miRNA in a multiomic context for diagnosis, treatment monitoring and personalized management of metastatic breast cancer

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Metastatic breast cancer is characterized by aggressive spreading to distant organs. Despite huge multi-level research, there are still several important challenges that have to be clarified in the management of this disease. Therefore, recent investigations have implemented a modern, multiomic approach with the aim of identifying specific biomarkers for not only early detection but also to predict treatment responses and metastatic spread. Specific attention is paid to short miRNAs, which regulate gene expression at the post-transcriptional level. Aberrant miRNA expression could initiate cancer development, cell proliferation, invasion, migration, metastatic spread or drug resistance. An miRNA signature is, therefore, believed to be a promising biomarker and prediction tool that could be utilized in all phases of carcinogenesis. This article offers comprehensive information about miRNA profiles useful for diagnostic and treatment purposes that may sufficiently advance breast cancer management and improve individual outcomes in the near future.

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Breast cancer

Statistics

Breast cancer (BC) has been the most common cancer (25.2%) and the leading cause of cancer-related death (14.7%) in female populations worldwide for several years [1]. Despite the huge screening programs applied for early diagnosis and significant improvements in treatment algorithms, there are still about 1.67 million new cases with over a half of million disease-related deaths registered annually worldwide. These alarming data led the scientific community to recognize BC as a modern epidemic in the early 21st century [1–3]. Certainly, currently applied screening programs have contributed to more diagnoses of BC, which increase the overall prevalence recorded for the disease. However, permanently increasing BC incidence is a great worry to researchers, care givers

and society at large. Noteworthy, BC is frequently linked to lifestyle-related risk factors that are modifiable, which opens a great deal of room for strongly under-represented targeted preventive measures in currently applied BC management [3]. On an optimistic note, advances in early cancer detection and modern adjuvant treatment have led to decreased disease-related mortality rates [4,5].

Furthermore, several epidemiological studies confirmed an increasing incidence of metastatic BC (MBC) in young women, which is a very new phenomenon that needs, multiprofessional expertise, multicenter studies and innovative screening programs as clearly demonstrated by recently performed pilot projects [6–8]. Expressed in figures, for example, in the USA the increase of BC incidence in 25- to 39-year old women was from 1.53/100,000 in 1976 up to 2.90/100,000 in 2009 [6]. Data from Europe also show an increase in the number of new malignant BC cases in women under 45 years of age from 16.6 to 37.7% between the years 2002 and 2011 [9].

BC characteristics

BC is a heterogeneous group of neoplastic disorders with various morphologic signs and, therefore, has different clinical behaviors and outcomes [10]. Breast carcinomas can be classified according to the WHO's recommendations using histologic patterns as: invasive ductal carcinoma (80%), followed by lobular carcinomas (10–15%), whereas some of the others are rare [11]. The classification of these rare breast carcinomas is based on the dominating growth pattern and could be mucinous, papillary, medullary or cribriform [12]. The second classical morphologic prognostic factor is histologic grading, which is more important than the morphologic type for clinical management of patients with BC [13]. The most widely used system is Bloom and Richardson's histologic grade, modified by Elston and Ellis in 1991 [14].

It is known that histopathological appearance alone does not reliably predict clinical behavior of the disease in individual patients [15]. Other pathologic features of BC provide additional insights into probable clinical behavior, including hormone receptors status, tumor size, nodal status and invasion of the lymphatics or blood vessels.

Very important factors included in therapeutic prediction are hormone receptors (estrogen receptor [ER] and progesterone receptor [PR]) and HER2, which must be measured on all newly diagnosed primary invasive BCs [16]. The majority of BCs are ER and/or PR (75%) positive [16–18] and about 10–15% of BCs also express the receptor protein HER2. It is a tyrosine kinase receptor involved in regulation of cellular growth and, therefore, HER2 is regarded as a prognostic and predictive factor in the context of anti-HER2-targeted therapy [19]. The extent of heterogeneity of BCs was enlarged by microarray techniques that interrogate concurrent expression patterns across a large number of transcripts and characterized the molecular signature of BC subtypes [20–23]. Nowadays, individual molecular BC subtypes are characterized as luminal A (50–60% of BCs), luminal B (10–20%), HER2 enriched (15–20%), triple-negative BC (TNBC; basal-like; 10–20%), claudin-low and a normal breast-like type [21,24–26]. All the entities have shown significant differences in terms of their incidence, risk factors, patient stratification, prediction, prognosis and treatment selection, and sensitivity to hormonal, chemotherapeutic and HER2-targeting therapy [15,27–30]. These intrinsic subtypes are identified using a combination of specific immunohistochemical analyses of hormone receptors, HER2 expression status and Ki-67 proliferative index as: luminal A-like type (ER-positive and/or PR-positive, HER2-negative, low proliferation), luminal B-like type (ER-positive and/or PR-positive, HER2-negative, high proliferation; or hormone receptor [HR]-positive and HER2-positive), HER2-positive type (HR-negative and HER2-positive) and TNBC type (HR-negative and HER2-negative) [31].

Luminal subtypes tend to have slower metastatic spread, lower recurrence rates and better outcomes than HER2-positive or basal subtypes [32–37]. The comparison of median survival (from time of first distal metastasis) showed that luminal types display longer overall survival (2.2 for luminal A and 1.6 years for luminal B) compared with HER2-positive subtype (1.3 years). The worst overall survival rate (0.7 years) was detected in the basal subtype [38]. The relapse rates also vary considerably between subtypes, with the highest rate for HER2-positive (51.4%), followed by luminal B (42.9%), basal (35.1%) and luminal A (27.8%) [32]. Despite advances in treatment, up to 30% of patients with early BC experience distant disease relapse [39].

Metastatic breast cancer

BC metastasis accounts for the majority of deaths from BC as MBC is often incurable and about 5% of patients already have distal metastases (M1) at the time of diagnosis [40,41]. Moreover, distal metastases occur in 10–15% of patients within the first 3 years. Distal metastases also developed in 33% of BC with no lymph node involvement at the time of diagnosis. However, the metastasis has a complex flow starting with local invasion of

the surrounding tissue, followed by spreading into the blood or lymphatic vessels and ending with dissemination in distal organs [38,40,42].

MBC is characterized by aggressive spread of metastases to distant organs, namely the liver, lung, brain and bones [32,43,44]. Bone metastases remain the most common metastatic site in luminal A and B BCs compared with other subtypes. Luminal subtypes of BC tend to also have an increased risk of lung and distant nodal recurrence and a lower rate of CNS metastases. HER2-positive tumors show a significantly higher risk for distant relapse compared with HER2-negative tumors and have higher metastatic potential in the CNS, despite the implementation of trastuzumab-based treatments for HER2-positive breast and lung cancers in the late 1990s [32,43]. On the other hand, it has low risk of bone metastasis [39]. Basal cancers were found to primarily metastasize to the lungs [39]. Although in BC-free populations primary brain tumors is a rare disorder, brain metastases are reported for more than 20% of patients affected by some specific BC subtypes [45,46], such as the TNBC with more than 50% of patients who die within the first 6 months of MBC diagnosis [47]. Early detection of BC metastasis is extremely important for treatment management and prediction of BC progression [40].

Noteworthy, the younger the age of the BC patients, the more aggressive metastatic disease can be expected with correspondingly poor outcomes [45] that strongly motivates, on one hand, identification of the targeted phenotype of the patients predisposed to MBC and, on the other hand, for the detection of a molecular signature specific for aggressive BC and metastatic disease. Modern analyses of circulating tumor cells show promising results in predicting and identifying the early stages of BC metastasis [40].

Investigations dedicated to the specific molecular signature of MBC still demonstrate moderate progress, though they have been carried over several decades prevalently utilizing a genomic approach. In PubMed, we found 2916 items dedicated to ‘metastatic breast cancer Genomic’ appearing first in the 1980s and currently demonstrating about 250 articles per year. The history of the application of proteomic technologies in the area is much younger: altogether 282 items were found in PubMed regarding ‘metastatic breast cancer Proteomic’ appearing for the very first time at the beginning of the millennium, which indicate the field is still in its infancy. Nevertheless, the early achievements are very promising. The proteomic approach seems to be capable to detect protein patterns characteristic for MBC versus non-MBC cell lines [48], metastatic lymph node tissues [49], differentiation between MBC and non-MBC forms *in situ*, and even to suggest protein targets for diagnostic and treatment purposes [50,51].

miRNAs in cancer

Biogenesis & role of miRNAs

miRNAs are short, noncoding RNAs of typically 22 nucleotides in length, which regulate gene expression at the post-transcriptional level and, thus, are responsible for proteome shaping [50,52] by binding to the 3'-untranslated regions (3'-UTR) of mRNA [53,54]. miRNAs are encoded by genomic DNA and are located mostly in intergenic regions (about 52%), intronic regions of genes (40%) and within exons (8%) [55,56]. They represent the human genome information previously considered junk DNA, nowadays believed to be the hidden treasure regarding their potential relevance in diagnosis, prognosis, treatment and follow-up of cancer [57].

The biogenesis of miRNA is localized to the nucleus and cytoplasm and consists of several steps. In the nucleus, miRNA genes are transcribed mainly by RNA polymerase II and form primary transcripts with a hairpin structure (pri-miRNA). These pri-miRNAs are processed by the RNase III enzyme Drosha and its cofactor DGCR8 into a 70–100 nucleotide precursor pre-miRNA. Some pre-miRNAs are produced, alternatively, by the mirtron splicing pathway [50,58]. The pre-miRNAs are further exported to the cytoplasm by Exp5 and are processed by Dicer and RNase II enzyme that interacts with the 5'- and 3'-end of pre-miRNA and form an about 22 nucleotide double-stranded mature miRNA. The functional strand of mature miRNA is incorporated into the RNA-induced silencing complex, which contains Ago protein and TNRC6 (GW182 protein), and is ready to bind its target mRNA (Figure 1A) [27,50,59]. Translation and maturation of miRNAs can be altered by several genetic and epigenetic mechanisms involved in any phase of the biogenesis (Figure 1B).

The sequence that is crucial for miRNA–mRNA binding is known as the ‘seed sequence’ and is mostly located at a position 2–8 base pairs from the 5'-end of the miRNA. It is an evolutionary conserved part that is complementary to the 3'-UTR of the target-matching mRNA (Figure 2) [54,60,61]. These bindings are able to suppress gene expression by targeting partially complementary regions of mRNAs and inhibiting protein translation. This binding has a crucial role in determining specific miRNA function, which is generally very variable. At the cellular level, miRNAs regulate various physiological and pathological processes such as differentiation, metabolism, proliferation and apoptosis and thus, their alteration can lead to malignant transformation by initiating rapid proliferation, cell

Figure 1. miRNA biogenesis, translational alterations and export. (A) Biogenesis: miRNAs are canonically transcribed from DNA in the nucleus by RNAse II, forming pri-miRNA with a length of about 70 nucleotides (1); processed by the RNA-binding protein DGCR8 and RNase III Drosha into pre-miRNAs with a truncated hairpin that bears a hairpin structure with a 2 nt overhang at the 3'-end (2); sequestered into the cytoplasm by Exp5 and Ran GTPase (3) and further cleaved by Dicer into 21–23 nucleotide duplexes (4) from which one strand of mature miRNA is formed. The mature strand can either bind to the target mRNA or be released to the extracellular environment (5). To exert its effect, miRNA requires RISCs and Ago proteins. An alternative miRNA biogenesis pathway from mirtrons substitutes Drosha cleavage with splicing. **(B) Function:** miRNA expression can be altered by several mechanisms (see violet arrows): chromosomal aberrations, genetic and epigenetic mechanisms, transcription factors that change the expression or the structure of the hairpin, mutations in *EXPO5* leading to miRNA trapping in the nucleus, reduced Dicer expression and post-transcriptional editing (e.g., by ADARs). **(C) Export:** miRNAs can be transported to the extracellular environment either by being bound to RNA-binding proteins such as Ago2 (i), or by binding to lipoproteins (HDL or LDL) (ii), within microvesicles (iii) or loaded in exosomes that are released after endosomes, cytoplasmic membrane fusion (iv). ADAR: Adenosine deaminases acting on RNA; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; RISC: RNA-induced silencing complex.

migration and resistance to treatment [62]. The pivotal regulatory role of miRNAs in biological processes is well demonstrated in the literature [63,64]. To identify precise miRNA targets and to specify functional roles of miRNAs, several databases, algorithms and tools were developed: miRBase [65], miRWalk [66], mirDB [67], DIANA-TarBase [68], miRNA map [69], TargetScan [70], miRanda [71], PicTar [72] and others.

Figure 2. Binding of mature miRNA to the complementary 3'-UTR of targeted mRNA.
 ORF: Open reading frame; RISC: RNA-induced silencing complex; UTR: Untranslated region.

Table 1. Selected types of miRNAs in the breast cancer with their function in disease progression.

miRNA	Oncogenic miRNAs	Tumor-suppressor miRNAs
Function	Promote tumorigenesis by inhibiting tumor-suppressor genes	Inhibition of oncogenes expression
Types	miR-10b [77] miR-17-92 cluster [78] miR-125b [79] miR-18 [80] miR-19a [81] miR-20a [82] miR-155 [83] miR-569 [84]	Let-7 family [81] miR-145 [85] miR-15a [86] miR-16 [86]

A considerable amount of data confirmed the direct involvement of miRNA in the regulation of gene expression at the transcriptional level [73]. miRNAs may control both upregulation and downregulation of target gene transcription. Both these processes seem to be dependent on the presence of a DNA promoter region complementary to the seed sequence of a regulatory miRNA and on a noncoding transcript that overlaps the gene promoter itself [74]. The recognition between the complementary sequence on the noncoding RNA and miRNA arises through classic Watson and Crick base pairing. Currently, this mechanistic model is the most widely favored; however, more recent knowledge proposes that miRNA conducted recognition and interaction with promoter sequences may be accomplished via a direct interaction between RNA and single-stranded DNA complementary sequences. This alternative model of the role of miRNAs in regulation processes of gene expression points to direct and specific binding to DNA promoters, like transcription factors, and provide a platform for the localization of transcriptional regulators and chromatin modifying proteins at genomic loci [73,75].

Regulatory role of miRNA in cancer biology

miRNAs play a vital role in tumor initiation, progression, drug resistance and metastatic disease (Table 1) [76–86]. Moreover, the tissue and cancer specificity of several miRNAs allows for detecting miRNA fingerprints for individual cancer subtypes [54,87–89] that can be instrumental for screening programs, better patient stratification, therapy targeting and efficacy monitoring [50,54,90].

Dysregulation of expression of miRNAs causes altered functionality. As the predominant function of miRNA is, generally, to decrease target mRNA expression, upregulated miRNAs often act as oncogenes, oncomiRs (e.g., *miR-10b*, *miR-21* and *miR-155*), as they downregulate tumor-suppressor genes involved in cell differentiation. This leads to tumor formation, proliferation and invasion. On the other hand, tumor-suppressor miRNAs (e.g., *miR-31*, *miR-125b*, *miR-200* and *miR-205*) downregulate proteins with oncogenic activity and exhibit lower expression in cancer cells [52,53]. However, under certain cellular conditions, miRNA–mRNA binding could lead to increased expression [27,50]. Moreover, some miRNAs could act as an oncogene in one type of cells and as a suppressor in another as they are involved in various mechanisms and pathways [27,52,91,92]. Regarding cancer aggressiveness,

several miRNAs also act as prometastatic factors, called *metastamiRs* (e.g., miR-10b, miR-21 and miR-373), or like *metastasis suppressors* (e.g., miR-31, miR-125b, miR-200 and miR-206) [53]. Here, the importance of miRNAs in cancer spread rises with the knowledge that not only oncogenic and tumor-suppressive transcription factors but also several epigenetic mechanisms like miRNA hypermethylation can modify miRNA profiles crucial for metastatic progression [52]. Consequently, individualized miRNA profiles are decisive for both tumor progression and suppression [27,50].

miRNAs in body fluids as promising biomarkers

Apart from tumor microenvironment, miRNAs can be found and isolated from various body fluids including serum, plasma, saliva, urine, breast milk, seminal fluid, cerebrospinal fluids and others [93–95]. These miRNAs, called circulating miRNAs or extracellular miRNAs (ECmiRNAs), are remarkably stable and resistant to RNases, fluctuations in pH, long storage periods and to multiple freeze/thaw cycles [93,96]. The stability is most probably caused by the transport mechanisms as miRNAs are conjugated in complexes, providing them with protection. ECmiRNAs can be released and transported: in membrane-derived vesicles, either in microparticles (microvesicles) or in smaller exosomes; in HDL or LDL lipoprotein complexes; in the Ago protein complex; or wrapped in large apoptotic bodies (Figure 1C) [97–99]. Exported ECmiRNAs can be taken up by a variety of recipients, even distant cells, where they can alter target gene expression. ECmiRNAs, thus, represent cell–cell communication that contributes in carcinogenesis to tumor progression, metastasis and therapy resistance [50,52,93,100–102]. Export of miRNAs most probably has a selective pattern and is not only passive. Cells secrete specific miRNAs due to cellular signals or environmental cues and load them into specific vesicles. Moreover, some miRNAs seem to be expressed only to be exported, as they were not detected in the parent cell, only in extracellular vesicles [97,103].

Due to characteristics of the ECmiRNAs and noninvasive method of the acquisition mentioned in the ‘miRNAs in cancer’ section, miRNAs represent excellent detectable biomarkers and promising targets in cancer management [100,104,105].

Exploitation of miRNAs in BC prediction & diagnostics

Specific miRNA profiles are capable of differentiating between the individual breast tumor subtypes, namely luminal A, luminal B, basal-like, HER2-positive and normal breast-like [106–108], and possibly discriminate between HER2-positive and HER2-negative tumors [109]. Furthermore, miRNA profiles are differentially associated with lymph node metastasis, vascular invasion and/or higher proliferation index (members of let-7 family, miR-9–3 and miR-145), different tumor stage (miR-21 and miR-145) or metastatic spreading (miR-10b) [110–112]. To this end, plasma concentrations of miR-16, miR-107, miR-130a, miR-146a, miR-214 and miR-520 are associated with lymph node status, and miR-17, miR-21, miR-34a, miR-155, miR-210 and miR-1258 are linked to disease spread and metastases. The levels of miR-10b, miR-146a and miR-373 correlate with both features [113,114]. Noteworthy, miRNA profiles demonstrate some overlaps between Ki-67-positive/HER2-positive/ER-positive/PR-positive groups. Consequently, subtype-specific miRNA patterns should be analyzed for precise diagnosis rather than single miRNAs [115].

miRNA profiles characteristic for individual BC subtypes

Endocrine receptors positive BC subtypes

Approximately 70% of breast malignancies are endocrine receptor (ER/PR) positive, controlling expression of downstream proteins and pathways [27,116]. In the invasive luminal subtypes, miRNA profiles include upregulation of miR-29c-5p. DCIS signature comprises 27 miRNAs in total including upregulated miR-21-5p and the miR-200 family and downregulated let-7 family members [117]. miRNA profiles in cancer tissue with ER-positive (miR-135b, miR-190, miR-217, miR-218, miR-299 and miR-342), PR-positive (miR-377, miR-520g, miR-520f–520c and miR-527–518a) and HER2 receptor status (miR-30e, miR-181c, miR-302c, miR-376b and miR-520d) have been identified. Thereby, miR-342 expression demonstrated the highest levels in ER-positive and HER2-positive luminal B tumors but the lowest ones in the triple-negative tumors; therefore, representing an attractive target for diagnostic, stratification and therapeutic purposes [118].

Furthermore, miRNA blood profiles seem to be dependent on the tumor’s endocrine status, being differentially expressed (miR-155 and miR-182) in serum of women with hormone sensitive versus resistant BC and demonstrating lower levels for miR-155 but higher levels for miR-182 in PR-positive cases [119,120]. The molecular portrait of the luminal A-like BC subtype is further supported by the miR-29a, miR-181a and miR-652 signature [121].

HER2-positive BC subtype

HER2-positive BC is characterized by increased tumor aggressiveness and poor prognosis. miR-9, miR-125a, miR-125b, miR-200 and miR-3622b-5p are involved in regulating pathology relevant ERBB2 and ERBB3 and corresponding protein signaling pathways [122,123]. Thereby, upregulation of miR-210 is associated with trastuzumab resistance, being an important predictive biomarker for the therapeutic response [27,124]. Similarly, miR-9 has targeting potential to HER2 mRNA and increased responsiveness of BC to docetaxel and cyclophosphamide treatments [125]. Characteristics for the luminal tumor subtype plasma miR-130a, miR-146a and miR-373 are also differentially expressed in HER2-positive versus negative tumors [114,126]. To this end, miR-375 is the best representative reflecting better clinical outcomes under neoadjuvant chemotherapy with no relapse in HER2-positive cancers. Increased levels of circulating miR-375 are also characteristic for ER/PR-negative status and inflammatory BC [127].

TNBC & miRNAs

TNBC demonstrates highly heterogeneous profiles at clinical, pathophysiological and molecular biological levels, demonstrating negative hormone receptor status and highly aggressive tumors and early metastatic disease with poor prognosis. Currently, there are no specific diagnostic markers or effective therapeutic approaches elaborated that altogether result in lowering survival rates within the entire BC patient cohort. Subtype-specific miRNA profiles seem to be a powerful instrument to improve the overall situation in TNBC management, due to their ability to regulate pathways fundamental for its pathology, such as cellular growth, proliferation, cellular movement and migration, and extracellular matrix degradation [128–130].

It has been concretely shown that miR-9 and miR-155 are related to epithelial–mesenchymal transition in BC, which is more relevant for TNBC compared with other subtypes. However, their expression profiles inversely correlate with epithelial–mesenchymal transition phenotype; consequently, whereas high miR-9 expression is predictive of distant metastasis and poor disease-free survival, increased miR-155 indicates more positive outcomes [131]. Fingerprints of miR-16, miR-55, miR-125b, miR-374a, miR-374b, miR-421, miR-497 and miR-655 can be further predictive of distant metastasis-free survival in patients under 50 years of age, who are strongly affected by particularly poor outcomes of TNBC. Plasma-based screening may become possible utilizing miR-16, miR-21, miRNA-30a, miR-195-5p, miR-199a-5p and miR-495 profiles for early detection of TNBC [132,133]. Moreover, plasma miR-199a-5p expression levels significantly differ between pre- and postoperative stages, suggesting miR-199a-5p to be highly specific for TNBC as a potential diagnostic marker and therapeutic target [134]. Table 2 summarizes miRNA profiles specific for individual BC subtypes [27,38,53,78,121,135–138].

Primary tumor versus serum: miRNA profiles

Although circulating miRNAs do not reflect the miRNA profiles within the tumor [139], it is a robust indicator for the tumor onset. miRNAs have been proven to be selectively secreted from malignant cells in the mammary gland, and expressed differently in the blood of healthy individuals and patients affected with BC [98]. Therefore, miRNAs hold promise as a future screening marker for BC, because circulating miRNAs associated with neoplasia have the potential to detect cancer even in its earliest stages, and they can differentiate between various tumors, for example, in compliance with their histological features such as hormone receptors and the state of lymph nodes [140].

Only a few studies have evaluated and compared miRNA expressions between tumor tissue and serum. Chan *et al.* [141] aimed to compare miRNA profiles between tumor and serum samples in patients with BC. MiRNA profiles from paired BC tumors, normal tissue and serum samples taken from 32 patients were assessed. The authors found different expressions of the 20 most significant miRNAs in BC tumors including miR-10b, miR-21 and miR-145, which have been previously shown to be dysregulated in BC. Only seven miRNAs have been detected in increased levels in both tissues and serum, suggesting that miRNAs may be released into the serum selectively. Moreover, 16 of the 20 most significant miRNAs found differentially expressed in the serum were novel. In another clinical study, Zhu *et al.* [142] found that over 174 miRNAs were expressed differently between BC tumors and normal tissue, and only 109 miRNAs' expressions differed between serum from BC patients and healthy individuals. This comparison showed only ten common miRNAs. In addition, the study revealed that the changes in miRNA expressions between healthy and BC individuals were opposite in serum and tissue samples for 28 miRNAs. Due to inconsistencies in expression levels of miRNAs in different sample mediums, it seems that miRNA biomarkers (signatures) should be developed as tissue-specific. Recently, the article of Bahrami *et al.* [143] summarized preclinical and clinical investigations and described a list of several tissue-specific miRNAs: miR-7, miR-21, miR-145, miR-

Table 2. Selected circulating breast cancer miRNAs[†] and their respective status of expression in specific molecular subtypes of breast cancer.

Molecular subtype of breast cancer	Expression of nonmetastatic miRNAs		Expression of metastatic miRNAs	
	Upregulated	Downregulated	Upregulated	Downregulated
Luminal A	miR-106a	miR-125a-5p	let-7a	miR-107
	miR-130a	miR-139-5p	let-7b	miR-145a
	miR-99b	miR-155	let-7c	miR-148a
	miR-182	miR-195	let-7f	
	miR-30a-3p	miR-219-5p	miR-155	
	miR-30a-5p	miR-24	miR-103a-3p	
	miR-342-3p	miR-451	miR-126	
	miR-425	miR-99a	miR-16	
	miR-501-5p		miR-17	
	miR-92a		miR-200c	
	miR-30a-3p		miR-107	
	miR-30a-5p		miR-26a	
TNBC	miR-106a	miR-135b	miR-17	
	miR-150	miR-500a-3p	miR-200a	
	miR-15a		miR-200b	
	miR-18a		miR-20a	
	miR-501-5p		miR-519a	
	miR-92a			
HER2 positive	miR-150	miR-125a-5p	miR-155	miR-148a
		miR-125a	miR-21	
		miR-125b	miR-19a	
			miR-375	
Normal-like	miR-130a	miR-100-5p	miR-143	miR-215
		miR-139-5p	miR-299-5p	
		miR-195	miR-335	
		miR-432-5p	miR-411	
		miR-497-5p	miR-452	

[†]Metastatic or nonmetastatic.
 TNBC: Triple-negative breast cancer.
 Data taken from [27,38,53,78,121,135–138].

155/154, miR-182, miR-203 and miR-213, and abnormal activation in BC patients extending to the circulation: miR-16, miR-18a, miR-21, miR-145, let-151a and miR-155, suggesting their values as noninvasive markers and, in addition, as a potential approach to overcome chemoresistance.

Primary tumor versus distant metastases: miRNA profile

As was described earlier, intrinsic (molecular) BC subtypes show a site-specific pattern of metastases formation [144,145], which occur predominantly in the brain and bones. Probably due to the difficulty to obtain metastatic samples, up to now only a few studies compared miRNA expression profiling of either primary breast tumors and their distant metastases or circulating miRNA and metastases [146–148]. Generally, it seems that the miRNA expression pattern of primary and metastatic samples is similar, but with higher expression of metastatic miRNAs [146]. Based on the results of Schrijver *et al.* [146], miR-106b-5p could be an independent predictor of lung and GI metastases, miR-7-5p of skin and miR-1273g-3p of ovarian metastases. As these data represent just the first studies and preliminary results, several aspects remain to be elucidated and methods validated.

Methodological challenges of miRNA profiling

The earlier-mentioned data point out the necessity for the development of standard protocols for blood specimen collection and processing, as well as disclosure of detailed clinical patient information in reports. Many of the

Table 3. miRNAs profile and expressions under chemotherapy and antihormonal treatment.

miRNA	Source	Drug	Expression	Effect	Ref.
let-7	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-10b	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-21	Patient	Epirubicin, cyclophosphamide	High	Not stated	[154]
miR-34	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-34a	Patient	Anthracycline	High	Antitumor effect	[153]
miR-122	Patient	Anthracycline	High	Antitumor effect	[153]
miR-125b	Cell line	Letrozole, anastrozole, paclitaxel	High	Resistance	[79,156]
miR-134	Cell line with artificially enhanced miR-134	Cisplatin	–	Sensitivity to cisplatin-induced apoptosis	[157]
miR-155	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-181	Cell line	Doxorubicin	High	Chemoresistance, cancer cell survival	[158]
miR-195	Patient	Epirubicin, cyclophosphamide	High	Not stated	[154]
miR-200c	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-205	Patient	Epirubicin, cyclophosphamide	Low	Not stated	[154]
miR-205	Cell line	Letrozole, anastrozole	High	Resistance	[79]
miR-221	Patient	Epirubicin, cyclophosphamide	High	Not stated	[154]
miR-424	Cell line	Letrozole, anastrozole	Low	Resistance	[79]
miR-451	Patient	Doxorubicin/cyclophosphamide, taxane	Low	Resistance, worse survival	[155]
miR-3200	Patient	Taxane	Low	Higher residual cancer burden and relapse rates	[155]

discrepancies observed in results within clinical research can be attributed to this early step in the process [149]. Considerable effort has been made in the methodological progress of circulating miRNA detection profiling. Currently, several methodological approaches have emerged to evaluate circulating miRNA levels including quantitative real time PCR (qRT-PCR), microarrays and next-generation sequencing (NGS). Each method has its advantages and disadvantages regarding simplicity, quantification and validity. Concerning the significance of quality control in qRT-PCR analysis or in mRNA microarray, the quality and quantity of the circulating miRNA strongly affect the detection level of analysis. Several techniques, such as phenol–chloroform-based RNA extraction or extraction kits, are available; nonetheless, there is no gold standard for assessing circulating miRNA. Prospective studies are needed to find a consensus on this important issue [149].

The discussion about the advantages and disadvantages of qRT-PCR, microarray and NGS methods is necessary to consider the complexity, throughput, sensitivity/specificity, time factor, required RNA input and expected costs. qRT-PCR is the most useful for assessing several known specific miRNAs. It is easily and quickly performed; moreover, it is characterized by the highest sensitivity, specificity, accuracy and reproducibility compared with other methods. Microarray and NGS are used for high throughput or unknown targets; however, the accuracy, reproducibility and costs may be a problem. In contrast to qRT-PCR, microarrays cannot be used for absolute quantification of miRNA levels because of their lower sensitivity and specificity [150]. Similarly, validation of the NGS method is ongoing to determine its specificity and sensitivity in detecting circulating miRNA [151].

Despite recent reductions in the total costs for microarrays and NGS methods and their improved computational accuracy, qRT-PCR remains the most widely used method for validating the results from microarray and NGS, because it still shows the highest relative sensitivity and specificity. Further effort is required to establish standard protocols for blood specimen collection, processing and analysis of circulating miRNAs regarding BC patients.

miRNAs in therapy

miRNA profiles in response to chemotherapy: therapy prediction, monitoring & prognosis

miRNA profiles sufficiently vary under chemotherapy applied to BC patients, impacting survival rates and disease-free survival [152]. Consequently, they affect the reaction toward treatment and therapy resistance [153,154]. Plasma miRNA profiles vary dynamically during neoadjuvant chemotherapy and antihormonal treatment, differentially correlating with the therapy prognosis in individual BC subtypes [79,155–158]. Table 3 summarizes preliminary data, which should be validated in multicentered, large-scale studies.

Table 4. Circulating miRNA profiles (signatures) as a molecular model for breast cancer treatment monitoring

Breast cancer subtype	Number of cases	Influenced parameter of the treatment monitoring	miRNA signature	Ref.
Early stage, ER+	23	Disease recurrence	miR-377-5p, miR-633b, miR-548t and miR-3648	[159]
ER+	Meta-analysis of five studies	Disease recurrence and survival	↓miR29c, miR30 family and let-7a, ↑miR221/222, miR22 and miR224	[160]
HR+ HER2-	611	Disease recurrence	miR-21, miR-30c, miR-181a, miR-181c, miR-125b, miR-7, miR-200a, miR-135b, miR-22 and miR-200c	[161]
HER2+	110	Disease-free survival	miR-4734 and miR-150-5p	[162]
HER2+, inflammatory breast carcinoma	113	↑Progression-free and overall survival time	↑miR-19a	[163]
TNBC	130	Disease recurrence and overall survival	miR-18b, miR-103, miR-107 and miR-652	[164]
Both TNBC and HR+	90	Disease recurrence	↑miR-21-5p, miR-375, miR-205-5p and miR-194-5p, ↓miR-382-5p, miR-376c-3p and miR-411-5p	[165]
TNBC – BRCA1 or sporadic	50	Overall survival	↑miR-214	[166]
Metastatic CTC-positive or negative	269	The onset of metastasis up to 2 years prior to clinical diagnosis, overall survival	miR-141, miR-144, miR-193b, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-215, miR-365, miR-375, miR-429, miR-486-5p, miR-801, miR-1260 and miR-1274a. Moreover, miR-200a, miR-200b, miR-200c, miR-210, miR-215 and miR-486-5p	[167]

CTC: Circulating tumor cell; ER+: Estrogen receptor positive; HR+: Hormone receptor positive; TNBC: Triple negative breast cancer.

Thus, circulating miRNA profiles provide a very promising tool for the prediction of the most optimal therapeutic modalities and evaluation of therapeutical efficacy. Table 4 shows specific miRNA signatures for treatment monitoring, reflecting BC subtypes.

Beside the association analyses, several preclinical and clinical studies are focused on miRNA detection and aberrant expression patterns during BC treatment [168]. These studies analyze miRNA changes either generally in the context of sensitivity of BC patients to neoadjuvant and adjuvant chemotherapy [169], or are focused on the specific treatment, for example, with herceptin combined with other chemotherapy [170], anthracyclines [171], application of tamoxifen or antiaromatases [172].

Results of these studies together with pharmacological efforts and technological possibilities have great potential for clinical applications in the near future.

miRNA as targets & tools in clinical therapy

Since miRNAs participate in cellular proliferation, differentiation, migration and apoptosis, dysregulation of miRNA expression is involved in tumorigenesis and metastatic activity, correlating with disease progression and recurrence [53,173–175]. Consequently, miRNAs can be used as novel targets in BC treatment [54,59] and several methods for that have been suggested (Figure 3). The first method for inhibiting miRNA in BC utilizes sponges, transcripts with antisense sequences of miRNA, which can bind endogenous miRNAs in cells and inhibit their function in cancer progression. Sponges can be delivered *in vitro* or *in vivo*. For *in vitro* delivery, they are transfected via plasmids or via viral particles [54,176]. Synthetic sponges for miR-183, miR-96 and miR-182 have been created using lentiviral vectors for delivery of cluster sponges and demonstrating a significantly decreased expression of the targeted miRNA cluster [177].

The second method is based on inhibition of miRNAs by delivery of antisense synthetic 17–22 nt oligonucleotides that are designed to bind endogenous mature miRNAs. These antisense synthetic 17–22 nt oligonucleotides are highly unstable due to degradation by nucleases. Therefore, short synthetic oligonucleotides were additionally modified, for example, by changing their phosphate-backbone chemistry to stabilize them in the cell. They are known as ‘*antagomiRs*’ and may be delivered *in vivo*, for example, using viral particles, a liposomal delivery system or nanoparticles-mediated delivery [54,174,178]. AntagomiRs very effectively inhibit tumor growth, migration and invasion [179–181].

The third method, called Inforna, is based on the repression of transcription or transcript processing of miRNAs acting as oncomiRs (oncogenic miRNAs) by binding of small lead molecules to the Dicer or Drosha processing part. Velagapudi *et al.* constructed small bioactive molecules and by their application inhibited the miRNA hairpin precursor of miR-98m, which resulted in downregulated expression of the oncomiR. This methodology is

Figure 3. Methods applied to inhibit cancer progression by dysregulation of mRNA translation by miRNA. (A) Normal miRNA:mRNA binding resulting in translational repression, **(B)** miRNA mimics substitute downregulated miRNAs and bind to target mRNA, which results in translational repression, **(C)** antagomiRs bind to miRNA, thus inhibiting miRNA:mRNA binding leading to mRNA translation, **(D)** synthetic miRNA sponges scavenge complementary miRNA resulting in normal translation of targeted mRNA and **(E)** inhibition of miRNA transcription and biogenesis by small molecules. RISC: RNA-induced silencing complex.

similarly sensitive to using antisense oligonucleotides but has no problems with delivery into cells or with immune responses [182].

The fourth technology applied in cases when miRNAs are downregulated is based on the supply of short nucleotide sequences similar to miRNA. As these synthetic RNA molecules mimic endogenous mature miRNA, they are called miRNAs 'mimics'. In the cancer tissue where the expression of tumor-suppressor miRNAs is low, these synthetic miRNAs can restore antitumor activity [27,54]. The first cancer-targeted miRNA drug is a liposome-based miR-34 mimic already entering Phase I clinical trial [54].

Challenges of miRNA-based therapy delivery

Delivery of miRNA into cancer cells is a challenging step in the overall approach of the targeted therapy [54,183]. The main problems to be resolved are: the poor penetration of miRNA into cancer tissues, degradation of antagomiRs and miRNA mimics without modification in tissues or plasma, miRNA-induced immune responses, neurodegeneration, neurotoxicity, low efficiency of gene silencing, and off-target effects of miRNAs in cancer cell (unmatching) [183,184]. Several technologies have been proposed (Figure 4) considering two main approaches for targeted delivery of miRNAs based on either viral or nonviral systems [185]. Although nonviral delivery systems are less effective, they are less immunogenic, highly biocompatible and unlimited in nucleic acid-loading capacity [186–188]. One method of chemical nonviral delivery is based on synthetic polymers or dendrimers; another system utilizes lipoplex (liposome-based system) preventing degradation of miRNA inside the complex [186]. This system is characterized by low toxicity and has been demonstrated to be efficient in a mouse BC model [189] and MBC [190]. Inorganic carriers, such as carbon or gold nanoparticles, are utilized as an alternative nonviral delivery system, which have been demonstrated to be beneficial, nontoxic and nonimmunogenic [187], offering targeted inhibition of cancer progression [191].

Viral delivery systems utilize several types and subtypes of the viral vectors (retrovirus, adenovirus, adeno-associated virus and lentivirus) with high transfecting ability and expression levels targeting delivery of antagonist miRNA or miRNA mimics into cancer cells [180,192]. miRNA viral-based delivery systems are effective in inhibiting MBC but suffer from high immunogenicity and potential mutagenesis by viral particles [183,185,193]. The review by Abba *et al.* provides comprehensive information about the challenges of miRNA therapeutic techniques [54].

What approach is currently applied?

BC diagnostics are currently based on clinical and physical examinations, histopathology, imaging mammography and ultrasound MRI. Each method has several advantages and disadvantages. The noninvasive ultrasound is considered to be a safe tool, but it cannot be used to screen many of the cancers, mostly at ages above 40 years. Mammography screening is frequently not efficient enough for early tumor detection. Histopathological biopsy analysis is an invasive approach and needle biopsy extracts represent small amounts of the malignant tissue, which is not representative for the entire tumor heterogeneity, and the metastatic spread is not considered at all by this operation. Surgically resected primary tumor alone does not provide sufficient information about future disease development, seeding and specific biological characteristics of (potential) metastases, which are decisive for individual outcomes.

Therefore, there is an enormous effort to find noninvasive biomarkers that could be utilized not only in early diagnostics but could also specify tumor type, predict response to treatment and occurrence of metastases. Significant progress in understanding the cancer-related mechanisms has been achieved in epigenomics by the discovery of the central role of miRNAs in regulating protein expression. Currently, the miRNA signature is believed to be an important biomarker in each phase of carcinogenesis [194,195].

With the rapid development of NGS, massive multi-omics data for BC have been generated in the last years. The systematic integration of multi-omics data seems to be a very effective tool in the diagnosis of specific cancer subtypes and finding novel biomarkers [196]. In this regard, the Multi-Omics Breast Cancer Database (MOBCdb), a simple and easily accessible repository that includes genomic, transcriptomic, epigenomic, clinical and drug response data of different BC subtypes, was recently introduced. MOBCdb allows users to retrieve miRNA expression, but also simple nucleotide variation, gene expression, DNA methylation and specific drug response data by various search modes. The genome-wide browser of the MOBCdb offers an interface for visualizing multiomics data of multiple samples simultaneously. Moreover, the survival module shows survival analyses for all or some of the samples by using data of the three -omics [197]. MOBCdb is a useful tool for clinicians and scientists, which enables them to identify and translate potential novel biomarkers into predictive, preventive and personalized medicine.

Figure 4. Delivery strategies for miRNAs in cancer treatment. (A) Polymer (PLGA or PEI)-based delivery system, **(B)** liposome-based delivery system, **(C)** viral vector and **(D)** gold nanoparticle. PEI: Polyethylenimine; PLGA: poly(lactide-co-glycolide).

Conclusion & future perspective

Individualized patient profiles are crucial for effective BC prediction, prevention and personalization of the services provided to improve BC management. Huge multiomic research, such as genomics, proteomics, metabolomics, epigenomics, including miRNAs, has been focused on complex understanding of biological functions and dynamics of specific diseases, including BC. Revealed results represent a very promising base for early and innovative screening programs, prediction of disease and its development, and specific characterization of individual BC subtypes that should assist in optimized individual-targeted therapy selection with minimal side effects and high efficiency. By early screening, precise diagnostics and individual follow-up, we should significantly decrease BC incidence, prolong life and, most importantly, improve quality of life and treatment outcomes for women.

Executive summary

- Growing evidence demonstrates that the miRNA profiles play a central role in the regulation of the functional pathways, tumor biology and metastatic breast cancer (MBC).
- Aberrational miRNAs have the potential to induce cancer initiation, increase tumor cell proliferation, progression, invasion, migration, dissemination and metastatic spread or drug resistance.
- Individual miRNA profiles hold promise as a multifunctional biomarker for MBCs.
- miRNAs provide information for identifying and characterizing individual breast cancer (BC) subtypes, which is particularly important for accurate prediction of aggressively metastasizing subtypes such as triple-negative BC and inflammatory BC.
- The specific miRNA profiles have been linked to all stages along the metastatic cascade in BC.
- It has been shown that miRNAs may be instrumental for identifying a subtype-specific response to treatment and have the potential to predict the risk of recurrence.
- The plasma miRNA profiles could be used as a robust biomarker for screening, prognosis, treatment management, recurrence prediction and follow-up.
- The altered miRNA profiles reliably indicate the risk of BC clinical onset within 18 months of observation.
- It is suggested that specific miRNA profiles are an essential tool in individualized patient profiling for prediction, prevention and personalized treatment in overall BC management.

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