast cancer, previous study has documented by Guo et. al (175) using a custom made stem-loop PCR to quantify miR-181a/miR-16 levels in 152 breast cancer patients and 75 controls. They also concluded that miR181a may represent a new diagnostic biomarker for primary breast cancer as well as for early stage breast cancer.

Concerning, miR-652-3p, our results are in agreement with one report that indicated a decreased level in patients with Luminal A-like breast cancer in comparison with controls (162). Conversely, Cuk et al. showed increased levels of miR-652-3p, likely because the study was performed on plasma instead of serum

samples (120). In support of this suggestion, analysis of a small number (n = 20) of plasma samples from our breast cancer cohort revealed that miR-652-3p was indeed increased in breast cancer patients compared with controls (data not shown). Finding an opposite trend in serum or plasma samples is not new (158, 165) and is strongly connected to the genesis of circulating miRNAs (176).

To what concern miR-148b-3p and miR-425-5p, the published literature reports opposite results to ours, as both miRNAs were found to be higher in breast cancer patients than in healthy controls. However, studies on miR-148b were all performed by using plasma (120, 140, 161), whereas the miR-425 study was performed with serum, but normalization was based on the mean of assays performed on all samples (160); thus, these results are not directly comparable with ours.

Many researchers are concentrating in finding effective blood based biomarkers for early breast cancer detection. Similarly, there is a strong need for prognostic and predictive markers for breast cancer for patient follow-up and to guide the selection of the most appropriate therapy for individual patients. Here, in the search for possible correlations with clinico-pathological features, we found that miR-10b-5p levels were increased in the serum of patients with a high cancer stage or grade, or with presence of lymph node metastases. In notion with previous findings, our result suggests the overexpression of miR-10b may play an important role in breast cancer metastasis. Albeit not statistically significant, the average expression of miR-10b-5p was also higher in cases with ER/PR-negative tumours and in cases with HER2-positive tumours.

Together, these findings indicate that miR-10b-5p represents a biomarker of tumour aggressiveness. This suggestion is also supported by other studies that indicated higher levels of circulating miR-10b in patients with metastatic breast cancer and worsening clinical stage (103, 114, 127). It is notable that the role of miR-10b in invasion and metastasis has been thoroughly investigated and its importance proven (177-180). MiR-10b has also been found to be highly expressed in the vasculature of high-grade breast cancer (181). Generally, these findings suggest that tumour and microenvironment features may be directly responsible for the increased levels of circulating miR-10b in blood of patients with advanced stage breast cancer and may represent a new therapeutic target.

The possibility of assessing the absolute levels of miR-10b-5p in the serum of patients by using a robust technique such as ddPCR could represent a potential new approach for monitoring disease behaviour in breast cancer patients.

# 5.Conclusion

The discovery of miRNAs brought forward a new understanding of the basic mechanisms of oncogenesis and circulating miRNAs opened up exciting perspectives for diagnostic and prognostic applications.

Although still a new field, with much to be explored, the hope is to apply circulating miRNAs to cancer diagnosis, prognosis and prediction. However, before novel biomarkers can be routinely used in a clinical setting, standardized detection procedures of circulating miRNA should be established.

On the whole, this study establishes the basis for using EvaGreen dye–based assays on a ddPCR system for quantifying circulating miRNA biomarkers in human biofluids.

EvaGreen dye–based and TaqMan probe–based assays can be equally used with the ddPCR system to quantify circulating miRNAs in human plasma and serum in miRNA biomarkers studies.

Taken together, using ddPCR quantitative approach, our panel of circulating miRNAs were successfully derived and validated. Very good agreement between two independent cohorts was revealed in terms of comparable absolute miRNA concentrations and consistent trends of dysregulation in breast cancer patients versus controls.

On the whole, this study extends the findings of previous studies and confirms the potential of circulating miRNAs, namely, miR-148b-3p, miR181a-5p and miR-652-3p as minimally invasive biomarkers for breast cancer detection, on one hand, miR-10b-5p as potential prognostic biomarker.

In conclusion, clinical use of this panel as non-invasive biomarker is promising, and could be further validated in large scale independent clinical studies.

### Future perspectives

Several initial results and discoveries have come out on circulating miRNA as biomarker for breast cancer detection as well as prognosis and follow-up of patients. Undoubtedly, there is a long way to go before circulating miRNAs emerge as tools for routine clinical use, as the levels of circulating miRNAs are affected by various physiological or pathological conditions.

The reference range of miRNA levels that characterize the healthy state should be determined. Several retrospective studies on many markers have been conducted; nevertheless, there is strong need for prospective assessments in marker validation studies. There is also the need to explore the influence of tumour dynamics in terms of tumour resection and adjuvant chemotherapy on the levels of candidate miRNAs, to assess significance on their role in monitoring treatment response.

Future studies in circulating miRNA biomarkers may focus on combining the expression profiles of circulating miRNAs from all common diseases to obtain specific biomarkers for unique disease detection, which could be used for breast cancer patients follow-up or screening healthy subjects looking for an early and non-invasive diagnostic tool.

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