AMERICAN UNIVERSITY OF BEIRUT

POTENTIAL VALUE OF CIRCULATING MICRORNA AS DIAGNOSTIC BIOMARKERS FOR BREAST CANCER IN LEBANESE WOMEN

by
MAHA MOHAMMAD ITANI

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AN ABSTRACT OF THE PROJECT OF

Maha Mohammad Itani for Master of Science
Major: Physiology

Title: Potential Value of Circulating microRNA as Diagnostic Biomarkers for Breast Cancer in Lebanese Women

Breast cancer (BC) is the most predominant type of cancer among women. Lebanon is one of the countries in which the incidence of breast cancer is increasing tremendously. The Lebanese Ministry of Public Health recommends mammography for women of age 40 and above for breast cancer screening, yet 20.75% of breast cancer patients in Lebanon are younger than 40 years. Therefore, it is critical to find new biomarkers that can help in early detection of breast cancer. Plasma microRNA are stably circulating in body fluids and differentially expressed in tumor versus normal samples making them promising diagnostic biomarkers for breast cancer. Using microRNA microarray profiling, we previously showed dysregulation of 173 mature microRNA in tumors from early breast cancer patients as compared to normal adjacent tissues. A set of these microRNAs including miR-145, miR-425-5p, miR-139-5p, miR-125b, miR-100, and miR-182 were further validated by quantitative real-time polymerase chain reaction (qRT-PCR). Our aim is this study is to investigate the expression of a signature of circulating microRNA (miR-21, miR-130a, miR-155, miR-195, miR-23a, miR-451, miR-145, miR-425-5p, miR-139-5p, miR-125b, miR-100, and miR-182) in the plasma of newly diagnosed early breast cancer patients having Invasive Ductal Carcinoma (IDC) BC. Accordingly, total RNA was extracted from the plasma of Lebanese breast cancer patients. cDNA of specific microRNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit. Then, the expression levels of miRNA of interest were measured using real-time PCR. Finally statistical analysis including Wilcoxon's signed-rank test, Mann-Whitney U test, Linear regression, Logistic regression, and ROC curve were applied to identify microRNA molecules significantly dysregulated in early breast cancer patients, detect any correlation between the expressed microRNAs and breast cancer risk factors and clinical traits, and to evaluate the role of microRNAs as early breast cancer diagnostic biomarkers. Our results showed that 7 microRNAs are significantly upregulated and 1 is significantly downregulated in plasma of early breast cancer patients compared to healthy controls. miR-23a and miR-145 had a significant positive fair correlation with tumor size. The combined diagnostic potential of miR-145, miR-425-5p, and miR-139-5p was more accurate (AUC= 0.929) than that of each microRNA alone. In this context, circulating microRNA would be potential non-invasive diagnostic biomarkers for early breast cancer.

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ABBREVIATIONS

- 3'UTR — 3' Untranslated Region
- AGO2 — Argonaute 2
- AJCC — American Joint Committee on Cancer
- ASR — Age-Standardized Incidence Rate
- ATM — Ataxia Telangiectasia Mutated
- AUBMC — American University of Beirut- Medical Center
- AUC — Area Under the Curve
- Bim — Bcl2 Interacting Mediator
- BRCA1/2 — Breast Cancer type 1/2
- BSE — Breast self-examination
- BT-IC — Breast Tumor Initiating cell
- CBE — Clinical Breast Examination
- CDH1 — cadherin 1
- CHEK2 — Checkpoint Kinase 2
- CK14 — Cytokeratin 14
- CK5 — Cytokeratin 5
- Ct — Cycle Threshold
- CXCR4 — C-X-C Motif Chemokine Receptor 4
- DCIS — Ductal Carcinoma In Situ
- DGCR8 — DiGeorge Syndrome Critical
- EGF — Epidermal Growth Factor
- EMT — Epithelial-Mesenchymal Transition
- ER — Estrogen Receptor
- FFPE — Formalin Fixed Paraffin Embedded
- FOSL-1 — Fos Like Antigen 1
- FOXM1 — Forkhead Box M
- FOXO3a — Forkhead Transcription Factor O3a
- HER2 — Human Epidermal Growth Factor
- IDC — Invasive Ductal Carcinoma
- ILC — Invasive Lobular Carcinoma
- IRB — Institutional Review Board
- LCIS — Lobular Carcinoma In Situ
- LMIC — Low- and Middle- Income Countries
- MiRISC — microRNA-Induced Silencing Complex
- MMP16 — Matrix Metallopeptidase 16

- MRE microRNA Response Element
- MRI Magnetic Resonance Imaging
- mRNA Messenger RNA
- MSH2 MutS Homolog 2
- NBN Nibrin
- NCT Neoadjuvant Chemotherapy
- NGS Next Generation Sequencing
- NSCLC Non-small Cell Lung Cancer
- PALB2 Partner and Localizer of BRCA2
- PDCD4 Programmed Cell Death 4
- POLH DNA Polymerase Eta
- PR Progesterone Receptor
- pre-miRNA Precursor microRNA
- pri-miRNA Primary microRNA
- PTEN Phosphatase and Tensin Homolog
- PTPRJ Protein Tyrosine Phosphatase Receptor Type J
- RAB5A Ras Analog in Brain
- RNA pol II RNA Polymerase II
- ROC Receiver Operating Characteristic
- RTq-PCR Quantitative Real Time- Polymerase Chain Reaction
- SOCS1 Suppressor of Cytokine Signaling 1
- STK11 Serine/threonine Kinase 11
- TARBP2 Transactivation-responsive RNA-binding Protein 2
- TDLU Terminal Ductal-Lobular Units
- TGF-B1 Transforming Growth Factor
- TIMP3 Matrix Metalloproteinases 3
- TN Triple Negative
- TP53 Tumor Protein 53
- TPM1 Tropomyosin 1
- ZO-1 Zonula Occludins-1

CHAPTER I

INTRODUCTION

A. Breast Cancer

1. Incidence and Mortality Rates

a. Worldwide

Breast cancer (BC) is one of the most commonly diagnosed types of cancer worldwide, and the leading cause of cancer related deaths among women [1]. Between 1990 and 2017, the number of newly diagnosed BC cases has risen from 870.2 thousand to 1937.6 thousand women, worldwide [2]. According to GLOBOCAN, the incidence rate of BC among women was expected to further increase to reach 2.1 million newly diagnosed cases in 2018. Similar to incidence rate, the mortality rate of BC is increasing globally where it constitutes 15% of all female cancer deaths (Figure 1) [3]. Importantly, the mortality rate of BC patients is decreasing in high-income countries while increasing in low and middle-income countries. Mortality-to-incidence ratio is high in low- and middle- income countries (LMIC), meaning that patients diagnosed with BC have higher chance of dying from this disease in LMIC vs. high-income countries [4].This variation in mortality-to-incidence ratio is due to the presence of screening and early detection tools and improved treatment in high-income countries [5].

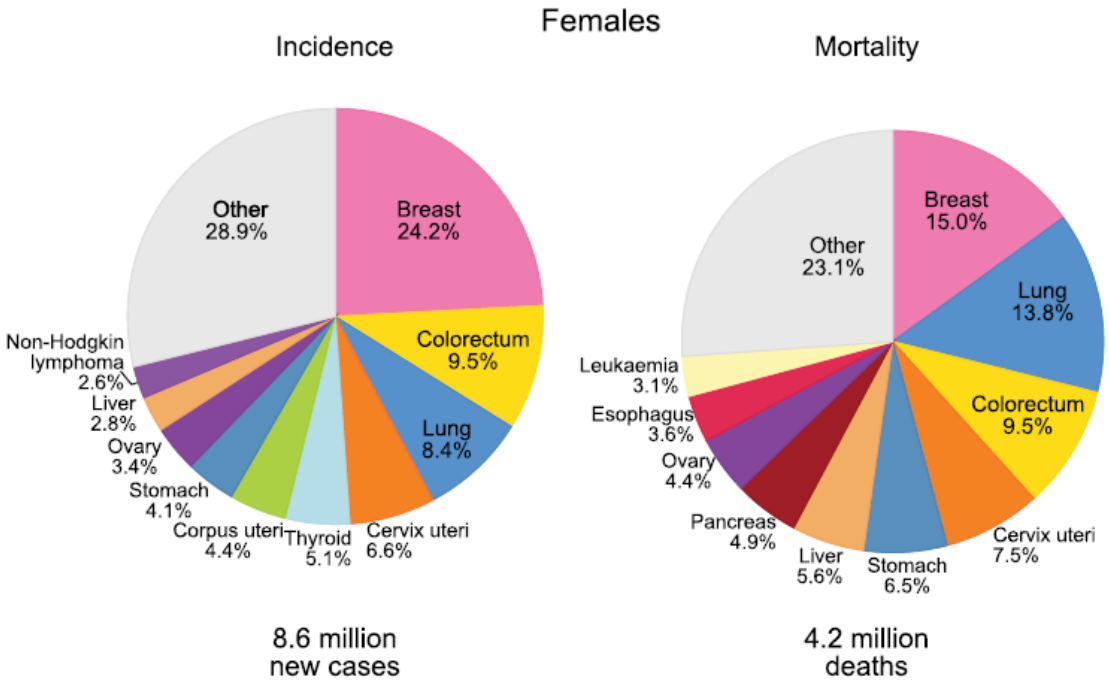


Figure 1. Pie chart showing the distribution of incidence and mortality of the top 10 common cancers worldwide, among women, in 2018. Globally, breast cancer has the highest incidence and mortality rates among women. Source: GLOBOCAN, 2018.

b. In Lebanon

On the report of GLOBOCAN 2018, in Lebanon, similar to all countries, breast cancer is the most prevalent type of cancer constituting 37.9% of all female cancer cases and the leading cause of cancer death among women (920 death cases in 2018) (Figure 2). In order to compare BC incidence in Lebanon with other countries having different population pyramid, the age-standardized incidence rate (ASR) is the best variable to utilize. In a study analyzing the epidemiology of female BC in Lebanon over 7 years, BC ASR remained in first position among all types of cancers affecting women and showed increase from 71.0 per 100,000 in 2004 to 105.9 per 100,000 in 2010 [6]. According to Fares *et al.* (2019), between 2005 and 2015, Lebanon ranked the first position among the regional countries with respect to the breast cancer ASR (91.7 per 100,000), followed by Malta (79 per 100,000) and ranked the second position among randomly selected countries, preceded by Denmark (97.3 per 100,000) [7]. The mean age at diagnosis of female BC in Lebanon was 54.6±13.4 years with 43% diagnosed before the age of 50 years between 1990 and 2013 [8]. However, the incidence rate of BC among females continues to increase with ages. It was nearly null below the age group (25-29) and more than 90% of BC patients were 35 years and older [7]. Based on the Lebanese Ministry of Public Health recommendations to start mammography screening at age of 40 years, 20.75% of Lebanese BC patients aging between 0 and 39 years (GLOBOCAN 2018) are not eligible to mammography. Thus, it is of great importance to find reliable, sensitive, and minimally invasive biomarkers, which help in BC early detection especially for patients younger than 40 years.

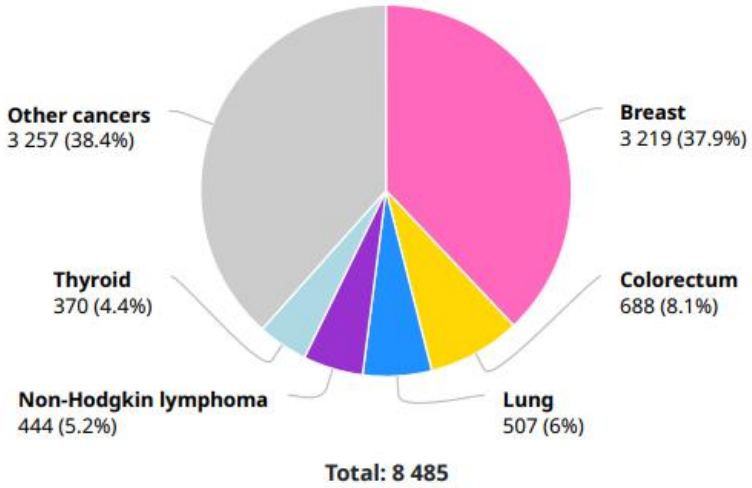


Figure 2. Pie chart representing the numbers and percentages of the 5 most common cancer types among females in Lebanon, 2018. Breast cancer, mostly widespread, attributes to 37.9% of all cancer cases in Lebanese women. Source: GLOBOCAN, 2018.

2. Breast Cancer Risk Factors

Identifying the risk factors of breast cancer provides women with opportunities to promote its prevention. Breast cancer risk factors could be categorized as nonmodifiable intrinsic factors (such as age, sex, race, genetic makeup) or modifiable extrinsic factors (such as lifestyle and long-term medications) (Figure 3) [9]. 4.7% of female BC cases reported at the American University of Beirut-Medical Center (AUBMC) between 1983 and 2000 were below 30 years of age while 28.3% and 26.3% were 40-49 and 50-59 years of age, respectively [10]. In addition to that, a study by Shamseddine *et al.* concluded that a population with high proportion of elderly suggests further increase in cancer cases, including BC [11]. Recently published paper projecting geriatric cancer trends in Lebanon until 2025 claimed that cancer is expected to increase in individuals between 65 and 74 years of age [12]. Having an unhealthy lifestyle is one of the documented risk factors such as active and passive smoking [13, 14], lack of physical activities [15] and shift from healthy Mediterranean diet to Westernized food [16], alcohol consumption [17], reduced breastfeeding [18], and usage of hormone replacement therapies [19]. A study comparing the risk factors of developing BC in Lebanese vs. Lebanese-American women showed that Lebanese women smoke more, exercise less, breastfeed for shorter period of time, and have less knowledge of the benefits of BC screening tools making them at higher risk of developing breast cancer [20]. Reproductive factors (early menarche, delayed menopause, delivery after 35 years, and infertility) affect the risk of developing BC [21-24]. In addition to that, women who had a history of breast carcinoma *in situ* or invasive breast carcinoma have higher risk

of second cancer, especially breast cancer [25]. Finally, yet importantly, breast cancer incidence is affected by the genetic factor such as family history and genetic mutations. A positive association has been reported between family history of BC and the development of any of the 4 BC subtypes (Luminal A, Luminal B, HER2+, and triple negative BC) [26]. The risk of breast cancer was shown to vary based on type and location of BRCA1/2 mutations [27], however these mutations alone didn't support the high percentage of breast cancer in Lebanon and the Arab countries [28] indicating that there are other mutations aiding in the increase of BC cases such as CDH1, TP53, MSH2, ATM and POLH which are found to be associated with several hereditary cancerous syndromes among the Lebanese population [29].

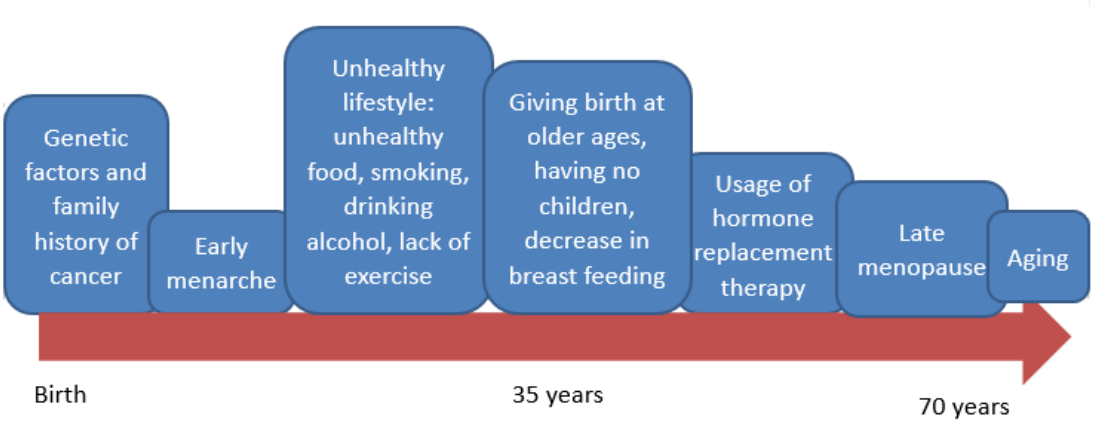


Figure 3. Lifetime of a women and the risk factors contributing to the development of breast cancer at each stage of her life. At birth, the risk factors making women predisposed to BC are inheriting mutation related to BC and/or having family history of BC or any type of cancer. Risk factors for developing breast cancer in women varies throughout her life. The final and general risk factor for BC development is aging.

3. Anatomy, Physiology, and Tumorigenesis of Mammary Gland

The breast, also known as the mammary gland, is a unique organ for the mammalian reproductive system. It is generally the first female secondary sexual characteristic to develop at puberty. A refresher on the anatomy and physiology of the mammary gland is necessary to classify BC subtypes and understand BC pathology.

Mammogenesis starts during fetal stage (at week 4 of gestation) and pauses during childhood. At puberty, mammary gland development terminates in males but continues in females. The key anatomy of the breast includes parenchyma (ducts and lobes) and supporting stroma (fats, connective tissues, nerves, lymphatics, arteries and veins). The ductal system possesses branching epithelial structure in which the primary ducts undergo extensive branching until the formation of terminal ductules. At the tip of the terminal ductules, there are small secretary sac like structures called alveoli. The glandular tissue of the mammary gland is organized in 15-20 lobes. Each lobe is composed of several lobules, which are grape like clusters of mammary alveoli. At the cellular level, the ducts are lined by two cell layers: the myoepithelial cells (facing the basement membrane) and the luminal cells (facing the lumen of the ducts). The mammary stroma is a highly fibrous structure in which the glandular and ductal system are embedded [30, 31]. The functional unit of the breast is called terminal duct lobular unit (TDLU) which is a collection of acini arising from one terminal ductule and the surrounding interlobular stroma [32]. The mammary gland reaches its mature structure only during pregnancy/lactation cycle, in which it undergoes micro- and macro-modifications to become a fully functional milk-secretary organ [30].

The mammary gland structure is organized to fulfill its physiological function, milk production, secretion, and ejection for the nourishment of neonates. Mammary epithelial cells are responsible for milk proteins and lactose synthesis and secretion under the effect of prolactin. In addition to that, they have oxytocin receptors which when activated; the milk will be expelled out of the alveoli into the ducts and then the lactiferous sinus. The myoepithelial mammary cells obtain asynchronous contractile activity. In response to oxytocin release, myoepithelial cells start contracting causing an increase in the intraductal pressure, ultimately leading to milk ejection [33].

Genetic and epigenetic alterations in a single cell of the mammary gland are at the basis of BC initiation. Mutations which activate specific proto-oncogenes and/or inhibit specific tumor suppressor genes expression, enable the cells to break free of mechanisms which normally control their proliferation, differentiation, migration, and death [34]. With these mutations, the breast cancer progresses through pathological and clinical stages. At first, luminal cells start hyperproliferation leading to an increase in the cells of the mammary ducts or lobules. Next stage is the development of carcinoma in situ in either the duct or lobule. In carcinoma in situ, myoepithelial cells become altered phenotypically and epigenetically thus losing their arrangement and the basement membrane start to be degraded, resulting in the progression of carcinoma in situ to invasive carcinoma [35]. The last stage in breast cancer progression is metastasis depicted by migration of tumor cells to other organs through the lymphatic or blood circulations [35].

4. Breast Cancer Classification

Breast Cancer has been shown to be a collection of biologically and molecularly distinct diseases rather than being a single disease. Therefore, breast cancer could be classified and subclassified into several types based on histopathology, immunopathology, and molecular aspects. Figure 4 represents the mostly widespread BC subtypes, their characteristics, prognosis, and grade.

a. Histopathological Subtypes

Based on the classical histopathological classification, breast carcinoma is categorized into non-invasive (in situ carcinoma) or invasive (infiltrating carcinoma). Furthermore, these classifications are subdivided based on the location where the tumor arise, whether in the ducts or lobules. It is worthy of note that histopathological classification of BC has prognostic implication. Ductal carcinoma in situ (DCIS), the most prevalent in situ carcinoma category, is considered to be an obligate precursor lesion to invasive breast cancer as compared to lobular carcinoma in situ (LCIS) which is a non-obligate precursor lesion [36, 37]. Among the invasive carcinoma category, the invasive ductal carcinoma (IDC) is the most common type, accounting for 55% of breast cancer incidence upon diagnosis, and invasive lobular carcinoma (ILC) ranked the second major biologically distinct invasive mammary carcinoma [37, 38]. Other histological subtypes that include apocrine, mucinous, papillary, tubular, micropapillary, and neuroendocrine carcinomas are less common.

b. Immunopathological Subtypes

Immunopathologically, BC is classified based on specific markers found on the tumor cells. The markers include estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2). ER status helps in predicting the efficiency of hormonal therapy more than PR status which has less clinical significance. Expression of the combination of these three receptors, results in 4 BC subgroups: ER^+ ($ER^+/HER2^-$), $HER2^+$ ($ER^-/HER2^+$), triple negative (TN; ER^-/PR^- /HER2), and triple positive ($ER^+/PR^+/HER2^+$) each with different overall prognosis and therapeutic response [39]. BC cases with ER^+ and /or PR^+ status showed better prognosis than BC cases that are ER^- and/or PR^-[40, 41], HER+ tumors showed more aggressive clinical behavior but better response to targeted therapy than HER2- tumors [41], and TNBC cases showed significantly poorer prognosis[42].

c. Molecular Subtypes

The first description of molecular classification of BC was in 2000, whereby each individual tumor was demonstrated to have its own unique global molecular profile [43]. Based on ER and HER2 expression, 4 main groups of BC were identified: Luminal, HER2 enriched, basal-like, and normal breast like carcinomas [44]. Luminal breast cancers are enriched for ER^+ tumors and subclassified into two main subgroups: Luminal A (low grade with excellent prognosis, $ER^+/ PR^+/HER2^-$, and low expression of proliferation related genes) and Luminal B (high grade with low prognosis, PR^- and/or $HER2^+$, and high expression of proliferation related genes) [45]. HER2-enriched

subtype is an ER negative cancer. Most tumors in this subgroup show HER2 gene amplification or HER2 protein overexpression. HER2-encriched tumors had a large improvement in event-free survival with the addition of HER2 targeted therapy, trastuzumab [45]. Basal-like subtypes are typically high-grade TNBC characterized by up regulation of high molecular weight cytokeratins (CK5 and 14), P-cadherins, and epidermal growth factor receptor (genes expressed by basal/myoepithelial cells) [46]. The fifth group (normal-like subtype) has gene expression pattern similar to that of a normal breast tissue [47], however, it has been dropped as it has been thought to represent contamination by normal gland [45].

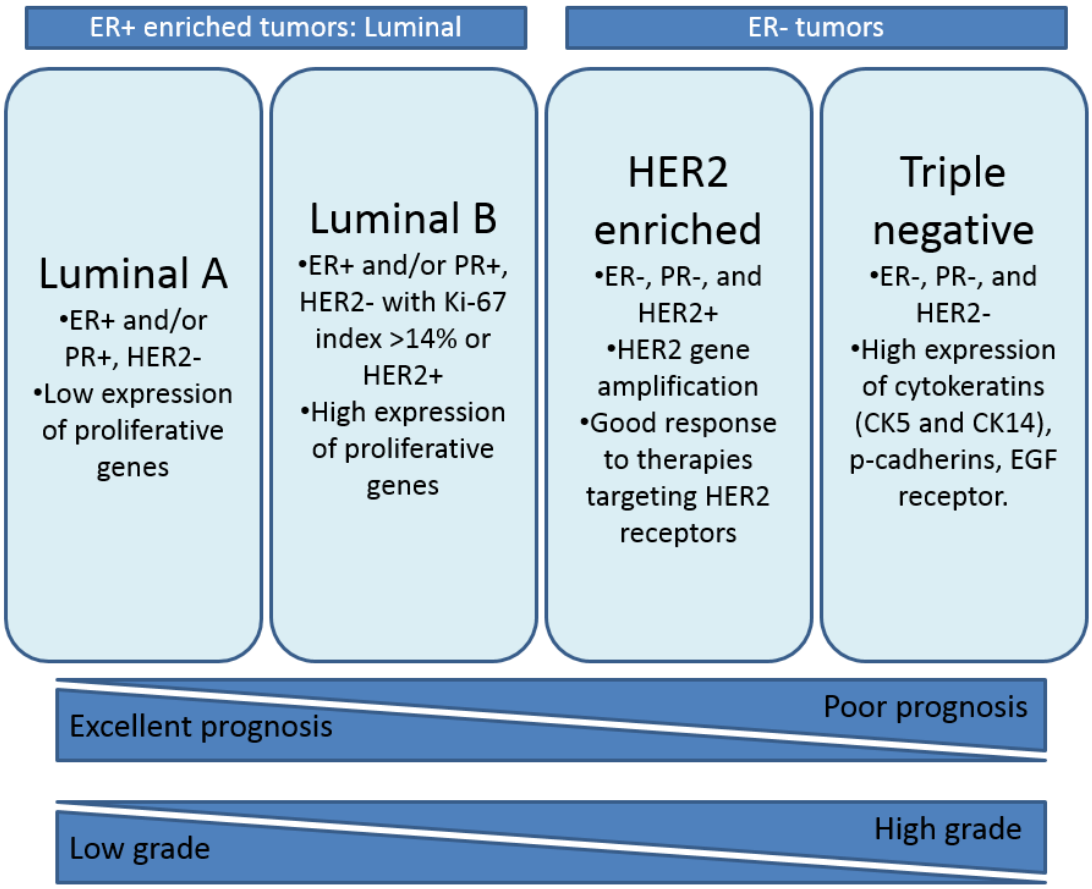


Figure 4. Molecular breast cancer subtypes and their corresponding prognosis and grade. The receptors expressed on the breast cancer cells (estrogen receptor;ER, Progesteron receptor; PR, and Human epidermal growth factor receptor-2; HER-2) are major biomarkers in classifing breast cancer cases into the 4 major molecular subtypes: Luminal A, Luminal B, HER-2 enriched, Triple negative breast cancer. Luminal A breast cancer has excellent prognosis and low grade, while Triple negative breast cancer has poor prognosis and high grade.

5. Breast Cancer Prevention and Diagnosis

Breast cancer prevention plays a major role in reducing its mortality and morbidity. The preventative methods include primary prevention (avoiding the editable risk factors that are lifestyle and habitual related such as exercising more, quitting all forms of smoking, consuming low-fat diet, and decreasing alcohol intake) and secondary prevention (raising awareness and launching campaigns targeting the importance of early detection of BC through breast self-examination (BSE), clinical breast exam (CBE), mammography, and ultrasound imaging) [48].

BC early detection is a critical stage in the process of treatment and survival. There are several strategies utilized to diagnose BC in its early stages. BSE was considered to be part of breast cancer screening, until 2009 where it was excluded from the screening program in the US [49]. 2 randomized clinical trials in China and in Russia, claimed that BSE increases the numbers of unnecessary biopsies for benign breast lesions without any decrease in mortality [49]. In addition to that, BSE has low sensitivity (12-14%) and high index of false-positive results [48]. Ginsburg *et al.* explained the phases of early BC detection program, starting with strategies for clinical breast assessment (such as taking medical history and applying physical examination, including clinical breast examination (CBE) followed by imaging and tissue sampling) [50]. Imaging techniques have been labeled as powerful tools for BC detection and monitoring [51]. The gold standard imaging technique for BC detection is mammography. Although this technique is the primary approach in BC detection due to its easy implementation, easy standardization, and minimal technical set-up yet it is

associated with pain, anxiety, and radiation exposure risk [52]. Not to mention that some of the potential harms of mammography include false-positive results, recall for additional imaging, missed BC (false-negative), and overdiagnosis [53]. Moreover, mammography screening is of limited utility for some women especially those younger than 40 years since they tend to have denser breast tissue. In short, the superposition of fibroglandular tissues may obscure small tumors in dense breast tissues [54]. As such, mammography sensitivity decreases from 90-95% to 60-75% in high density breast tissues [55]. Ultrasound techniques (Ultrasound contrast and Elastography) are another important BC tumor detection methods especially among young women, pregnant, and breast-feeding women since it doesn't utilize ionized radiation and is not limited to low breast densities [56]. One of the factors which block Ultrasound technique from being the perfect screening tool is that it cannot identify small and/or deep lesions, thus giving false-negative results [57]. Another imaging technique used for many aspects of BC is MRI which help in monitoring response to therapies and assessing BC metastasis and provide several data for clinicians (e.g., BC staging and premalignant lesions) [58, 59]. The specificity of MRI technique according to literature varies between 37% and 97% [57]. MRI technique cannot differentiate between malignant lesions and benign lesions, leading to false positive results and is not found in every medical center [60]. A study evaluating the efficacy of mammography, ultrasound, and MRI techniques, reported that mammography had the lowest sensitivity and MRI technique had low specificity. Moreover, a cross-sectional study evaluated the accuracy of the same three screening through reviewing the records of 32 female BC patients [61]. The results showed that

mammography, ultrasound, and MRI techniques had sensitivity of 56.2%, 75%, and 100%, specificity of 87.5%, 18.8%, and 50%, and accuracy of 71.8%, 46.9%, and 75%, respectively [57]. Economically, mammography and Ultrasound techniques are inexpensive and require5-10 minutes as total imaging time while MRI technique is expensive and requires longer total imaging time (20-30 minutes) [61]. Table 1 shows the mostly used imaging techniques for early detection of breast cancer.

An increasing demand led to the development of new framework for screening incorporating molecular diagnostics which increase the benefit and decrease the harm [62, 63]. Genetic testing is available for individuals who are at higher risk of BC and might carry the BRCA gene mutation, however, this screening method is highly expensive [63]. Myriad Genetics company first offered the BRCA testing [64, 65]. Later, several laboratories started offering the BRCA testing plus other predisposition gene tests [66]. Some of the genetic tests available on the market which target high-risk individual (family history of breast cancer) are: BreastNext (screens for mutations in 17 cancer related genes using blood or saliva specimens), CancerNext (screens for mutations in 28 cancer related genes using blood or saliva specimens), OncoGeneDx Custom Panel (screens for mutations in 28 cancer related genes using blood or oral rinse specimens), and Breast/Ovarian Cancer Panel (screens for mutations in 21 cancer related genes using blood or oral rinse specimens). Next-generation Sequencing (NGS) is a promising method which can identify genes associated with breast cancer risk. Using NGS, Pinto *et al.* analyzed a panel of 17 genes, in which 10 of them (ATM, BRCA1, BRCA2, CDH1, CHEK2, NBN, PALB2, PTEN, STK11, and TP53) are

suspected to be associated with the development of hereditary BC. The results showed that there are 4 genetic mutations (ATM, CHEK2, PALB2, and TP53) other than BRAC1 and BRCA2 predisposing to BC. Most recently, microRNA which is the core of our study (discussed in further sections) and exosomes are showing to be promising diagnostic biomarkers for early BC detection [67].

Diagnostic Test	Imaging/ Genetic based	Disadvantages	Advantages	Genes it tests for
Mammography	Imaging based	Pain, anxiety, radiation exposure	Easy implementation, easy standardization, minimal technical set-up, false positive and false negative results, and affected by breast density.	
Ultrasound		No ionized radiation used, not limited by breast density	Cannot identify small and deep lesions and give false negative results	
MRI	Imaging based	Help in identifying premalignant lesions	Give false positive results, low specificity	
BreastNext	Genetically based	Some panels provide a wide variety of genes	Wide variety of cut-offs in research studies instead of 1, increased prevalence of VUS, and tests for genes the patient don't want to test for	Total 17 gene including: ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MRE11A, MUTYH, NBN, NF1, PALB2, PTEN, RAD50, RAD51C, RAD51D, TP53
CancerNext				Total 28 genes including: BRCA1, BRCA2, RAD51C, RAD51D, NTHL1, CHEK2, PALB2
OncoGeneDx Custom Panel				Total 28 gene including: Ki67, STK15GRB2, HER2, ER, STK15, MMP11, GSTM1, ACTB, GAPDH
Breast/Ovarian Cancer Panel				Total 21 gene including: ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, NBN, PALB2, PMS2, PTEN, RAD51C, RAD51D, TP53

Table 1. List of the mostly spread imaging techniques for early breast diagnosis. All the techniques showed to have advantages and disadvantages.

B. microRNAs

microRNAs (miRNAs or miRs) are short (made up of ~22 nucleotides), single stranded, non-coding, and silencing RNA molecules [68]. They play a fundamental role in gene expression regulation in almost all key cellular processes [69]. The first microRNA, lin-4, was described in 1993 in *C. elegans*, suggesting later that it regulates lin-14 translation via an antisense RNA-RNA interaction [70]. Since then, more miRNAs and their role in regulating plant and animal gene expression were established. In 2019, newer version of miRbase (v22), a database for miRNA annotation and sequence, was released and it included 48,860 mature miRs from 271 organisms and 2,654 mature miR sequences in the human genome [71].

1. microRNA Biogenesis

microRNA biogenesis is a multi-step process, starting with a coding gene sequence and ending up with a mature miR molecule capable of targeting hundreds of mRNA. The genes encoding for microRNAs are located in all human chromosomes. Specifically, some miRs are transcribed from a non-coding gene whose only transcriptional product is microRNA, while others are transcribed from the introns or untranslated region of a protein coding gene such as the intron of protein coding gene MCM7 which encodes for a cluster of 3 miRs [72].

The biogenesis of miRs, illustrated in figure 5, starts in the nucleus, where miRNA genomic sequence is transcribed by RNA polymerase II into a long primary transcript, called pri-miRNA. Next, the pri-miRNA binds to the Drosha-DGCR8 protein

complex through its stem loop to be micro-processed into precursor miRNA (pre-miRNA). Some miRNAs are byproducts of introns splicing and they bypass the Drosha splicing step. These miRNAs are called mirtrons and they follow the non-canonical biogenesis pathway [73]. To be further processed, pre-microRNA is exported from the nucleus into the cytoplasm with the help of enzyme Exportin 5/RanGTP. In the cytoplasm, pre-miRNA undergoes a process called dicing in which it is cleaved by the DICER complex (RNase III enzyme Dicer 1 in union with transactivation-responsive RNA-binding protein 2 (TARBP2) and Argonaute 2 (AGO2)). The product of the dicing process is a double stranded miRNA-miRNA* duplex. After that, the double strand miRNA unwinds asymmetrically leading to the separation of the two strands [74, 75]. Finally, one of the strands which act as mature functional miRNA strand (guide strand) and the AGO proteins are incorporated into the RNA-induced slicing complex forming the miRNA-induced silencing complex (miRISC) capable of reaching the complementary mRNA sequence of the target gene, while the other microRNA strand (passenger strand) can be loaded in the RISC complex or degraded.

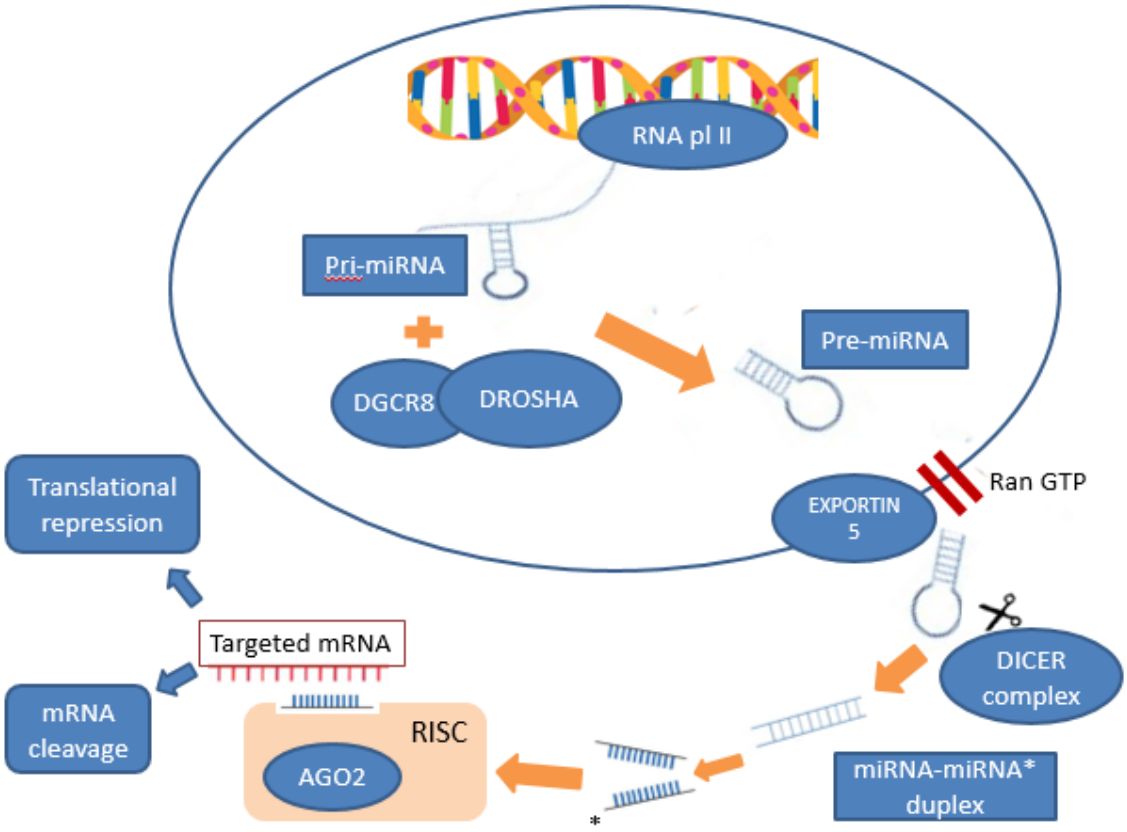


Figure 5. microRNA molecules canonical biosynthesis process. The synthesis of microRNA starts in the nucleus. miRNA intronic and intergenic genes are transcribed by RNA polymerase II (RNA pol II) enzyme forming primary miRNA (pri-miRNA). pri-miRNA is then cleaved by the DROSHA and DGCR8 complex forming the precursor microRNA (pre-miRNA). With the help of Exportin-5 the pre-miRNA exits the nucleus into the cytoplasm in a Ran-GTP dependent manner. In the cytoplasm, the DICER complex (RNase III enzyme Dicer 1 in union with transactivation-responsive RNA-binding protein 2 (TARBP2) and Argonaute 2 (AGO2)), cleaves the pre-miRNA resulting in the microRNA-microRNA duplex. Then the duplex unwinds resulting in 2 microRNA strands. The guide strand microRNA incorporates into the RNA-induced silencing complex (RISC) and interacts with the targeted mRNA, leading to translational repression or mRNA degradation. * denote the passenger strand. Blue ovals denote enzymatic proteins, while blue rectangles denote the produced precursors.

2. microRNA Mode of Action and Biological Function

Each mature miRNA has unique 6-8 nucleotide sequence at their 5'end, called the "seed" sequence which binds complementarily to the 3' untranslated region (3'UTR) of the targeted mRNA [75, 76] leading to translational repression or mRNA degradation and thus altering the expression of the corresponding gene [77]. Some studies indicated that miRNA could bind to other regions on the mRNA including 5'UTR, coding region (silence mRNA and gene expression [78, 79]), and promoter region (induce transcription and gene expression [80]). One miRNA can target several mRNAs thus affect the expression of many genes involved in functional cellular pathways, simultaneously. The degree of complemanterity between the miRISC and MRE (microRNA response element; the complementary sequence on the mRNA) initiate either AGO2-dependent slicing of the targeted mRNA or miRSC-mediated translational inhibition and mRNA decay [81]. A fully complementary interaction between miRISC and MRE leads to AGO2 endonuclease activity and mRNA cleavage [81]; which is not the case in animals, due to a central mismatch in the MRE region [82].

MicroRNAs are involved in a variety of biological functions including cell/tissue development, differenciation, homeostasis, metabolism, proliferation and appoptosis, and immune response [83]. The biological importance of miRNAs was first demostrated in mouse model deficient for Dicer and DGCR8. Results showed that the loss of miRNA biogenesis lead to embryonic lethality in mice [84, 85]. Moreover, tissue-specific knock out of either gene lead to developmental defect in that tissue [86]. These studies, highlight the need for functional miRNA in the developmental process of

mammalian tissue. Besides the developmental process, miRNAs regulate the biological homeostasis process. Chandrasekarana *et al.* explored the role of miRNA in maintaining kidney homeostasis through regulating blood pressure, hormone, water, and iron balace [87]. Another biological function in which miRNAs are involved is differentiation. miRNAs have been identified in several differentiation processes including hematopoeisis, myogenesis, cardiogenesis, and neurogenesis [88]. The normal role of miRNAs could be affected by several factors including epigenetic modification and hereditary mutations in the miRNA seed region leading to pathological processes (such as cancer development) [89].

3. microRNA Role and Regulation in Cancer

Some effective technologies (including multiplex PCR, microarrays, and most recently next-generation sequencing) have identified the expression profile of several microRNA in different human pathologies [90, 91]. With these technologies, a correlation between altered miRNA expression profile and numerous malignancies was achieved. miRs are sorted based on the target gene they are regulating into "*oncomiRs*" or "*Tumorsuppressor-miRs*". OncomiRs and tumorsuppressor-miRs coding genes are located in a fragile region of the genome facilitating microRNA aberrant expression or deletion [92].

a. OncomiRs

microRNAs that are known to promote tumorigenesis and growth are termed "*oncomiRs*". *OncomiRs* are usually upregulated in cancer cells and tissues and target tumor-suppressor protein-coding transcript [93]. The first validated oncomiR was miR-17/92 cluster (oncomiR-1) whose overexpression leads to more proliferation and less cell death of the lymphocytes via targeting the pro-apoptotic protein, Bim [94]. Other miRNAs that were established as oncomiRs and shown to be upregulated in several hematological and solid tumors are: miR-21 and miR-155. Together, miR-21 and miR-155 were shown to be upregulated in patients with chronic lymphocytic leukemia [95]. Furthermore, miR-21 acts as an oncomiR in breast cancer, where it downregulates the expression of the metastasis suppressor protein PDCD4 [96]. Its overexpression was associated with advanced clinical stages, lymph node metastasis, and poor prognosis among BC patients [97]. In addition to that, miR-155 showed to act as an oncomiR in colorectal cancer by targeting the tumor suppressor PTPRJ [98] and in Breast cancer by targeting the suppressor of cytokine signaling 1 gene [99].

b. Tumorsuppressor-miRs

On the contrary to *"oncomiRs", tumorsuppressor-miRs* regulate the expression of oncogenes through targeting their oncogenic protein-coding mRNA transcript. In cancerous cells and tissues, *oncosuppressor-miRs* are downregulated [93]. miR *let-7* is one of the tumorsuppressor-miRs that is highly expressed in differentiated adult tissues but is lost in cancers [100, 101]. The decreased expression of let-7 results in oncogenic

loss of differentiation. In humans, let-7 is located at a chromosomal region that is usually deleted, leading to the loss of this tumorsuppressor-miR and initiation of cancer [102]. In addition to let-7, some studies showed that miR-9 functions as tumor suppressor in colorectal cancer by targeting CXCR4 [103] and miR-505-5p is a tumor suppressor which regulates tumor cell proliferation, migration, and invasion in cervical cancer [104]. In breast cancer, miR-145 exerts a tumor suppressive effect by targeting several genes leading to: inhibition of breast tumor-initiating cells (BT-IC) differentiation [105], inhibition of breast cancer cell growth [106], induction of apoptosis [107], suppression of cell invasion and lung metastasis [108], and inhibition of breast cancer cell EMT [109].

As indicated previously, one miR can target the expression of hundreds of genes, and one single miR can act as both an oncomiR and Tumorsuppressor-miR, such as miR-125b. The targets of miR-125b include mRNAs encoding anti-apoptotic factors (MCL1, BCL2L2, BCL2), pro-apoptotic factors (TP53, BAK1, BMF, BBC3, MAPK14), pro-proliferative factors (JUN, STAT3, E2F3, IL6R, ERBB2/3), metastasis promoters (MMP13, LIN28B, ARID3B), metastasis inhibitors (STARD13, TP53INP1, TP53), and factors involved in hematopoietic differentiation (CBFB, PRDM1, IRF4, IL2RB, IL10RA). Therefore, the balance between the expression of the targeted oncogenic/tumor suppressor genes determines the role of miR-125b as oncomiR or tumorsuppressor-miR, depending on the context. miR-125b has a net oncogenic effect in hematological malignancies, where as in solid tumors, it has a net tumor-suppressive

effect [93]. In breast cancer, miR-125b shows a tumor-suppressive function and is reported to be down-regulated in breast cancer biopsy specimens and cells [110].

4. Circulating microRNA as Cancer Biomarkers

Deregulation of miRNAs has been observed throughout the progression of cancer from early stages, till late stages (after metastasis). Circulating microRNA found in plasma, serum, urine, saliva or any fluid in the body exhibit promising potential for cancer screening.

Etheridge *et al.* indicated that an ideal biomarker must be specific, sensitive, predictive, robust, translatable, and non-invasive [111]. Hence, with the fulfillment of preceding criteria, circulating miRs provide a potential clinical role as biomarkers for cancer diagnosis, prognosis, and therapy prediction. The presence of miRNA in serum was firstly observed in 2008 in patients with diffuse large B-cell lymphoma [112]. Later, another study reported that miRNA in human plasma is stable because these tiny molecules are protected from endogenous RNase activity [113]. Yu et al claimed that miRNA in circulation are relatively stable, very accessible, less invasive, and easily testable, hence they are potential unique biomarkers to detect and monitor cancer [114].

a. Diagnostic Role

Circulating miRs were considered as potential diagnostic biomarkers of numerous malignancies, some of which are discussed in this section [89]. One of the

world's most common malignancies is breast cancer. Circulating microRNA help in distinguishing early breast cancer (EBC) patients from healthy individuals. Most recently, Guo et al concluded that miR-1273g-3p (upregulated in plasma) could represent a potential biomarker for early breast ductal cancer diagnosis (AUC: 0.6333, 95% CI: 0.5114-0.7552, p=0.0414) [115]. Besides, several studies performed on different ethnic groups and using distinct experimental methodologies, confirmed that miR-21 and miR-155 serve as diagnostic biomarkers for EBC [116]. A 9-serum miRNA signature (miR-425, miR-365, miR-145, miR-143, miR-139-5p, miR-133a, miR-107, miR-18a, and miR-15a) was able to identify ER+ EBC patients from healthy individuals with an AUC range of 0.61-0.66 and predicted (73% probability) BC development within one year in women [117, 118]. Moving to another malignancy, miR-485-5p, miR-361, miR-326, miR-487b were the first serum diagnostic biomarkers for colorectal cancer (CRC) [119]. After that, studies started reporting the role of more circulating microRNA as CRC diagnostic biomarkers (miR-17-3p, miR-92, miR-134, miR-146a, miR-17-3p, miR-181d, miR-191, miR-221, miR-222, miR-223, miR-225, miR-229, miR-320 and miR-92a) [120, 121].

b. Prognostic Role

Despite having the same malignancy and similar tumor stage, patients can vary considerably with their prognosis depending on tumor type, histopathological subtype, molecular changes, and other subclassifications. Some microRNAs were identified as cancer prognostic biomarkers leading to more appropriate treatment and better clinical

outcomes. Several bioinformatics tools estimate cancer patients' prognosis through the follow-up information (overall survival; OS, disease free survival; DFS, relapse free survival; RFS, metastasis free survival; MFS, progression free survival; PFS, and disease specific survival; DSS) they provide [122]. Circulating miRNAs were established as prognostic biomarkers in breast cancer patients with miR-21 the most studied miR, according to Nassar et al [116]. A meta-analysis showed that elevated expression of miR-21 in breast cancer significantly predict poor OS and lymph node metastasis [123]. In other studies, miR-181b, miR-155, miR-24, and miR-19a were significantly higher in the serum of EBC patients with high-risk recurrence than in the low-risk recurrence group after tumor removal and therapy [124, 125]. Other prognostic miRNAs are miR-23b, miR-200b, and miR-200c which were shown to be higher in metastatic BC (MBC) compared to EBC. miR-200b predicted for shorter OS in MBC patients and in $HER2^-$ BC patients and both, miR-23b and miR-190, were associated with shorter PFS in *de novo* metastatic patients [126]. A 4-serum microRNA signature (miR-652, miR-107, miR103, and miR-18b) discriminates tumor relapse in BC and is associated with worse survival in TNBC [127]. Some miRs have been shown to be prognostic biomarkers in lung cancer. For instance, high serum miR-125b was shown to predict poor survival in NSCLC patients of various stages [128]. Additionally, NSCLC patients with high plasma miR-18a, miR-20a, miR-92a, miR-126, miR-210, and miR-19a expression levels had worse DFS, while patients with high plasma miR-18a, miR-20a, miR-92a, miR-126, and miR-210 levels had shorter OS in comparison to NSCLC patients with low expression levels of these miRs [129]. miR-590-5p also showed to be

a prognostic biomarker in which its low expression is associated with low, median survival rate among NSCLC patients [130]. Shifting to another malignancy, circulating microRNA act as prognostic biomarkers in colorectal cancer. High serum levels of miR-200c was correlated with LN metastases, recurrent disease, distant metastases, and short OS in all stages CRC patients [131]. Besides, Yuan et al showed that postoperative plasma miR-31, miR-141, and miR-16 are biomarkers for disease recurrence after the surgical resection [132]. The correlation of circulating miRNAs as prognostic biomarkers with other malignancies has also been analyzed including, osteosarcoma [133], acute myeloid and chronic lymphocytic leukemias [134, 135], prostate [136, 137] pancreatic [138, 139], and adrenocortical cancer [140].

c. Therapy Predictive Role

There are several strategies for cancer treatment such as, chemotherapy, radiotherapy, hormonal therapy, immunotherapy and surgical ablations. However, in order to provide the most efficient treatment to a cancer patient, there is a strong need for a reliable therapy response predictive biomarker. microRNAs were shown to predict the sensitivity or resistance of patients towards a specific therapy. High levels of miR-125b predicted poor response of breast cancer cells to taxol-based treatments [141, 142]. Another cohort study reported that low serum levels of miR-125b and miR-21 during neo-adjuvant chemotherapy (NCT) predicted favorable treatment response in stage II/III BC patients [143]. Additionally, variation in the expression of miR-222, miR-451, miR-20a, and miR-34a in BC patients could discriminate responder from non-responder

subjects to NCT [144]. Similar to BC, NSCLC patients with high serum miR-125b levels showed resistance to chemotherapy [145]. miR-613, miR-495-3p, miR-302e, and miR-98-5p elevated levels indicated sensitivity to radiotherapy in NSCLC patients [146]. In CRC, Kjersem et al identified 3 plasma miRs (miR-160a, miR-130b, and miR-484) whose upregulation indicates lack of response to 5-fluorouracil (5-FU) and oxaliplatin [147]. The down regulation of miR-15b and miR-122 showed an increase in the resistance of CRC cells to 5-FU treatment [148, 149]. Moreover, Nikzad et al concluded that miR-222 and miR-155 predict CRC cells resistance to radiation by targeting PTEN and FOXO3a genes, respectively [150]. Besides their role as therapy predictive biomarkers, miRNAs could be used as an alternative approach for tackling drug resistance. For example, miR-205 ectopic expression improved the responsiveness of BC cells to tyrosine kinase inhibitors through direct targeting of HER3 [151]. Inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine in cholangiocarcinoma cell lines [152].

C. microRNA and Breast Cancer

microRNAs are new promising diagnostic biomarkers in which their expression varies depending on the state of human health. Based on their role in modulating various cellular and signaling pathways, microRNA molecules have been linked to carcinogenesis including breast cancer [153, 154]. As demonstrated in the following sections, gene expression profiling studies comparing breast cancer to healthy

subjects have identified significant changes in the expression profile of several tissue and circulating miRNAs.

1. Tissuelar miRNAs

Tissuelar microRNA expression profile analysis have been utilized extensively in biomedical research to explore breast cancer malignancy. It was shown that microRNA expression profiling derived from FFPE BC tissues could be more reliable than mRNA expression profiling [155]. microRNA are stable enough to be preserved for long time in FFPE tissue and quantified using qRT-PCR compared to mRNA molecules which are unstable to be suitable for qRT-PCR [156].

microRNA expression in breast tumor tissue may vary depending on the age of the group and tumor subtype, and their mode of dysregulation would help in malignancy prediction and management. For example, 8 miRs were down regulated in breast tumors from very young BC patients, while another 3 miRs were downregulated in that of young BC patients, 1 specific miR was downregulated in premenopausal and no unique miR observed in postmenopausal breast tumors [157]. Besides, differential expression of miRs in breast tumor samples was associated with hormone receptor status and tumor subtypes. For example, miR-375 and miR-17 were upregulated in ER^+ and ER^- tumors, respectively. miR-30c was upregulated in HER2-negative, while miR-155 was upregulated in HER2-overexpression breast tumor tissues [157]. A study assessing the expression of microRNA molecules in 35 biopsies of tumor tissues of various molecular genetics subtypes concluded that possibility of using the level of

expression of microRNAs in tissue samples to assess the malignancy of breast carcinoma [158]. miR-222 expression in breast cancer tissues is also helpful in breast cancer diagnosis [159]. A study by Tfaily et al investigating the expression of miRNA in advanced Algerian BC tissues reported that miR-21, miR-183, miR-182, miR-425-5p and miR-200c were significantly upregulated [160]. In addition to that, several studies reporting the dysregulation of miRNA in BC tissues compared to normal controls have been included in a systematic review. The review concluded that at least 3 studies agreed on the consistent upregulation of 2 miRs and downregulation of other 6 miRs with miR-21, the most consistently reported miRNA, was upregulated in six profiling studies [56].

2. Circulating miRNAs

One of the prevalent challenges in managing BC is to achieve a sensitive and minimally invasive biomarker that can facilitate BC early detection and prediction. Another study determined the expression level of miR-122 in the blood of 90 BC patients and 60 healthy controls and concluded that higher levels of miR-122 could distinguish diseased subjects from healthy ones and predict metastasis [161]. Moreover, 7 plasma miRNAs (miR-23a-3p, miR-130a-5p, miR-144-3p, miR-148a-3p, and miR-152-3p) were shown to be down regulated in the plasma of BC patients vs. healthy controls [162]. Therefore, circulating microRNA molecules are showing a fundamental role as cancer diagnostic biomarkers.

D. microRNA and Lebanese BC Patients

As a refresh to what was previously mentioned, BC is the leading cause of death among the Lebanese women, accounting for 930 (23.1% of all cancer related deaths among Lebanese women) deaths in 2018 (Globocan, 2018). The Lebanese Ministry of Public Health (MOPH) recommends women to start mammography screening for BC at age of 40 years [7], yet 44.2% of all new cancer cases among Lebanese women aged 0-39 are BC cases (Globocan, 2018). Moreover, Lebanese BC patients diagnosed at younger age (35 years and younger) showed poor survival and more aggressive disease [163]. Therefore, the introduction of the concept of microRNA molecules as BC diagnostic, prognostic, and/or therapy predictive biomarkers is a promising solution for early breast cancer detection and prediction among Lebanese Women.

Our lab was the first lab to report differential miRNA expression pattern in Lebanese breast cancer patients and deduced that these tiny molecules could be used as potential early breast cancer detection biomarkers [164]. Nassar et al investigated the dysregulation of 5 miRNAs among Lebanese BC patients, where most of these patients were of young age. Results showed that the investigated miRs miR-148b, miR-21 and miR-155 were significantly upregulated, miR-10b was significantly down regulated, while miR-221 was insignificantly dysregulated in breast tumor compared to adjacent normal tissue. By analyzing the obtained data and comparing it to the literature, Nassar et al showed an inconsistency in the mode of dysregulation of miR-148b and miR-221 suggesting a potential ethnic difference in miRNA expression profile [164]. After that,

in 2017, Nassar *et al.* conducted comparative analysis of Lebanese and American miRNA profile and showed variation between the two patient sets. miRNA microarray analysis showed that 173 miRNAs were significantly dysregulated in 45 early BC compared to 17 normal adjacent breast tissues. Of the 173, 74 miRNAs were differentially expressed (either upregulated or downregulated) with expression fold change greater than 2. Furthermore, RT-qPCR was performed to validate the differential expression of 10 randomly selected miRNA in 20 tumor and 10 normal adjacent breast tissues. The mode of dysregulation observed in RT-qPCR experiment validated the microarray data results where miR-139-5p, miR-145, miR-100 and miR-125b were significantly down-regulated, while miR-183, miR-182, miR-155, miR-210, miR-200c and miR-425-5p were significantly up-regulated [165].

In conclusion, further investigations are needed to identify specific circulating miRNAs and determine their role and dysregulation in BC in order to apprehend their role in early BC onset and detection among Lebanese women.

CHAPTER II

AIM

Recently, breast cancer research has been focusing on the discovery of sensitive, reliable, easily accessible, and minimally invasive biomarkers to detect breast tumors in their early stages. MicroRNAs circulating in the blood of BC patients were showing to be unique molecules belonging to a potential class of diagnostic biomarkers. Previous work by our lab, validated the results of microRNA microarray by RTq-PCR and showed a significant mode of dysregulated expression of mir-145, mir-425-5p, mir-139-5p, mir-125b, mir-100, mir-182, and 155 in breast tissue samples from breast cancer patients. So, we chose these 7 miRs to study their expression in the circulation, 5 miRs were also added to this study as they are mostly studied as potential circulating miRs diagnostic biomarkers according to literature. In Lebanon, breast cancer with invasive ductal carcinoma (IDC) and estrogen and progesterone receptors positive status are the most common histotype and receptor profile tumors. Therefore, the main aim of this study is to investigate the potential value the 12 circulating microRNA (mir-145, mir-425-5p, mir139-5p, mir-125b, mir-100, mir-182, mir-195, mir-155, mir-21, mir-130a, mir-23a, and mir-451) in diagnosing early stage Lebanese BC patients of invasive ductal carcinoma (IDC) histotype with positive estrogen and progesterone receptor profile. In order to fulfill the main aim, 3 sub-aims were assigned. First, we aimed to detect the expression of the chosen circulating miRs in the plasma of early stage BC patients and compare it to that of healthy subjects. The second sub-aim in this thesis is to analyze the correlation between microRNAs expression and some BC risk factors

and clinical features. With this correlation testing, we can specify some miRs to be utilized as diagnostic biomarkers in EBC patients with specific BC clinical feature and/or BC risk factors. The third sub-aim is to explore the potential value of microRNA molecules as promising diagnostic biomarkers. For this part of the study, combination of miRs is established to detect their diagnostic accuracy of early breast cancer among Lebanese women.

CHAPTER III

MATERIALS AND METHODS

A. Sample Collection

Blood samples from 41 breast cancer patients diagnosed in 2012-2014 were collected at the American University of Beirut Medical Center (AUBMC). All participants were of Lebanese nationality. The study was approved by the Institutional Review Board (IRB) at AUB and an informed consent form was obtained from all participants. The average age of the participants at diagnosis was 53 years ranging from 30-84 years. It is noteworthy to indicate that the pathology type of all participants included in this study is IDC and all of them are positive for estrogen and progesterone receptors. The normal controls included in this study were 32 healthy females with average age 34.4 years ranging from 21-60 years. Plasma were received already isolated and stored at -80^{o}C.

B. Total RNA Extraction from Plasma

Total RNA was extracted from the plasma of breast cancer patients and healthy controls using Norgen kit, following the manufacturer's protocol. Towards the end of the procedure, the extracted RNA molecules were eluted in 30 µl of the elution solution. Then, the quality (using 260/280 ratio) and quantity (in ng/µl) of the extracted RNA molecules were assessed using spectrophotometric nanodrop machine from Denovix (DeNovix DS-11 FX Spectrophotometer) and then RNA samples were stored at -80^{o}C.

C. complementary DNA (cDNA) Synthesis from Specific microRNA

After the extraction of plasma total RNA, the complementary DNA (cDNA) of the microRNA of interest were synthesized on ice using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). 10 ng of the extracted RNA were added to Diethyl pyrocarbonate (DEPC) treated water to reach a total volume of 3.1 µl. Then, a master mix was made, following the manufacturer's protocol, containing DEPC treated water, 10X Reverse Transcription (RT) buffer, RNase inhibitor, 100mM dNTPs, 3 different microRNA primers, and Multiscribe RT enzyme. The primers (human hsa-miR-16, hsa-miR-145, hsa-miR-425, hsa-miR-139-5p, hsa-miR-125b, hsa-miR-100, hsa-miR-182, hsa-miR-195, hsa-miR-155, hsa-miR-21, hsa-miR-130a, hsa-miR-23a, hsa-miR-451) and their corresponding probes were purchased as part of the TaqMan® microRNA Assays Kit (Applied Biosystems, USA) with validated efficiency. 6 master mixes were prepared each containing hsa-miR-16 (endogenous control) and 2 other primers, hence creating 6 sets of reactions. In a cDNA reaction tube, 6.9 µl of the master mix were added and followed by the calculated 3.1 µl of the extracted RNA resulting in a total volume of 10 µl. Next, the reaction tubes were spinned down briefly and loaded into T100 thermal cycler from Bio-Rad using the following protocol: 30 minutes incubation at 16^{o}C, 30 minutes incubation at 42^{o}C, 5 minutes incubation at 85^{o}C, and an infinite hold at 4^{o}C. Finally, the cDNA synthesized was diluted by adding 57 µl of DEPC treated water and placed at -20^{o}C for long-term storage.

D- Quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) Analysis

For miRs expression detection, RT-qPCR was carried out in duplicates for each sample. First, a master mix containing: 5 µl of 2x TaqMan® Universal Master Mix with no Amperase Uracil N-glycosylase (UNG) (Applied Biosystems, USA), 0.5 µl of the corresponding 20x microRNA probe, and 2 µl of DEPC treated water was prepared for each well under the PCR cabinet. Second, the mixture was vortexed, spinned down, and distributed into the wells of a 384 well skirted PCR plate from Bio-Rad. Third, the 2.5 µl of the synthesized cDNA molecules was added into the wells resulting in a total of 10 µl volume in each well. A no template control [166] well was added for each miR, where the 2.5 µl of sample was replaced by DEPC treated water. After that, the plate was sealed and centrifuged briefly (for 1 minute) at 2500 rpm. The plate was loaded into real time Bio-Rad CFX384 machine and the following protocol was used: 10 minutes hold at 95°C then 40 cycles of: 15 seconds at 95°C (denaturing step) and 60 seconds at 60°C (annealing and extension step). Finally, the expression fold change of each microRNA in each sample was calculated in comparison to healthy controls using the delta delta Ct analysis.

The fold change of expression was calculated using the $2^{-\Delta\Delta Ct}$ rule, where ΔΔCt is calculated using the following formula:

$$\Delta\Delta Ct = (Ct_{\text{miR tested}} - Ct_{\text{miR-16}})_{\text{tumor sample}} - \overline{X}(Ct_{\text{miR tested}} - Ct_{\text{miR-16}})_{\text{healthy control}}$$

All Ct values obtained were normalized using miR-16 as an endogenous control. The mode of dysregulation reported is based on comparing the mean of the fold change of microRNA expression in plasma from EBC patients to that from healthy

controls. Using GraphPad Prism 6 software, Shapiro-Wilk normality test was used to test if the values came from a Gaussian distribution (Table 3), and then Wilcoxon's signed-rank test was applied to detect the miRs with significant different expression in plasma from healthy controls and EBC patients.

E- Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software to determine the dysregulation of miRNAs in the plasma of BC subjects in comparison to healthy subjects. The values were tested if they come from a Gaussian distribution by Shapiro-Wilk normality tests. The significance of the data obtained was determined by using the Wilcoxon's test. Also, Mann-Whitney U test was applied to detect any significant dysregulation of the miRs within a certain clinicopathological subgroup. p-values <0.05 were considered as statistically significant. In order to figure out the relation between 2 variables: fold change of miR expression and clinicopathological features of the patients, linear regression and correlation tests were utilized, using GraphPad Prism 6 and IBM SPSS version 22. An ROC curve was plotted to determine the single and combined diagnostic accuracy of the microRNA molecules tested.

CHAPTER IV

RESULTS

A. Patients Characteristics

A total of 73 female participants, 41 early stage breast cancer patients and 32 healthy controls were recruited at the American University of Beirut- Medical Center. The clinicopathological details for all patients have been retrieved and summarized in table 2. Pathologically, all cases were of IDC breast cancer type and had positive estrogen and progesterone receptor (ER and PR) status. 63.4% of the participants had HER-2 positive status. It is noteworthy to indicate that 41.5% of the participants were premenopausal at the time of diagnosis. All patients are distant metastases free (M0), therefore they are considered as early breast cancer (EBC) patients. None of the participated subjects has been exposed to radiation. More than half of the participants have tumor size ≤2cm (65.9%) and do not smoke cigarette (63.4%).

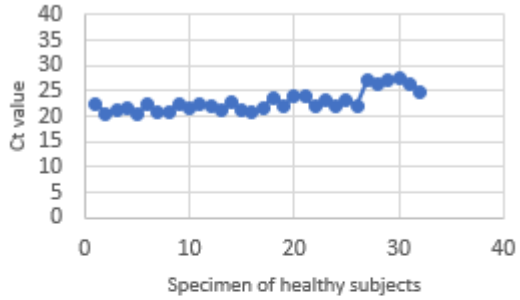
Clinicopathological Characteristics		Number of cases	Percentage of cases
Total number of patients		41	
Age (years)	Mean	53±11.88	
	Range	30.8	
Menopausal Status	Premenopausal	17	41.5%
	Postmenopausal	22	53.7%
	Unknown	2	4.9%
Tumor Size	1	27	65.9%
	2	13	31.7%
	3	1	2.4%
Lymph Node involvement	0	25	61.0%
	1	15	36.6%
	Unknown	1	2.4%
Histological Grade	I	17	41.5%
	II	16	39.0%
	III	7	17.1%
	Unknown	1	2.4%
HER-2 Status	Positive	26	63.4%
	Equivocal	12	29.3%
	Negative	3	7.3%
Distant Metastasis	Yes	0	0%
	No	41	100%
Age at Menarche (years)	≤12	17	41.5%
	13	12	29.3%
	≥14	10	24.4%
	Unknown	2	4.9%
Family History of BC	Yes	17	41.5%
	No	22	53.6%
	Unknown	2	4.9%
Radiation Exposure	Yes	0	0%
	No	41	100%
Cigarette Smoking	Yes	12	29.3%
	No	26	63.4%
	Unknown	3	7.3%
Waterpipe Smoking	Yes	5	12.2%
Alcohol Intake	No	33	80.5%
	Unknown	3	7.3%
Alcohol Intake	Yes	10	24.4%
	No	28	68.3%
	Unknown	3	7.3%
History of Oral Contraceptive Pills (OCP) Use	Yes	23	56.1%
	No	16	39.0%
	Unknown	2	4.9%
History of Hormone Replacement Therapy (HRT) Use	Yes	8	19.5%
	No	31	75.6%
	Unknown	2	4.9%
Body Mass Index (BMI)	Normal weight	20	48.8%
	Overweight/Obese	18	44.0%
	Unknown	3	7.3%

Table 2. Clinical and pathological characteristics of Lebanese breast cancer patients participated in this study. Tumor size categories: t1 ≤ 2cm, t2 >2 cm but ≤ 5 cm, t3 > 5 cm. Normal weight participants: BMI >20 kg/m^2 and < 24.9 kg/m^2; overweight/obese participants: BMI > 25 kg/m^2 (OCP; oral contraceptive pills, HRT hormone replacement therapy, BMI; body mass index; AJCC TNM staging system was used to indicate tumor size, lymph node involvement, and distant metastasis).

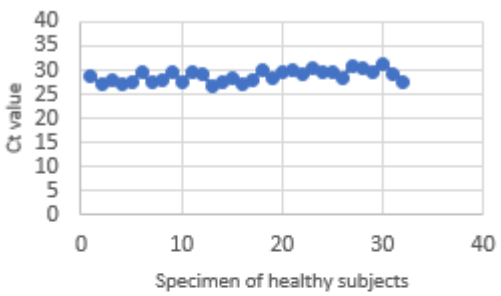
B. Consistency of miRs in Healthy Subjects

The plasma samples from healthy subjects were assessed for consistency in miR expression in order to ensure the validity of these controls. Figure 6 shows the Ct level at which the 12 miRs of interest (miR-16, miR-21, miR-130a, miR-155, miR-195, miR-23a, miR-451, miR-145, miR-425-5p, miR-125b, miR-139-5p, miR-182 and miR-100) are detected in the plasma of the healthy subjects. A Ct level is inversely proportional to the amount of the targeted nucleic acid. No variation was observed between healthy samples in expressing different miRs.

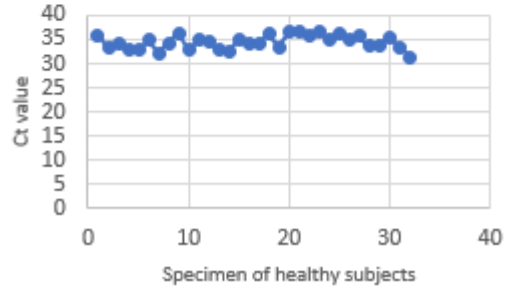
A)


miR-16
Ct value
Specimen of healthy subjects

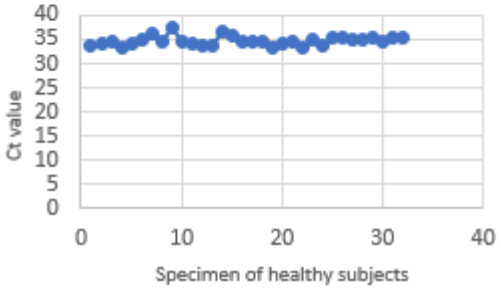
B)


miR-21
Ct value
Specimen of healthy subjects

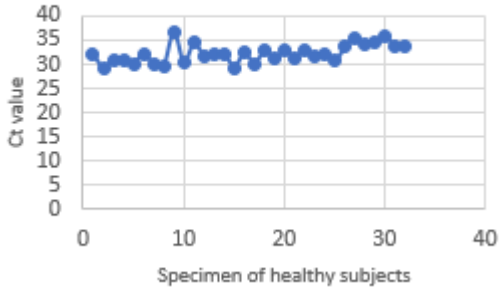
C)


miR-130a
Ct value
Specimen of healthy subjects

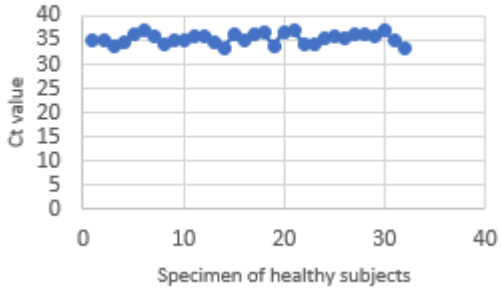
D)


miR-155
Ct value
Specimen of healthy subjects

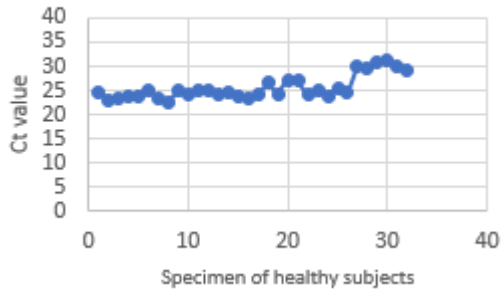
E)


miR-195
Ct value
Specimen of healthy subjects

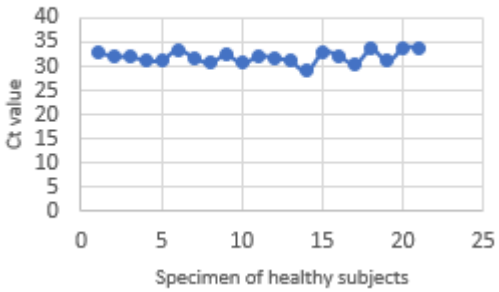
F)


miR-23a
Ct value
Specimen of healthy subjects

G)


miR-451
Ct value
Specimen of healthy subjects

H)


miR-145
Ct value
Specimen of healthy subjects

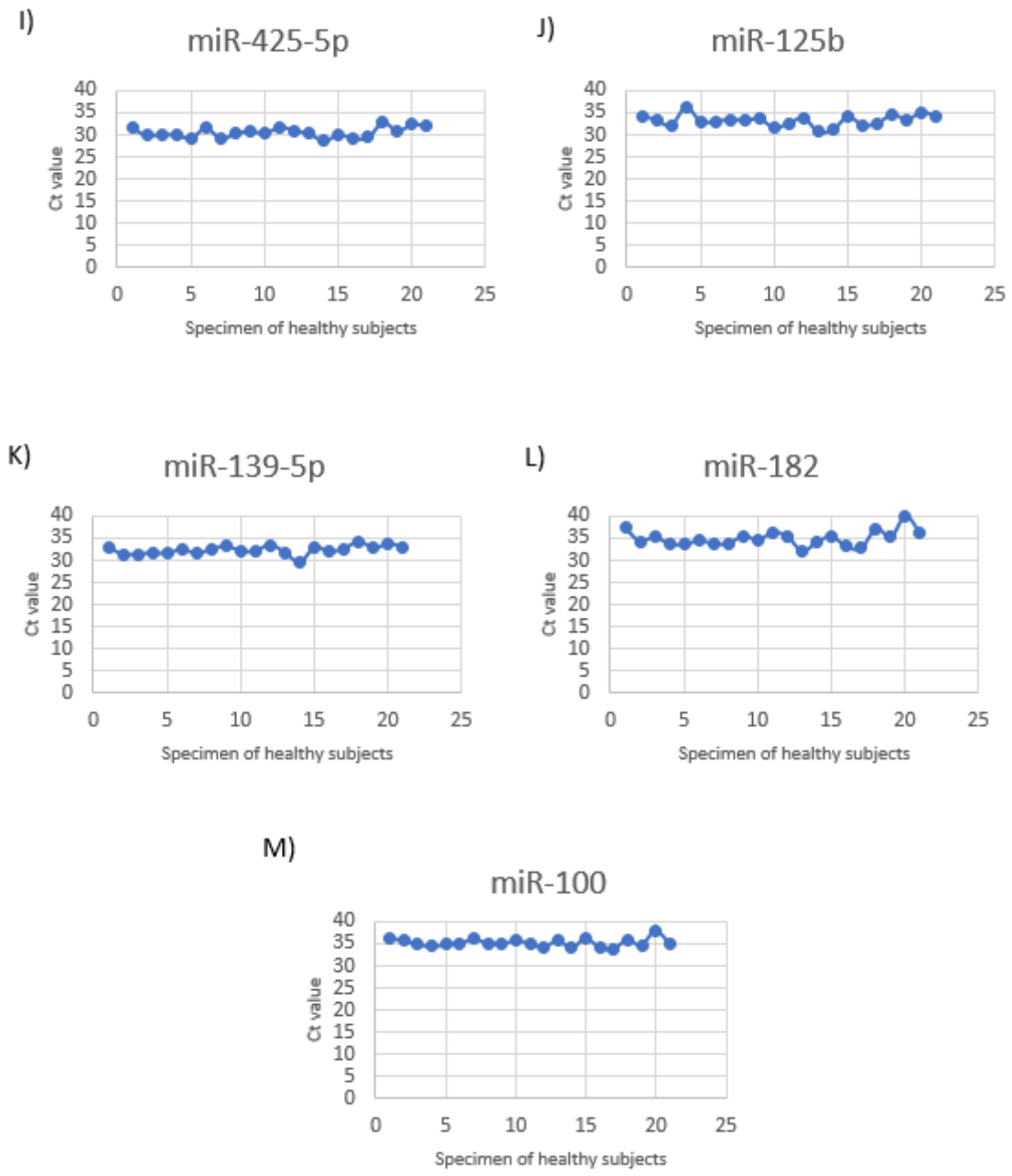


Figure 6. Consistency of Ct values (cycles) detecting the expression of 12 miRs (miR-21, miR-130a, miR-155, miR-195, miR-23a, miR-451, miR-139-5p, miR-125b, miR-425-5p, miR-145, miR-100, miR-182, and the endogenous control miR-16) in all healthy subjects. (A) n=32, 23.68 ±1.94. (B) n=32, 28.78 ±1.24. (C) n=32, 34.45 ±1.48. (D) n=32, 34.74 ±0.95. (E) n=32, 32.21 ±1.96. (F) n=32, 35.38 ±1.04. (G) n=32, 25.53 ±2.51. (H) n=21, 31.97 ±1.26. (I) n=21, 30.57 ±1.10. (J) n=21, 32.33 ±1.00. (K) n=21, 33.23 ±1.30. (L) n=21, 35.25 ±0.99. (M) n=21, 34.98 ±1.76. (Sample size, mean ± standard deviation).

In addition to that, in order to ensure data reproducibility, the detection of some miRs in plasma from healthy subjects were repeated twice, one time with 21 plasma samples and another time with the remaining plasma samples from EBC patients. Figure 7 shows the reproducibility of detecting the expression of 5 miRs (miR-16, miR-23a, miR-451, miR-21, miR-130a) at specific Ct values in plasma of healthy subjects.

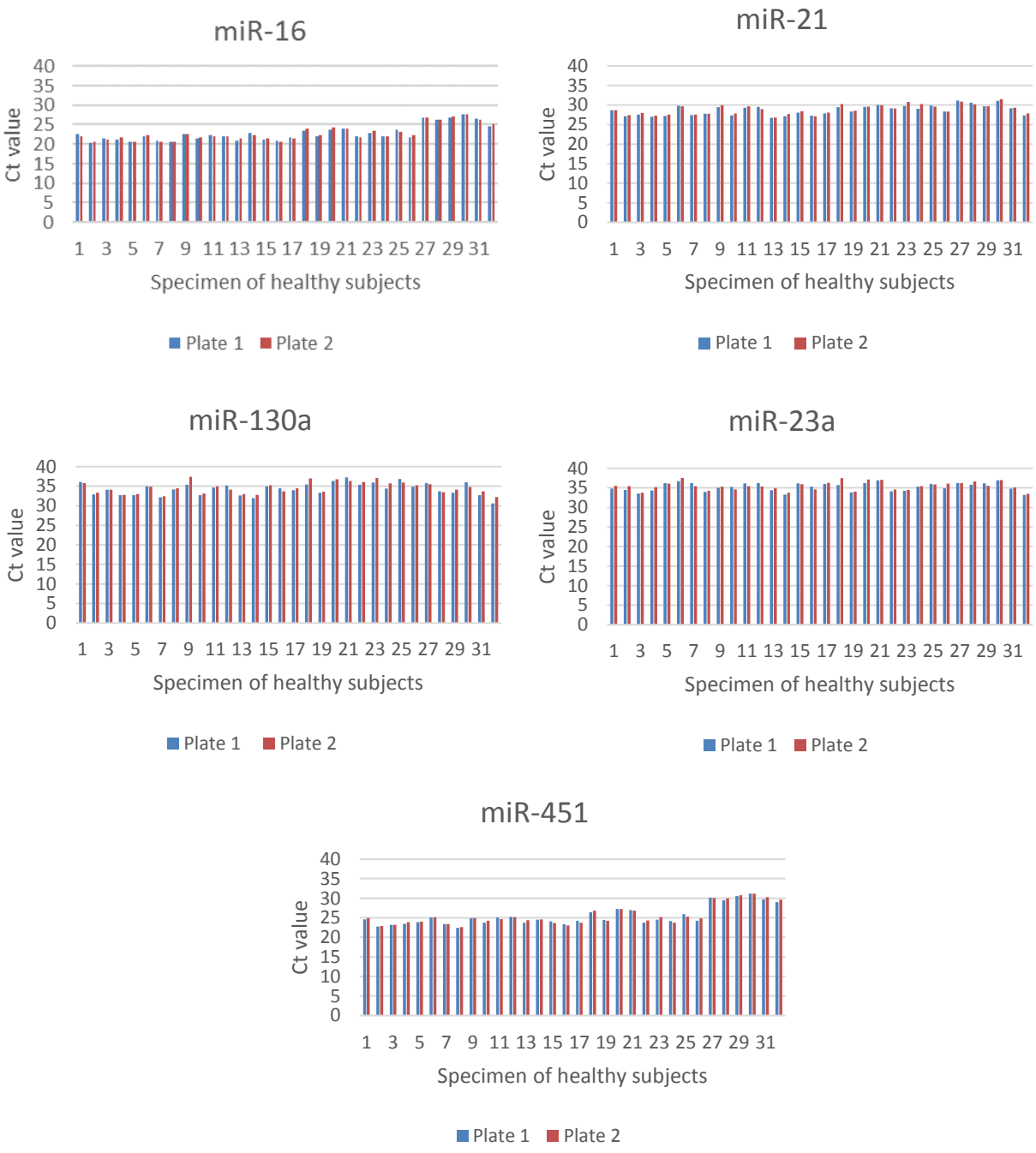


Figure 7. Reproducibility of Ct value expression of 5 miRs in the plasma of healthy subjects. Reproducible data when comparing the Ct values detecting the expression of 5 miRs (miR-16, miR-21, miR-130a, miR-23a, and miR-451) in the plasma of all controls in 2 different plates.

C. Validation of miR-16 as Endogenous Control

In this study, the level of expression of all miRs was normalized to the level of miR-16 which is widely used in the literature as an endogenous control in plasma samples [167, 168]. To validate the use of miR-16 as an endogenous control, the Ct level of miR-16 in plasma of healthy and tumor subjects was compared. Figure 8 shows the consistency in the expression of miR-16 in plasma samples among all healthy subjects and EBC participants.

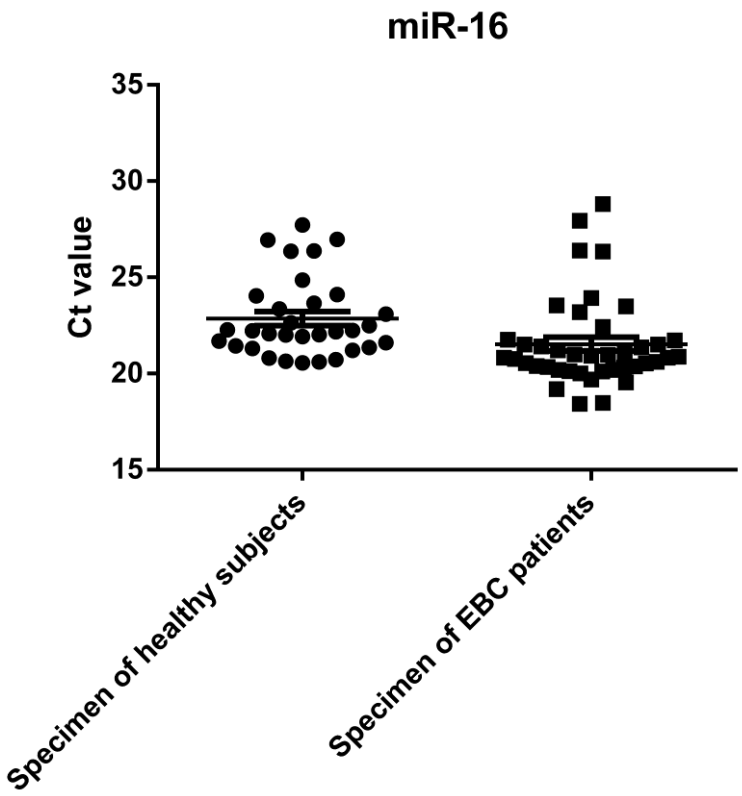


Figure 8. Consistency of Ct value of miR-16 endogenous control in the plasma of normal and breast cancer participants. Scatter plot showing the Ct value of the expression of miR 16 in specimen of healthy subjects and BC patients. The average cycle at which miR-16 expression is detected in plasma of normal subjects was 22.86063 (n=32) and that in the plasma of breast cancer subjects was 21.53073 (n=41).

D. miRNA Expression in the Plasma of Breast Cancer Patients in Comparison to Healthy Subjects

After extracting all RNA molecules from the plasma of 41 EBC patients and 32 healthy subjects, complementary DNA was synthesized, and real-time quantitative PCR was performed to detect the Ct value at which the miRs were expressed. The fold change of expression was calculated using the $2^{-\Delta\Delta Ct}$ rule, where ΔΔCt is calculated using the following formula:

$$\Delta\Delta Ct = (Ct_{\text{miR tested}} - Ct_{\text{miR-16}})_{\text{tumor sample}} - \overline{X}(Ct_{\text{miR tested}} - Ct_{\text{miR-16}})_{\text{healthy control}}$$

All Ct values obtained were normalized using miR-16 as an endogenous control. The mode of dysregulation reported is based on comparing the mean of the fold change of microRNA expression in plasma from EBC patients to that from healthy controls. Using GraphPad Prism 6 software, Shapiro-Wilk normality test was used to test if the values came from a Gaussian distribution (Table 3), and then Wilcoxon's signed-rank test was applied to detect the miRs with significant different expression in plasma from healthy controls and EBC patients. 7 miRs: miR-21 (*p*-value <0.0001), miR-130a (*p*-value <0.0001), miR-155 (*p*-value <0.0001), miR-23a (*p*-value <0.0001), miR-145 (*p*-value <0.0001), miR-425-5p (*p*-value <0.0001), and miR-139-5p (*p*-value <0.0001) are significantly upregulated (Figure 9), whereas 1 miR, miR-451, is significantly downregulated (*p*-value 0.0049) in plasma from EBC patients compared to that from healthy controls (Figure 10). The remaining miRs (miR-195, miR-125b, miR-100, miR-182) showed non-significant dysregulation (Figure 11).

miRs tested	Sample size	Shapiro-Wilk normality test
miR-21	Tumor: 41 Healthy controls: 32	No
miR-130a	Tumor: 41 Healthy controls: 32	No
miR-155	Tumor: 41 Healthy controls: 32	No
miR-195	Tumor: 41 Healthy controls: 32	No
miR-23a	Tumor: 41 Healthy controls: 32	No
miR-451	Tumor: 41 Healthy controls: 32	No
miR-145	Tumor: 37 Healthy controls: 21	No
miR-425-5p	Tumor: 37 Healthy controls: 21	No
miR-139-5p	Tumor: 37 Healthy controls: 21	No
miR-125b	Tumor: 37 Healthy controls: 21	No
miR-100	Tumor: 36 Healthy controls: 21	No
miR-182	Tumor: 36 Healthy controls: 21	No

Table 3. Results of the Shapiro-Wilk normality test using the fold change of expression of the miRs to test for normality. Shapiro-Wilk normality tested was used to test if the values come from a Gaussian distribution. There are 3 different sample size: for miR-21, miR-130a, miR-195, miR-155, miR-23a, and miR-451 the total sample size is 73, for miR145, miR-425-5p, miR-139-5p, and miR-125b the total population size is 58, and for miR-100 and miR-182 the total sample size is 57.

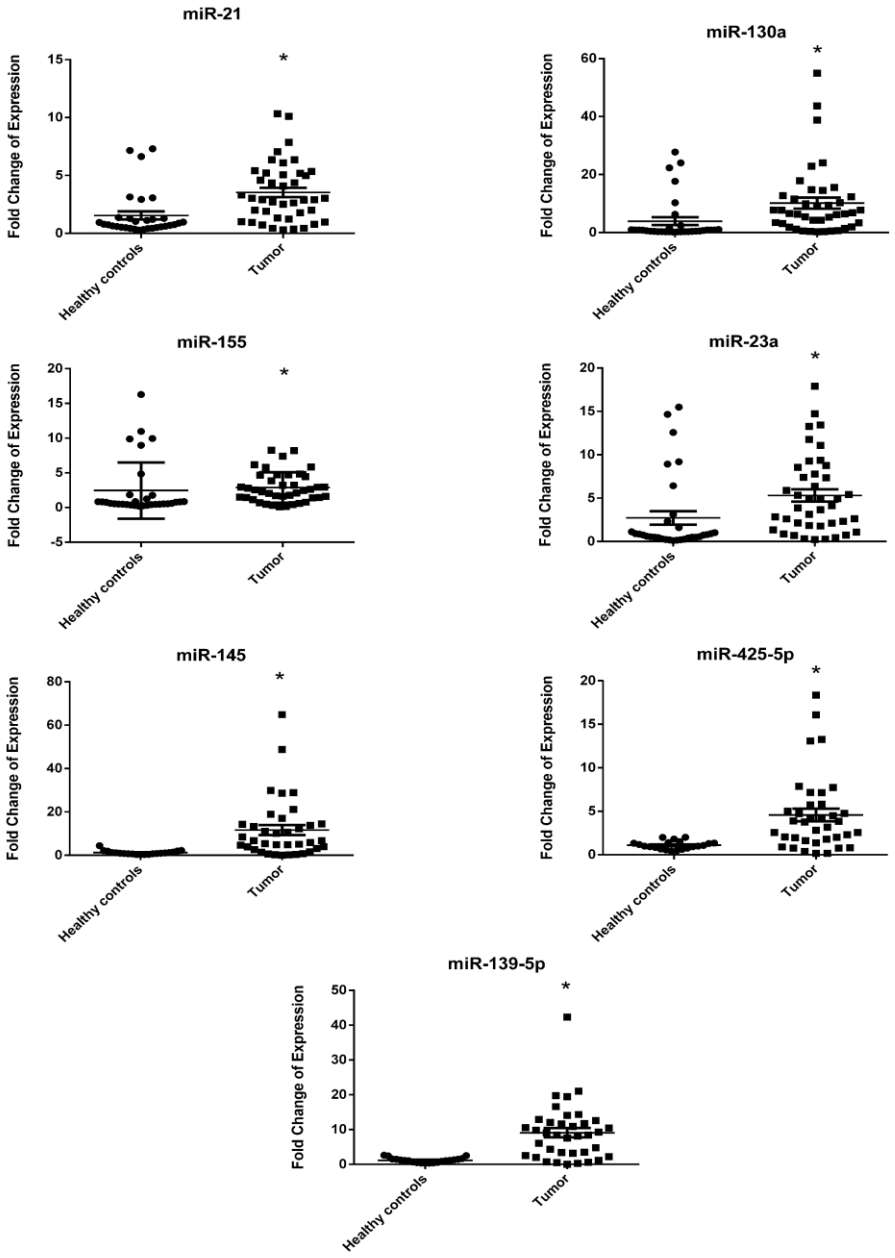


Figure 9. Fold change expression of the significantly upregulated miRs in the plasma of Lebanese women with EBC as compared to healthy controls. In the graphs showing the fold change of expression of miR-21, miR-130a, miR-155, and miR-23a, the total population size is 73 (32 healthy women and 41 women with EBC). In the graphs showing the fold change of expression of miR-145, miR-425-5p, miR-139-5p the total population size is 58 (21 healthy women and 37 women with EBC). The plot represents the mean (middle line) and the standard error of mean (error bars). * denotes *p-value* < 0.05 according to Wilcoxon's signed-rank test.

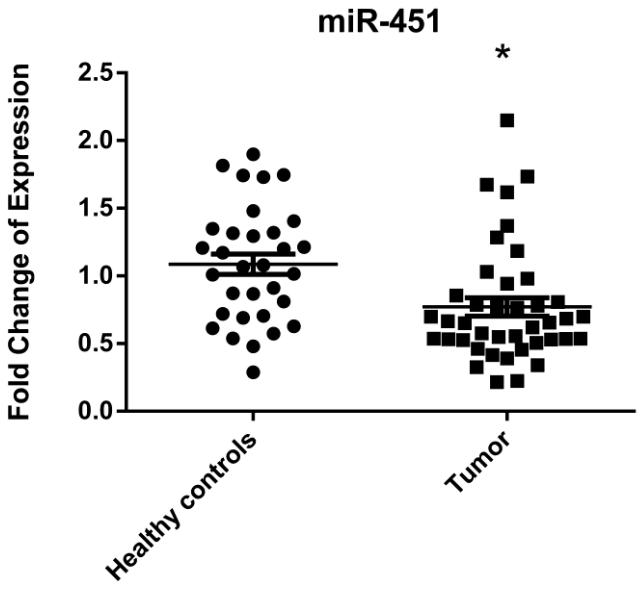


Figure 10. Fold change of expression of the significantly downregulated miR-451 in the plasma of Lebanese women with EBC as compared to healthy controls. Total population size is 73 (32 healthy women and 41 women with EBC). The plot represents the mean (middle line) and the standard error of mean (error bars). * denotes p-value < 0.05 according to Wilcoxon's signed-rank test.

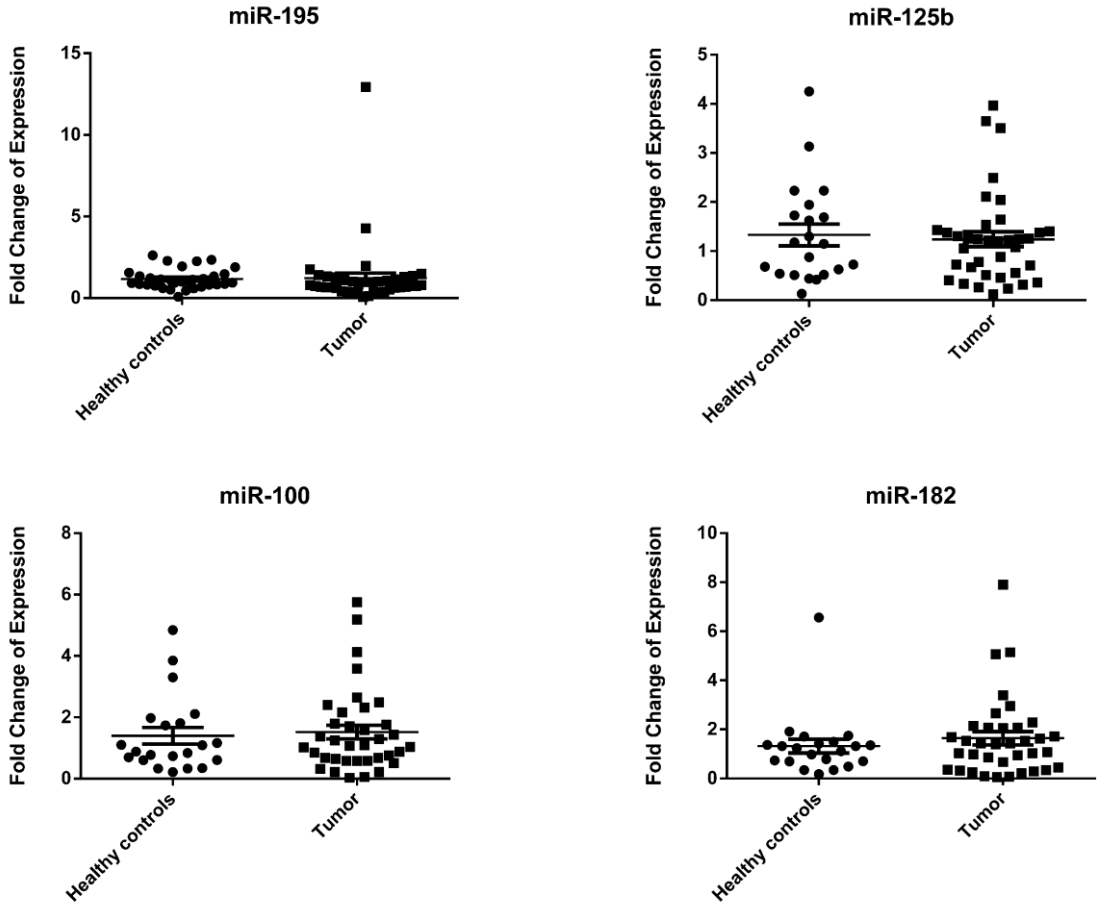


Figure 11. Fold change of expression of the non-significantly dysregulated miRs in the plasma of Lebanese women with EBC as compared to healthy controls. In the graph showing the fold change of expression of miR-195, the total population size is 73 (32 healthy women and 41 women with EBC). In the graphs showing the fold change of expression of miR-100 and miR-182, the total population size is 57 (21 healthy women and 36 women with EBC). In the graph showing the fold change of expression of miR-125b, the total population size is 58 (21 healthy women and 37 women with EBC). The plot represents the mean (middle line) and the standard error of mean (error bars).

E. Expression and Correlation of miRNAs in EBC Patients with Different Clinicopathological Features

The expression of circulating miRNA molecules in breast cancer subgroups was analyzed, using Mann-Whitney *U* and Kruskal-Wallis tests to correlate the expression of miRs with clinicopathological characteristics of BC patients. Patients were divided into subgroups according to 14 clinicopathological characteristics: menopausal status, tumor size, lymph node involvement, histological grade, family history of BC, cigarette smoking, waterpipe smoking, alcohol intake, history of OCP, history of HRT, and BMI. Table 4 shows the mean and the significance of the fold change of expression of 6 miRs (miR-21, miR-130a, miR-155, miR-195, miR-23a, and miR-451) in the subgroups. Similarly, table 5 shows those of miR-145, miR-425-5p, miR-139-5p, miR-125b, miR-100, and miR-182 in the subgroups.

Subgroups Characteristics	N (%)	miR-21		miR-130a		miR-155		miR-195		miR-23a		miR-451	
		mean	*p*-value	mean	*p*-value	mean	*p*-value	mean	*p*-value	Mean	*p*-value	mean	*p*-value
Menopausal Status	**39 (100%)**												
Premenopausal	17 (43.6%)	3.517	0.883	9.118	0.435	2.689	0.479	1.802	0.282	4.947	0.979	0.9142	0.082
Postmenopausal	22 (56.4%)	3.708		10.89		3.034		0.7790		5.720		0.6964	
Tumor Size	**41 (100%)**												
T1	27 (65.9%)	2.996	0.053	7.492	0.066	2.650	0.266	0.9088	0.591	4.137	0.013	0.8186	0.484
T2+T3	14 (34.1%)	4.570		15.29		3.335		1.815		7.566		0.6794	
Lymph Node Involvement	**40 (100%)**												
No	25 (62.5%)	3.878	0.387	8.461	0.852	3.135	0.539	1.471	0.788	5.772	0.572	0.7326	0.451
Yes	15 (37.5%)	3.167		13.62		2.625		0.8248		4.870		0.8483	
Histological Grade	**40 (100%)**												
G1	17 (42.5%)	3.820	0.878	9.639	0.870	2.957	0.967	1.633	0.543	5.584	0.939	0.7847	0.889
G2	16 (40%)	3.511		11.96		2.798		0.9994		5.566		0.7968	
G3	7 (17.5%)	2.981		7.326		2.948		0.7699		4.433		0.7244	
Family Hx of BC	**39 (100%)**												
No	22 (56.4%)	3.922	0.295	11.82	0.088	3.295	0.140	1.145	0.010	5.924	0.161	0.7083	0.264
Yes	17 (43.6%)	3.240		6.365		2.352		0.6357		4.682		0.8988	
Cigarette Smoking	**38 (100%)**												
No	26 (68.4%)	3.79	0.648	10.30	0.172	3.400	0.056	1.349	0.211	5.668	0.551	0.7212	0.051
Yes	12 (31.6%)	3.218		8.209		1.892		0.7483		4.606		0.9303	
Waterpipe Smoking	38 (100%)												
No	33 (86.8%)	3.526	0.350	9.327	0.068	2.665	0.028	1.187	0.776	5.072	0.252	0.8313	0.0050
Yes	5 (13.2%)	4.620		11.71		4.631		0.8676		7.889		0.5289	
Alcohol Intake	38(100%)												
No	27 (71.1%)	3.943	0.423	10.21	0.327	3.345	0.134	1.319	0.134	6.076	0.286	0.8181	0.9296
Yes	11 (28.9%)	3.000		8.251		1.888		0.7172		3.888		0.7261	
History of OCP Use	**39 (100%)**												
No	16 (41.0%)	3.508	0.905	9.406	0.772	3.207	0.272	0.806	0.298	4.400	0.431	0.7570	0.587
Yes	23 (59.0%)	3.706		9.472		2.659		1.516		6.067		0.8153	
History of HRT Use	**39 (100%)**												
No	31 (79.5%)	3.429	0.480	8.606	0.445	2.752	0.531	1.217	0.938	5.348	0.985	0.7764	0.665
Yes	8 (20.5%)	4.383		12.70		3.394		1.256		5.518		0.8491	
BMI	**39 (100%)**												
Normal weight	20 (51.3%)	3.459	0.961	10.40	0.518	2.490	0.136	1.662	0.286	4.704	0.722	0.8698	0.027
Overweight/Obese	19 (48.7%)	3.909		10.46		3.440		0.7513		6.395		0.6350	

Table 4. Analysis of the fold change of expression of miR-21, miR-130a, miR-155, miR-195, miR-23a, and miR-451 in plasma of Lebanese BC patients subgrouped into the different clinical and histopathology presentations. Tumor size categories: T1 ≤ 2cm, T2 >2 cm but ≤ 5 cm, T3 > 5 cm. Patients of BMI ranging from 20 kg/m^2 till 24.9 kg/m^2 belong to the normal weight category, while patients of BMI ≥ 25 kg/m^2 belong to the overweight/obese category. *p*-value was calculated using Mann-Whitney *U* or Kruskal-Wallis nonparametric tests. Kruskal-Wallis test was used in case of having more than 2 groups in one clinicopathological category such as histological grade. Highlighted values point to significant *p*-value (*p*-value < 0.05). OCP; oral contraceptive pills, HRT; hormone replacement therapy, BMI; body mass index.

Subgroups Characteristics	N (%)	miR-145		miR-425-5p		miR-139-5p		miR-125b		miR-100		miR-182	
		mean	*p*-value	mean	*p*-value	mean	*p*-value	mean	*p*-value	Mean	*p*-value	mean	*p*-value
Menopausal Status	**37(100%)**												
Premenopausal	16 (43.2%)	11.59	0.433	3.900	0.163	7.468	0.257	1.215	0.922	1.821	0.513	0.9142	0.245
Postmenopausal	21 (56.8%)	11.64		5.089		10.34		1.261		1.279		0.6964	
Tumor Size	**37 (100%)**												
T1	25 (67.6%)	8.983	0.023	3.453	0.087	8.960	0.648	1.368	0.427	1.428	0.295	1.326	0.139
T2+T3	12 (32.4%)	17.11		6.912		9.382		0.9774		1.729		2.363	
Lymph Node Involvement	**36 (100%)**												
No	25 (69.4%)	12.20	0.530	4.839	0.401	9.387	0.355	1.215	0.679	1.685	0.287	1.643	0.605
Yes	11 (30.6%)	11.22		4.211		9.066		1.187		1.153		1.731	
Histological Grade	**36 (100%)**												
G1	16 (44.4%)	11.42	0.944	4.585	0.703	8.000	0.697	1.148	0.123	1.523	0.916	1.812	0.858
G2	13(36.1%)	12.75		4.053		11.40		1.680		1.770		1.611	
G3	7 (19.4%)	10.67		5.455		6.777		0.744		0.9909		1.298	
Family Hx of BC	**37 (100%)**												
No	20 (54.1%)	13.58	0.116	5.446	0.068	11.22	0.105	1.168	0.293	1.950	0.028	1.895	0.101
Yes	17 (45.9%)	9.312		3.550		6.603		11.22		1.039		1.362	
Cigarette Smoking	**36 (100%)**												
No	25 (69.4%)	11.70	0.508	5.019	0.374	9.783	0.741	1.271	0.742	1.664	0.144	1.785	0.569
Yes	11 (30.6%)	12.13		3.911		8.186		1.225		1.228		1.451	
Water-pipe Smoking	36 (100%)												
No	31 (86.1%)	11.90	0.372	4.474	0.240	9.268	0.559	1.290	0.665	1.473	0.115	1.702	0.717
Yes	5 (13.9%)	11.42		5.963		9.461		1.055		1.949		1.505	
Alcohol Intake	35 (100%)												
No	25 (71.4%)	11.51	0.699	4.971	0.524	9.655	0.721	1.339	0.186	1.556	0.782	1.758	0.647
Yes	10 (28.6%)	12.68		3.925		8.359		1.045		1.456		1.485	
Hx of OCP	37 (100%)												
No	15 (40.5%)	14.32	0.275	4.483	0.566	11.06	0.486	1.234	0.440	1.681	0.892	1.617	0.755
Yes	22 (59.5%)	9.775		4.483		7.759		1.246		1.417		1.660	
Hx of HRT	**37 (100%)**												
No	30 (81.1%)	12.22	0.805	4.880	0.454	9.063	0.631	1.286	0.783	1.435	0.691	1.684	0.807
Yes	7 (18.9%)	9.024		3.267		9.244		1.047		1.871		1.474	
BMI	**36 (100%)**												
Normal weight	18 (50%)	12.411	0.831	4.337	0.438	8.505	0.600	1.137	0.737	1.651	0.750	1.603	0.678
Overweight/Obese	18 (50%)	1.47		5.045		10.19		1.408		1.467		1.779	

Table 5. Analysis of the fold change of expression of miR-145, miR-425-5p, miR-139-5p, miR-125b, miR-100, and miR-182 in plasma of Lebanese BC patients subgrouped into the different clinical and histopathology presentations. Tumor size categories: T1 ≤ 2cm, T2 >2 cm but ≤ 5 cm, T3 > 5 cm. Patients of BMI ranging from 20 kg/m^2 till 24.9 kg/m^2 belong to the normal weight category, while patients of BMI ≥ 25 kg/m^2 belong to the overweight/obese category. *p*-value was calculated using Mann-Whitney *U* or Kruskal-Wallis nonparametric tests. Kruskal-Wallis test was used in case of having more than 2 groups in one clinicopathological category such as histological grade. Highlighted values point to significant *p*-value (*p*-value < 0.05). OCP; oral contraceptive pills, HRT; hormone replacement therapy, BMI; body mass index.

From the results obtained in tables 4 and 5, miRs that show significant dysregulation between subgroups of EBC patients with different clinical and histopathology presentations were further analyzed, using correlation tests to assess the strength of the relation between the 2 variables. In comparison to healthy subjects, significant upregulation in the expression of miR-23a and miR-145 is observed in plasma from EBC patients, regardless of any characteristics. When comparing miR-23a and miR-145 expression in EBC patients with different tumor size, patients with larger tumor size (T2+T3; tumor size > 2cm) show significant upregulation compared to BC patients with smaller tumor size (T1; tumor size ≤ 2cm) (Figure 12a and b). When analyzing the inheritance factor, miR-195 and miR-100 differentiate EBC patients with BC family history from those without as they are significantly upregulated in patients with no BC family history. In comparison to healthy subjects, miR-195 is significantly downregulated only in patients with BC family history, while miR-100 is only significantly upregulated in EBC patients without BC family history (Figure 12c and d). Additionally, miR-155 is significantly upregulated in waterpipe smoking EBC patients compared to non-waterpipe smoking EBC patients. In comparison to healthy subjects, miR-155 is significantly upregulated only in non-waterpipe smoking EBC patients (Figure 12e). miR-451 also shows significant dysregulation among EBC patients of

different BMI. This miR is significantly downregulated in overweight/obese EBC patients compared to healthy subjects and normal weight EBC patients (Figure 12f). Regarding the rest of the miRs, they showed no significant differentiation in the rest of the subgroups, except miR-451 and miR-155 which show very close significant *p*-value, 0.051 and 0.056, respectively.

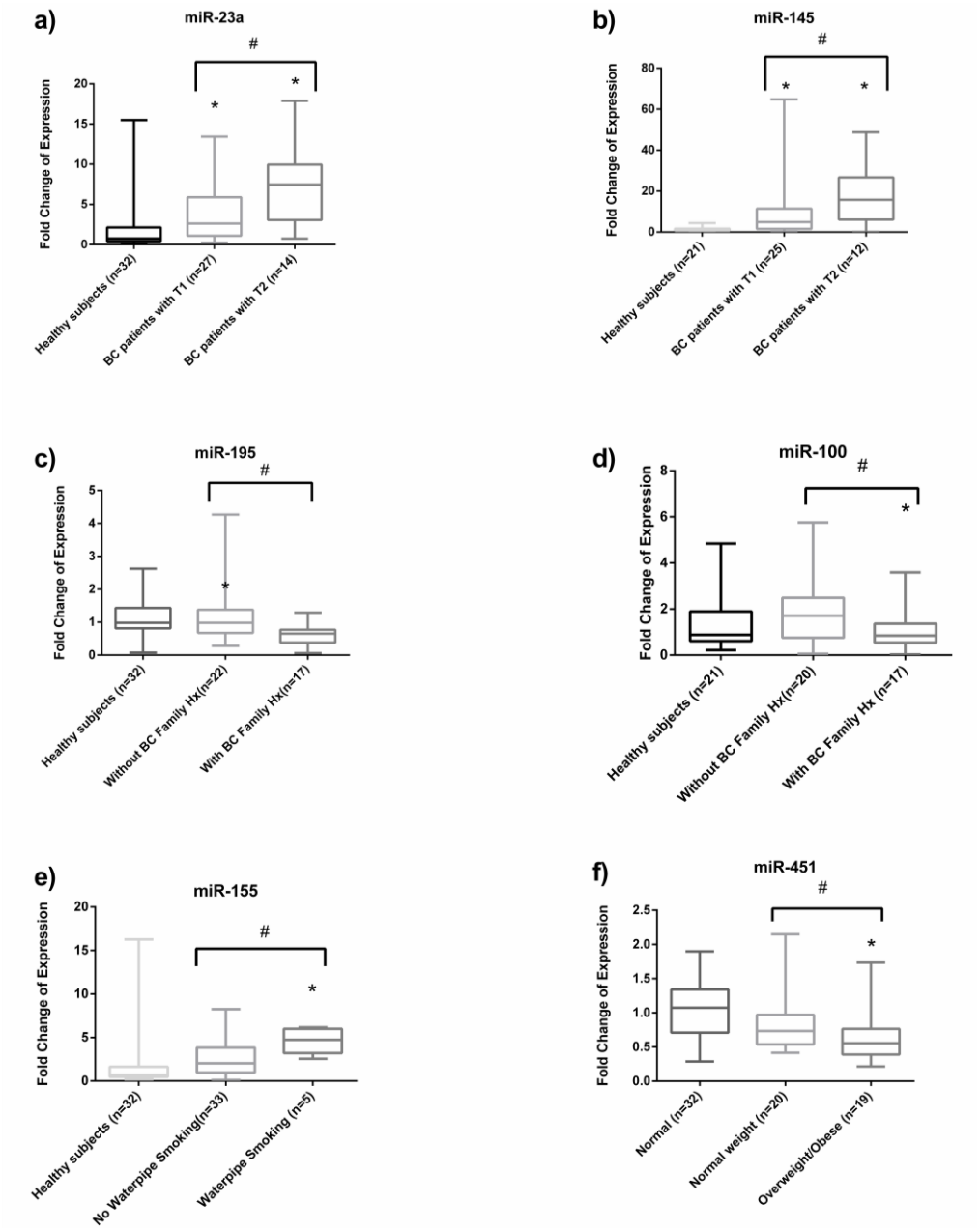


Figure 12. Fold change of expression of certain miRs in specific subgroups of BC patients as compared to healthy controls. This boxplot displays the fold change of expression of: (a and b) miR-23a and miR-145 in BC patients of different tumor size T1 and T2+T3, (c and d) miR-195 and miR-100 in BC patients with and without BC family history, (e) miR-155 in waterpipe smoking and non-waterpipe smoking BC patients and, (f) miR-451 in normal weight and overweight/obese BC patients. The fold change of expression of the miRs was measured using RT-qPCR using miR-16 as endogenous control. The whiskers represent minimum and maximum, the top, middle and bottom lines represent the 25% percentile, median, and 75% percentile, respectively. * denotes p-value <0.05, using Wilcoxon's signed-rank test. # denotes p-value <0.05 using Mann-Whitney U test or Kruskal-Wallis test if more than 2 subgroups.

Since specific miRNA were significantly dysregulated with tumor size and BMI, we were then interested to study the relation between miR-23a and miR-145 with tumor size (Figure 13a and b) and miR-451 with BMI of BC patients (Figure 13c). The nonparametric Spearman correlation coefficient "r" was calculated to measure the linear relationship between the 2 variables with respect to direction and strength. Another parameter, coefficient of determination, r^2, was calculated to measure how close each data point fits to the regression line. The correlation coefficient of fold change of expression of miR-23a and tumor size was 0.4130 with p-value 0.0073 and r^2= 0.05388, and that for miR-145 was 0.4648 with p-value 0.0022 and r^2= 0.1072, in BC patients which indicates that there is a significant, positive, and fair correlation between miR-23a and miR-145 expression in plasma of BC cancer to the breast tumor size. In addition to that, there is 5.388% and 10.72% that the change in the tumor size is due to the dysregulation of miR-23a and miR-145, respectively. This indicates that miR-145 affects the tumor size in EBC patients more than miR-23a. The correlation coefficient of fold change of expression of miR-451 and BC patients BMI was -0.2238 with p-value 0.1708 and r^2= 0.002369 which indicates that there is an insignificant negative correlation. In a nutshell, miR-23a and miR-145 expression in plasma from EBC patients significantly increase with tumor size with a significant fair correlation, while miR-451 decrease its expression as the BMI of EBC patient increases with insignificant poor correlation.

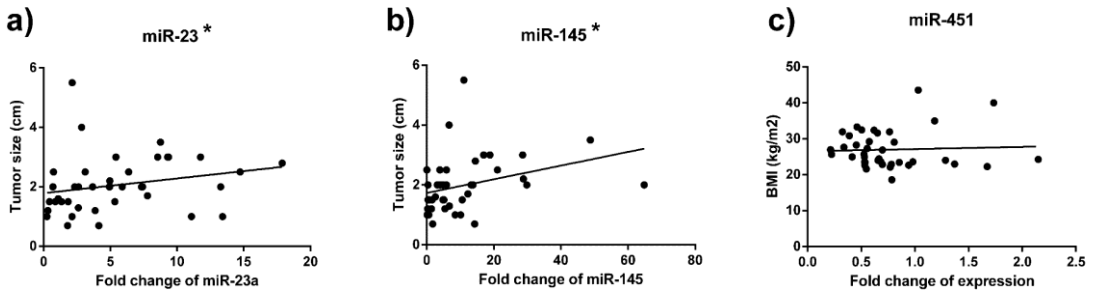


Figure 13. Linear regression model detecting the relation between a and b) fold change of expression of miR -23a and -145 with tumor size (cm) and c) fold change of expression of miR-451 with BMI (kg/m^2). The correlation coefficient was calculated based on non-parametric Spearman correlation. a) Best-fit line equation: Y = 0.04942*X + 1.789, the slope is not significantly deviated from zero. b) Best-fit line equation: Y = 0.02283*X + 1.733, the slope is significantly deviated from zero. c) Best-fit line equation: Y = 0.6105*X + 26.53, the slope is not significantly deviated from zero. * denotes *p*-value <0.05 and the correlation coefficient is significant.

Besides, a correlation test using IBM SPSS version 22 was done on the rest of the miRs (miR-1955, miR-100, and miR-155) because the dependent variables studied with these miRs are dichotomous. Correlation between fold change of expression of miR-195 and miR-100 and EBC patient BC family history on one hand, and between fold change of expression of miR-155 and waterpipe smoking status of the participant on the other hand was tested. miR-195 and miR-100 show negative poor correlation (r= -0.256 and -0.190, respectively) with family history of BC, while miR-155 showed positive poor correlation (r= 0.299) with waterpipe smoking status. The 3 miRs show insignificant correlation with their corresponding clinicopathological subgroups (p-value for miR-195, 0.115; miR-100, 0.260, and miR-155, 0.068). Therefore, correlation test show that the expression of miR-195 and miR-100 are not affected by family history BC risk factor, and miR-155 is not affected by waterpipe smoking BC risk factor.

F. Detection of the accuracy of the miR diagnostic ability

In order to identify the accuracy of the tested miRs in detecting early breast cancer cases, we plotted Receiver operating characteristic (ROC) curve. The horizontal axis represents specificity, the vertical axis represents the sensitivity, and the area under the curve [148] represents the degree of separability. The closer is the AUC to 1, the more accurate is the miR in diagnosing EBC. Figure 14 depicts the ROC curves plotted to illustrate the diagnostic ability of miRs that are significantly dysregulated in plasma from BC patient in comparison to healthy subjects.

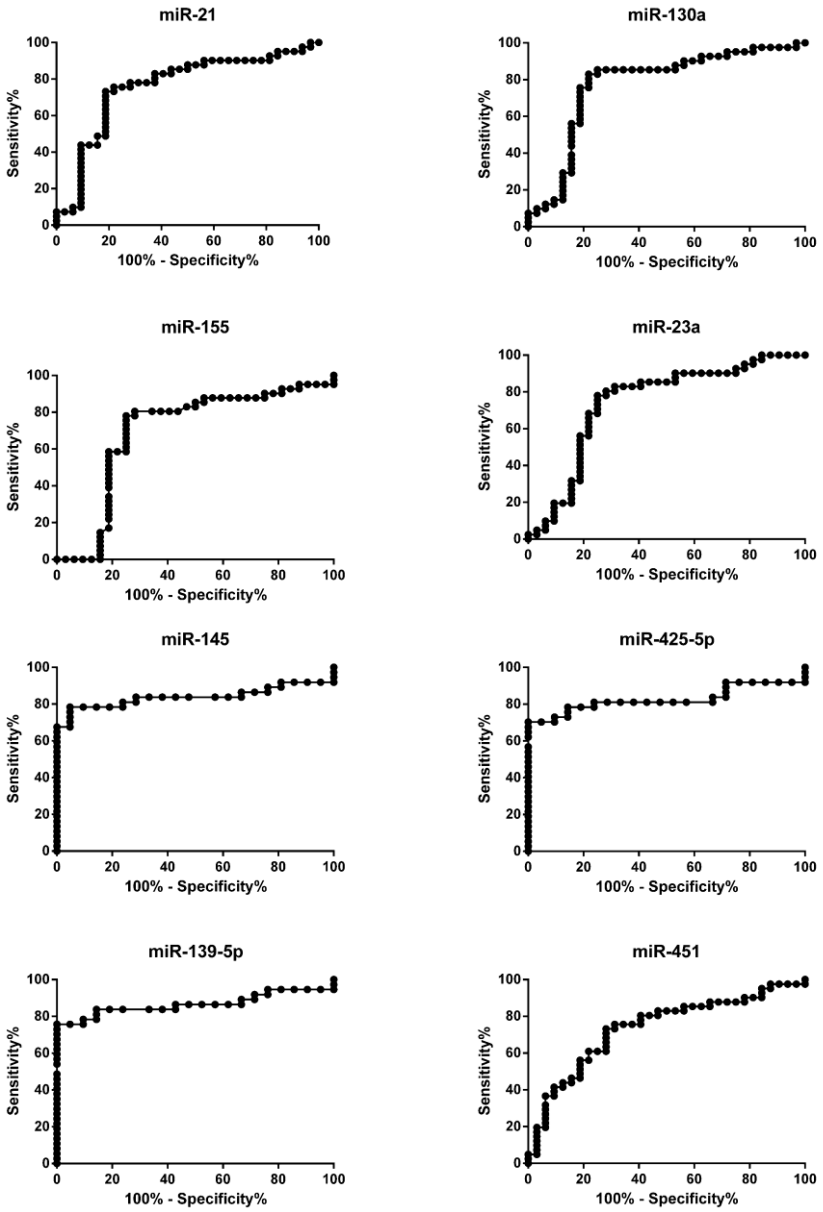


Figure 14. Diagnostic accuracy of miR-21, miR-130a, miR-155, miR-23a, miR-145, miR-425-5p, miR-139-5p, miR-451 for BC. ROC curve analysis was done to study the diagnostic accuracy of microRNA molecules in separating BC patients from healthy controls. miR-21 with AUC of 0.7591 (95% Cl: 0.6414-0.8769), miR-130a with AUC of 0.7736 (95%Cl: 0.6551-0.8922), miR-155 with AUC of 0.6951 (95% Cl: 0.5587 to 0.8316); miR-23a with AUC of 0.7431 (95% Cl: 0.6197 to 0.8666), miR-145 with AUC of 0.8391 (95% Cl: 0.7314 to 0.9468), miR-425-5p with AUC of 0.8263 (95% Cl: 0.7161 to 0.9364), miR-139-5p with AUC of 0.8662 (95% Cl: 0.7688 to 0.9635); miR-451 with AUC of 0.7355 (95% Cl: 0.6188 to 0.8522).

Furthermore, binary logistic regression model and ROC curve were utilized to study the combined diagnostic value of the variable biomarkers. Using the ROC curves generated, the combination of all 8 significantly dysregulated miRs showed the highest accuracy in diagnosing EBC patients with AUC of 0.929 (95% Cl: 0.868 to 0.990) (Figure 15), however the combination of the 3 miRs of highest AUC (miR-145, miR-425-5p, and miR-139-5p) showed the a diagnostic accuracy of 0.876 (95% CI: 0.784 to 0.969) in diagnosing EBC (Figure 16). Then our results indicate that the combination of the 8 miRs (miR-145, miR-425, miR-139-5p, miR-21, miR-155, miR-130a, miR-23a, and miR-451) have diagnostic accuracy higher than when each miR is used alone for EBC detection, even higher when we combine the 3 miRs of highest AUC.

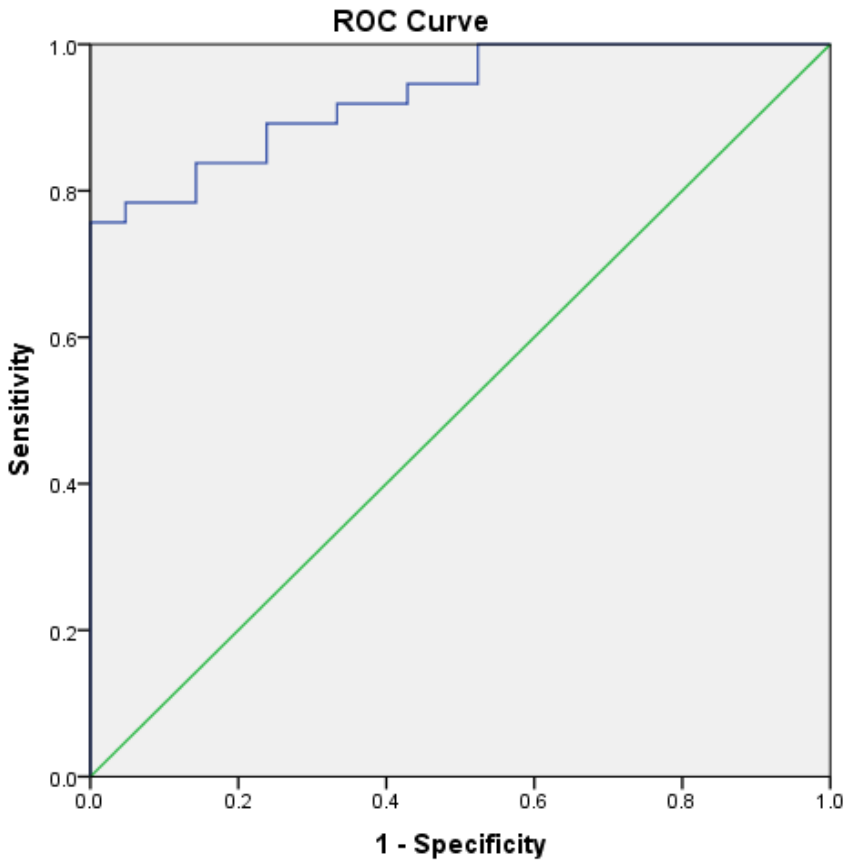


Figure 15. ROC curve showing the diagnostic accuracy of 8 significantly dysregulated combined miRs . MicroRNA signatures significantly distinguished EBC patients from healthy subjects (AUC= 0.929).

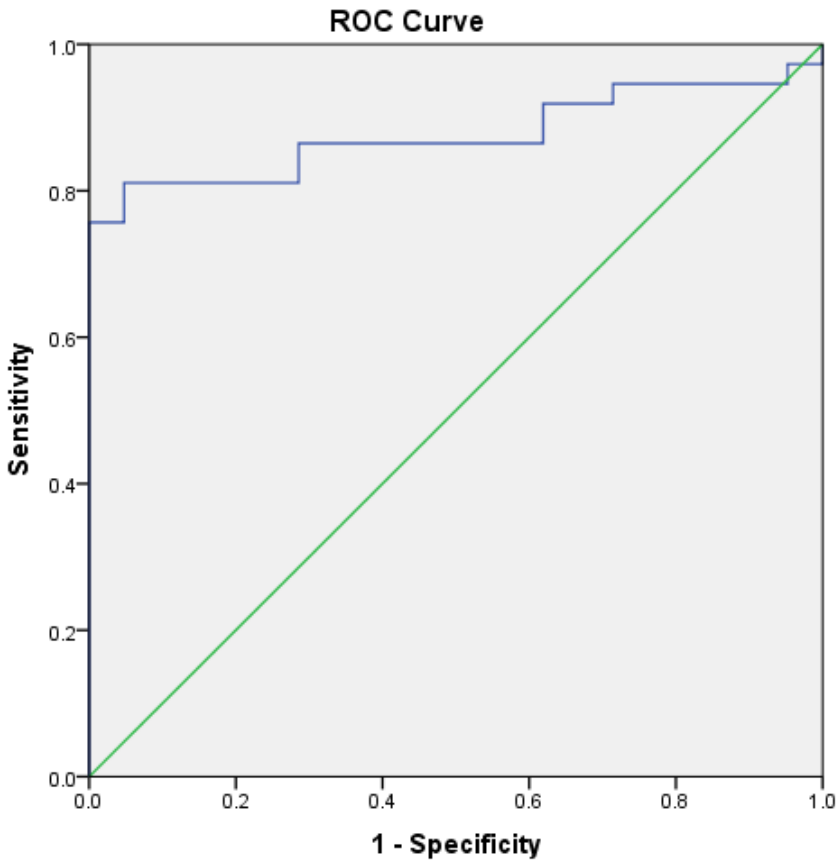


Figure 16. ROC curve showing the diagnostic accuracy of 3 combined miRs (miR-145, miR-425-5p, and miR-139-5p). MicroRNA signatures significantly distinguished EBC patients from healthy subjects (AUC= 0.876).

CHAPTER V

DISCUSSION

Breast cancer has the highest mortality rate among Lebanese cancer patients. This is despite advanced treatment options and the increased awareness about it and its current screening guidelines for mammography [169]. Early detection of BC could drastically help in disease outcome improvement [170]. Hence, the need for minimally invasive easily detected diagnostic biomarkers for BC diagnosis. Several studies have investigated the great potential of microRNA molecules as diagnostic biomarkers for BC patients in tissues and circulation [116, 171, 172]. In a previous work from our lab, we identified dysregulated microRNA expression profile of tissues taken from Lebanese EBC patients which was similar to some extent to that of American specimens [164, 165]. A correlation between the expression of microRNA molecules in different body fluids and their expression in tissue samples, in different types of cancer, including breast cancer was previously reported [173]. As such, in this work, the level of 12 candidate microRNAs (miR-21, miR-130a, miR-195, miR-155, miR-23a, miR-451, miR-145, miR-425-5p, miR-139-5p, miR-100, miR-125b, miR182) [174] were quantified by RT-qPCR in plasma from BC patients diagnosed with the most common histotype and receptor profile in Lebanon (IDC and ER+/PR+) compared to healthy controls to test their diagnostic ability and their correlation with clinical and pathological data.

Our results showed that out of the 12 studied miRs, 7 miRs (miR-21, miR-155, miR-130a, miR-23a, miR-145, miR-425-5p, and miR-139-5p) were significantly overexpressed, 1 miR (miR-451) was significantly underexpressed, and 4 miRs (miR-195, miR-125b, miR-100, and miR-182) were insignificantly dysregulated. Table 6 depicts the comparison between our results and that of some articles found in literature, using variety of sample types and different ethnic groups. miR-21 and miR-155 are extensively studied oncomiRs for EBC detection in a wide variety of ethnic groups. In consistency with our results, miR-21 and miR-155 were significantly over-expressed in several studies [175-178]. A study evaluating the biomarker potential of miR-21 and miR-155 in the tissue and plasma samples from Iranian Azeri breast cancer patients reported an upregulation of these 2 miRs in both experimental subjects [177]. Similarly, upregulation of miR-21 was observed in plasma from Spaniards breast cancer patients [178]. Another study reported the upregulation of miR-21 and miR-155 in serum in Chinese ethnic group [167]. In addition to that, circulating miR-155 and miR-21 in plasma of luminal A breast cancer patients was shown to be overexpressed as compared to healthy controls [179]. According to Dia *et al*, miR-21 overexpression promotes breast cancer progression and chemoresistance via TGF-β/ miR-21/ phosphatase and tensin homolog (PTEN) signaling axis suppression [180]. miR-21 also targets tissue inhibitor of matrix metalloproteinases 3 (TIMP3), programmed cell death receptor 4 (PDCD4), PTEN, tropomyosin 1 (TPM1), and reversion-inducing-cysteine-rich protein with kazal motifs (RECK) mRNA, therefore enhancing cell invasion, tumor metastasis, and angiogenesis [181]. As for miR-155, it promotes proliferation and

migration of BC cells through downregulating the suppressor of cytokine signaling 1 (SOCS1) and upregulating the matrix metallopeptidase 16 (MMP16) [182]. Our results didn't show any significant dysregulation of miR-21 in any of the clinicopathological subgroups (Table 4) even though miR-21 overexpression is reported to be associated with clinical characteristics of BC patients, including tumor grade and lymph node metastasis [180]. Interestingly, significant miR-155 overexpression was only found in the subgroup of waterpipe smoking patients compared to non-smokers.

Talking about miR-451 and miR-145, they are reported to be significantly downregulated in breast cancer tissue samples [183, 184]. miR-451 has been reported to play a role in influencing resistance of the Paclitaxel-resistant breast cancer cell line [183, 185]. In addition to that, transfecting 3 Paclitaxel-resistant breast cancer cell lines with miR-451 mimics, decreased Bcl-2 expression, increased the expression of caspase 3, and increased apoptosis [185]. One of the suggested mode of action of miR-145, is that it may inhibit transforming growth factor (TGF-β1) protein expression which contributes to tumor formation [184]. Regarding miR-145, it suppresses breast cancer cell migration by targeting FSCN and inhibiting epithelial-mesenchymal transition [109]. Regarding the expression of these 2 miRs in circulation, our results show that miR-451 is downregulated, while miR-145 is upregulated. According to literature, Ng *et al.* (2013), showed that miR-451 is overexpressed while miR-145 is underexpressed in the plasma of 170 Chinese BC patients compared to 100 normal controls [176]. However, miR-145 and miR-451 were significantly higher in the serum of Mexican BC patients [186] and Chinese BC patients [187], respectively. Regarding miR-451,

Motamedi *et al.* (2019) didn't observe any significant dysregulation in the expression of this miR in the plasma of Iranian BC patients [174]. Upon subgrouping, it is noteworthy to indicate that BC patients of normal weight showed an overexpression of miR-451 compared to the obese/overweight group. In addition to that, tumor staging differentiation was indicated by miR-145 expression.

The expression of miR-130a and miR-23a, was also analyzed in different sample types from different ethnic groups. A study by Pan *et al*. reported that miR-130a is a tumorsuppressor-miR as it is down-regulated in tissue samples from Chinese BC patients. Moreover, miR-130a inhibits cell proliferation, invasion, and migration by targeting Ras analog in brain mRNA (RAB5A) [188], reducing the expression of Fos like antigen 1 (FOSL-1), and suppressing the inhibition of zonula occludins-1 (ZO-1) [189]. On the contrary, miR-23a has an oncogenic role in breast cancer, in which its upregulation enhanced BC progression by directly activating forkhead box m (FOXM1 and histidine-rich glycoprotien (HRG) at RNA level [190]. Regarding the expression of miR-23a in circulation, it was reported by *Li et al.* its upregulation in the plasma of Chinese BC patients [162]. miR-23a and miR-130a were found to be downregulated in plasma of newly diagnosed Han Chinese BC patients, opposite to our results [191]. However in matching results, miR-23a show significant dysregulation in different BC patients with different tumor size [191]. Another study, profiling 6 cell-free miRs, showed that miR-130a levels in plasma were not able to discriminate normal controls from postoperative BC patients, before and after chemotherapy, however, miR-130a levels were able to differ between lymph node -positive and -negative patients (in

contradictory to our analysis which indicate an insignificant difference between the two groups) [192].

Regarding miR-139-5p and miR-425-5p, none have studied their mode of dysregulation in the plasma of BC patients; however one study have reported that miR-425-5p was upregulated while miR-139-5p was downregulated in the serum of Caucasian ER-positive EBC patients [118]. Another study assessed the level of miR-425-5p in the serum of BC patients, however non-significant results were obtained [193]. According to Krishnan et al. miR-139-5p has a significant functional role as tumorsuppressormiR.miR-139-5p was shown to prevent cell migration and metastasis by disrupting the TGFβ, Wnt, Rho, and MAPK/PI3K signaling cascade [194]. As for miR-425-5p, it was reported to have an oncogenic role in breast cancer by promoting cell invasion and migration via PTEN [195]

In this study, the plasma abundance of miR-125b, miR-100, miR-182, miR-195 displayed no remarked difference between breast cancer and healthy control cases. Similar to our results, non-significant dysregulation in the expression of miR-182 and miR-195 is observed in the plasma of BC patients [191, 196]. There are contradicting results regarding miR-195 expression in plasma samples as some studies showed that it is down-regulated in plasma samples [197, 198], however, miR-195 was elevated in plasma of Saudi TNBC patients . On the contrary, according to literature, miR-125b was reported to be abundant in the plasma from Spaniards breast cancer patients and serum of Mexican BC patients [178, 186]. When dividing the patients into subgroups, miR-195 and miR-100 were able to differentiate between BC patients with BC family

history and miR-182 was differentially expressed among patients with different parity. Hence each miR shows that it has its own role and importance in breast cancer initiation or suppression, therefore, each will have its own level of expression in different sample types and in different ethnic groups.

After analyzing the mode of dysregulation of the 12 miRs in the plasma of EBC Lebanese patients, linear regression and correlation analysis were done on 6 miRs (miR-23a, miR-145, miR-451, miR-195, miR-100, and miR-155) to assess the relation between their level of expression and some clinicopathological features. Using Linear regression, the relations between miR-23a and miR-145 expression and tumor size were identified, similarly the relation between miR-451 expression and patients BMI. It was clear that the expression of miR-23a and miR-145 significantly increases with tumor size. Mann-Whitney U test shows significant upregulation of miR-23a and miR-451 in plasma of EBC patients with tumor size ≥2cm compared to EBC patients with tumor size <2cm and compared to plasma of healthy subjects, and significant upregulation in specimen of EBC patients with tumor size <2cm compared to specimen of healthy subjects. In addition to that, linear regression model assured a significant, positive, and fair correlation between the miRs expression and the size of the tumor [199, 200]. It is noteworthy to indicate that, miR-145 is more affected by tumor size than miR-23a since it has higher correlation coefficient (r of miR-145= 0.4648 and r of miR-23a= 0.4130) and 10.73% of the overexpression of miR-145 are explained by increase in tumor size while that of miR-23a, it is 5.388% only. Shifting to miR-451, it was shown that its expression can differentiate between individuals with different BMI. Meaning that,

overweight/obese EBC patients tends to overexpress miR-451 vs. those having normal weight. Yet, linear regression didn't support the correlation between miR-451 expression and BMI. Regarding miR-195 and miR-100, Mann-Whitney U test showed that miR-195 is a specific biomarker for EBC in patients with BC family history, while miR-100 is a specific biomarker for EBC patients with no family history. These results explain the insignificant mode of dysregulation of the miRs when compared to all EBC patients. However, the correlation test was utilized to detect the strength of the relation between miR-195 and miR-100 expression and the BC risk factor: family history of BC, indicated a poor relation between the two factors. Finally, miR-155 was shown to be significantly upregulated in non-smokers EBC patients, while there was non-significant deregulation in smoking EBC patients. Yet, these 2 variables are poorly correlated with each other.

We then analyzed the ability of the 8 significantly dysregulated miRs (miR-21, miR-130a, miR-155, miR-23a, miR-451, miR-145, miR-425-5p, and miR-139-5p) in detecting early BC cases using ROC curve. The results show that miR-139-5p shows the highest accuracy, followed by miR-145 and miR-425 out of the 8 miRs, with AUC 0.8662 (95% Cl: 0.7688 to 0.9635) [201]. Other studies showed that, the combination of plasma miR-145 and miR-451 to be useful biomarker for breast cancer detection [176]. In this study, the combination of 3 miRs (miR-145, miR-425-5p, and miR-139-5p) showed even more accuracy in detecting EBC. This tells us that miR-145 has good association with tumor size, significantly upregulated in EBC patient's plasma, have a good diagnostic accuracy, and its combination with miR-139-5p and miR-425-5p

increases this accuracy. Therefore, miR-145 must be studied extensively and in larger experimental groups. Whereas a signature of dysregulated miRs in the plasma of Lebanese EBC patients show to have a potential role in diagnosing EBC cases, some discrepancy with previous literature exist and this can be due to difference in sample size, ethnic origin of the experimental subjects, different BC molecular and /or histopathological subtypes studied, different age groups involved, and inclusion of EBC patients who have received any treatments.

There are a few limitations to this study. First, the small sample size (N=41 EBC patients and N=32 healthy subjects), then, some samples were found to be contaminated with red blood cells as assessed by the ratio of the relative expression of the erythrocyte-specific miR-451 and the stable (hemolysis independent) miR-23a.

Another point to tackle, is the choice of endogenous control. Normalization of the experimental samples is an important concept to control any non-biological variation introduced during sample preparation [202]. RNU6B is commonly used for normalization in tissue samples, however it is not remarkably stable in plasma/serum. Studies on circulating microRNA in breast cancer normalized to wide variety of miRs including, miR-16, miR-23a, miR-191, miR-425, miR-484, let7a, miR-130a, miR-93, miR-10, and miR-30a [118, 124, 126, 146, 166, 178, 198, 203]. In this study, miR-16 was used as an endogenous control. Finally, the studied samples were all ER+ and it would be important to validate these findings in TNBC and HER2+ BC samples.

miRNA	Deregulation in our study	Deregulation in Literature	Sample type	Cohort Ethnicity	Reference
miR-21	Up-regulated	Up-regulated	Plasma and Tissue	Iranian	[177]
		Up-regulated	Plasma	Spaniards	[178]
		Up-regulated	Serum	Chinese	[167]
		Up-regulated	Plasma	Chinese	[176]
		Up-regulated	Plasma	American	[204]
miR-155	Up-regulated	Up-regulated	Plasma and Tissue	Iranian	[177]
		Up-regulated	Serum	Chinese	[167]
		Up-regulated	Serum	Chinese	[110]
		Up-regulated	Plasma	Iranian	[179]
miR-451	Down-regulated	Down-regulated	Tissue	Chinese	[183]
		Up-regulated	Plasma	Chinese	[187]
		Up-regulated	Serum	Chinese	[176]
miR-145	Up-regulated	Down-regulated	Tissue	Chinese	[184]
		Down-regulated	Plasma	Chinese	[176]
		Down-regulated	Serum	Mexican	[186]
miR-130a	Up-regulated	Down-regulated	Tissue	Chinese	[188]
		Down-regulated	Plasma	Chinese	[162]
miR-23a	Up-regulated	Up-regulated	Tissue	Not specified	[190]
		Up-regulated	Plasma	Chinese	[162]
miR-139-5p	Up-regulated	Down-regulated	Serum	Caucasian	[118]
miR-425-5p	Up-regulated	Up-regulated	Serum	Caucasian	[118]

Table 6. Comparison of the mode of expression of miRs inour study vs. Literature. miRs that showed non-significant mode of dysregulation were not added. Mode of dysregulation of the miRs varies with the variation of sample type and the ethnicity of the cohort, yet miR-21 and miR-155 are upregulated in all cases.

CHAPTER VI
CONCLUSION

In conclusion, our study is the first to investigate the potential of plasma microRNA molecules in detecting early onset of IDC breast cancer with ER-positive and PR-positive status, among Lebanese women. Breast cancer is the most widely spread malignancy among Lebanese women. There is a need for accurate and minimally invasive method to detect early breast cancer cases in order to improve patient's outcome. Importantly, we found that the significantly deregulated miRs (miR-21, miR-155, miR-130a, miR-451, miR-23a, miR-425-5p, miR-145, miR-425, miR-139-5p) could discriminate EBC from healthy controls and have potential EBC diagnostic ability. Furthermore, miR-23a and miR-145 were shown to be fairly related to the tumor size and the three miRNA panel (miR-145, miR-425-5p, and miR-139-5p) proved to have high accuracy in detecting EBC cases [201]. Our data provide the basis for further research on bigger sample size and diverse receptor status and pathological subtypes to validate these dysregulated circulating miRs in patients with early stages breast cancer.

BIBLIOGRAPHY

1. Ferlay, J., et al., *Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods.* International journal of cancer, 2019. **144**(8): p. 1941-1953.
2. Chen, Z., et al., *Trends of female and male breast cancer incidence at the global, regional, and national levels, 1990–2017.* Breast Cancer Research and Treatment, 2020. **180**(2): p. 481-490.
3. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA: a cancer journal for clinicians, 2018. **68**(6): p. 394-424.
4. DeSantis, C.E., et al., *International variation in female breast cancer incidence and mortality rates.* Cancer Epidemiology and Prevention Biomarkers, 2015. **24**(10): p. 1495-1506.
5. Azamjah, N., Y. Soltan-Zadeh, and F. Zayeri, *Global Trend of Breast Cancer Mortality Rate: A 25-Year Study.* Asian Pacific journal of cancer prevention: APJCP, 2019. **20**(7): p. 2015.
6. Lakkis, N.A., et al., *Sociological transition and breast Cancer in the Arab world: the experience of Lebanon.* Asian Pacific journal of cancer prevention: APJCP, 2017. **18**(5): p. 1357.
7. Fares, M.Y., et al., *Breast cancer epidemiology among Lebanese women: an 11-year analysis.* Medicina, 2019. **55**(8): p. 463.
8. Chahine, G., et al., *Characteristics of incident female breast cancer in Lebanon, 1990–2013: Descriptive study of 612 cases from a hospital tumor registry.* Cancer epidemiology, 2015. **39**(3): p. 303-306.
9. Kamińska, M., et al., *Breast cancer risk factors.* Przeglad menopauzalny= Menopause review, 2015. **14**(3): p. 196.
10. El, N.S., et al., *Age distribution of breast cancer in Lebanon: increased percentages and age adjusted incidence rates of younger-aged groups at presentation.* Le Journal medical libanais. The Lebanese medical journal, 2002. **50**(1-2): p. 3-9.
11. Shamseddine, A., et al., *Cancer trends in Lebanon: a review of incidence rates for the period of 2003–2008 and projections until 2018.* Population health metrics, 2014. **12**(1): p. 4.
12. Haddad, F.G., et al., *Geriatric cancer trends in the Middle-East: Findings from Lebanese cancer projections until 2025.* Journal of geriatric oncology, 2018. **9**(2): p. 120-123.
13. Gaudet, M.M., et al., *Active smoking and breast cancer risk: original cohort data and meta-analysis.* Journal of the National Cancer Institute, 2013. **105**(8): p. 515-525.
14. Strumylaite, L., et al., *Association between lifetime exposure to passive smoking and risk of breast cancer subtypes defined by hormone receptor status among non-smoking Caucasian women.* PloS one, 2017. **12**(2): p. e0171198.

15. de Boer, M.C., et al., *The mechanisms and effects of physical activity on breast cancer.* Clinical breast cancer, 2017. **17**(4): p. 272-278.
16. Naja, F., et al., *Nutrition in the Prevention of Breast Cancer: A Middle Eastern Perspective.* Frontiers in Public Health, 2019. **7**.
17. Bagnardi, V., et al., *Alcohol consumption and site-specific cancer risk: a comprehensive dose–response meta-analysis.* British journal of cancer, 2015. **112**(3): p. 580-593.
18. Freund, C., et al., *Breastfeeding and breast cancer.* Gynecologie, obstetrique & fertilite, 2005. **33**(10): p. 739-744.
19. Collaborators, M.W.S., *Breast cancer and hormone-replacement therapy in the Million Women Study.* The Lancet, 2003. **362**(9382): p. 419-427.
20. Badr, L.K., et al., *Breast cancer risk factors: A cross-cultural comparison between the West and the East.* Asian Pacific journal of cancer prevention: APJCP, 2018. **19**(8): p. 2109.
21. Cancer, C.G.o.H.F.i.B., *Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies.* The lancet oncology, 2012. **13**(11): p. 1141-1151.
22. Kouloulias, V., et al., *Physical activity, early first delivery and residence as parameters for breast cancer prevention: an observational study.* Journal of BU ON.: official journal of the Balkan Union of Oncology, 2019. **24**(4): p. 1512-1515.
23. Andarieh, M.G., et al., *Infertility as a risk factor for breast cancer: Results from a hospital-based case–control study.* Journal of cancer research and therapeutics, 2019. **15**(5): p. 976.
24. Albrektsen, G., et al., *Breast cancer risk by age at birth, time since birth and time intervals between births: exploring interaction effects.* British journal of cancer, 2005. **92**(1): p. 167-175.
25. Soerjomataram, I., et al., *Increased risk of second malignancies after in situ breast carcinoma in a population-based registry.* British journal of cancer, 2006. **95**(3): p. 393-397.
26. Barnard, M.E., C.E. Boeke, and R.M. Tamimi, *Established breast cancer risk factors and risk of intrinsic tumor subtypes.* Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 2015. **1856**(1): p. 73-85.
27. Rebbeck, T.R., et al., *Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer.* Jama, 2015. **313**(13): p. 1347-1361.
28. El Saghir, N.S., et al., *BRCA1 and BRCA2 mutations in ethnic Lebanese Arab women with high hereditary risk breast cancer.* The oncologist, 2015. **20**(4): p. 357.
29. Jalkh, N., et al., *Next-generation sequencing in familial breast cancer patients from Lebanon.* BMC medical genomics, 2017. **10**(1): p. 8.
30. Hassiotou, F. and D. Geddes, *Anatomy of the human mammary gland: Current status of knowledge.* Clinical anatomy, 2013. **26**(1): p. 29-48.

31. Jesinger, R.A., *Breast anatomy for the interventionalist.* Techniques in vascular and interventional radiology, 2014. **17**(1): p. 3-9.
32. Javed, A. and A. Lteif. *Development of the human breast*. in *Seminars in plastic surgery*. 2013. Thieme Medical Publishers.
33. Truchet, S. and E. Honvo-Houéto, *Physiology of milk secretion.* Best Practice & Research Clinical Endocrinology & Metabolism, 2017. **31**(4): p. 367-384.
34. Feng, Y., et al., *Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis.* Genes & diseases, 2018. **5**(2): p. 77-106.
35. Polyak, K., *Breast cancer: origins and evolution.* The Journal of clinical investigation, 2007. **117**(11): p. 3155-3163.
36. Morrow, M., S.J. Schnitt, and L. Norton, *Current management of lesions associated with an increased risk of breast cancer.* Nature Reviews Clinical Oncology, 2015. **12**(4): p. 227.
37. Makki, J., *Diversity of breast carcinoma: histological subtypes and clinical relevance.* Clinical Medicine Insights: Pathology, 2015. **8**: p. CPath. S31563.
38. Eheman, C.R., et al., *The changing incidence of in situ and invasive ductal and lobular breast carcinomas: United States, 1999-2004.* Cancer Epidemiology and Prevention Biomarkers, 2009. **18**(6): p. 1763-1769.
39. Bertos, N.R. and M. Park, *Breast cancer—one term, many entities?* The Journal of clinical investigation, 2011. **121**(10): p. 3789-3796.
40. Subramaniam, S., et al., *Breast cancer outcomes as defined by the estrogen receptor, progesterone receptor, and human growth factor receptor-2 in a multi-ethnic Asian country.* World journal of surgery, 2015. **39**(10): p. 2450-2458.
41. Ronchi, A., et al. *Current and potential immunohistochemical biomarkers for prognosis and therapeutic stratification of breast carcinoma*. in *Seminars in Cancer Biology*. 2020. Elsevier.
42. Nahid Nafissi, M., *Breast cancer survival analysis based on immunohistochemistry subtypes (ER/PR/HER2): a retrospective cohort study.* Archives of Iranian medicine, 2016. **19**(10): p. 680.
43. Perou, C.M., et al., *Molecular portraits of human breast tumours.* nature, 2000. **406**(6797): p. 747-752.
44. Rakha, E.A. and F.G. Pareja. *New Advances in Molecular Breast Cancer Pathology*. in *Seminars in Cancer Biology*. 2020. Elsevier.
45. Provenzano, E., G.A. Ulaner, and S.-F. Chin, *Molecular classification of breast cancer.* PET clinics, 2018. **13**(3): p. 325-338.
46. Weigelt, B., F.L. Baehner, and J.S. Reis-Filho, *The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade.* The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2010. **220**(2): p. 263-280.
47. Russnes, H.G., et al., *Breast cancer molecular stratification: from intrinsic subtypes to integrative clusters.* The American journal of pathology, 2017. **187**(10): p. 2152-2162.

48. Kolak, A., et al., *Primary and secondary prevention of breast cancer.* Ann Agric Environ Med, 2017. **24**(4): p. 549-553.
49. Jordan, V., M. Khan, and D. Prill, *Breast cancer screening: why can't everyone agree?* Primary care, 2018. **46**(1): p. 97-115.
50. Ginsburg, O., et al., *Breast cancer early detection: a phased approach to implementation.* Cancer, 2020. **126**: p. 2379-2393.
51. Alcantara, D., et al., *Molecular imaging of breast cancer: present and future directions.* Frontiers in chemistry, 2014. **2**: p. 112.
52. Becker, S., *A historic and scientific review of breast cancer: The next global healthcare challenge.* International Journal of Gynecology & Obstetrics, 2015. **131**: p. S36-S39.
53. Sitt, J.C., et al., *Understanding breast cancer screening—past, present, and future.* Hong Kong Med J, 2018. **24**(2): p. 166-74.
54. Wadhwa, A., J.R. Sullivan, and M.B. Gonyo, *Missed breast cancer: what can we learn?* Current Problems in Diagnostic Radiology, 2016. **45**(6): p. 402-419.
55. Çakır, M., et al., *Comparison of mammography sensitivity after reduction mammoplasty targeting the glandular and fat tissue.* Turkish Journal of Surgery/Ulusal cerrahi dergisi, 2015. **31**(2): p. 68.
56. Adhami, M., et al., *Candidate miRNAs in human breast cancer biomarkers: a systematic review.* Breast Cancer, 2018. **25**(2): p. 198-205.
57. Pereira, R.d.O., et al., *Evaluation of the accuracy of mammography, ultrasound and magnetic resonance imaging in suspect breast lesions.* Clinics, 2020. **75**.
58. Sardanelli, F., et al., *Magnetic resonance imaging of the breast: recommendations from the EUSOMA working group.* European journal of cancer, 2010. **46**(8): p. 1296-1316.
59. Mann, R.M., et al., *Breast MRI: guidelines from the European society of breast imaging.* European radiology, 2008. **18**(7): p. 1307-1318.
60. Mehnati, P. and M.J. Tirtash, *Comparative efficacy of four imaging instruments for breast cancer screening.* Asian Pac J Cancer Prev, 2015. **16**(15): p. 6177-6186.
61. Liu, H., et al., *Comparison of BSGI, MRI, mammography, and ultrasound for the diagnosis of breast lesions and their correlations with specific molecular subtypes in Chinese women.* BMC Medical Imaging, 2020. **20**(1): p. 1-10.
62. Onega, T., et al., *Breast cancer screening in an era of personalized regimens: A conceptual model and National Cancer Institute initiative for risk‐based and preference‐based approaches at a population level.* Cancer, 2014. **120**(19): p. 2955-2964.
63. Odle, T.G., *Precision medicine in breast cancer.* Radiologic technology, 2017. **88**(4): p. 401M-421M.
64. Park, S.S., *Gene patents and the public interest: litigating Association for Molecular Pathology v. Myriad Genetics and lessons moving forward.* NCJL & Tech., 2013. **15**: p. 519.
65. Sherkow, J.S. and C. Scott, *CASE STUDY: Myriad stands alone.* Nature biotechnology, 2014. **32**(7): p. 620-620.

66. Lynch, J.A., V. Venne, and B. Berse. *Genetic tests to identify risk for breast cancer*. in *Seminars in oncology nursing*. 2015. Elsevier.
67. Huang, T. and C.-X. Deng, *Current progresses of exosomes as cancer diagnostic and prognostic biomarkers.* International journal of biological sciences, 2019. **15**(1): p. 1.
68. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* cell, 2004. **116**(2): p. 281-297.
69. Herranz, H. and S.M. Cohen, *MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems.* Genes & development, 2010. **24**(13): p. 1339-1344.
70. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* cell, 1993. **75**(5): p. 843-854.
71. Kozomara, A., M. Birgaoanu, and S. Griffiths-Jones, *miRBase: from microRNA sequences to function.* Nucleic acids research, 2019. **47**(D1): p. D155-D162.
72. Hammond, S.M., *An overview of microRNAs.* Advanced drug delivery reviews, 2015. **87**: p. 3-14.
73. Rorbach, G., O. Unold, and B.M. Konopka, *Distinguishing mirtrons from canonical miRNAs with data exploration and machine learning methods.* Scientific reports, 2018. **8**(1): p. 1-13.
74. Lin, S. and R.I. Gregory, *MicroRNA biogenesis pathways in cancer.* Nature reviews cancer, 2015. **15**(6): p. 321-333.
75. Czech, B. and G.J. Hannon, *Small RNA sorting: matchmaking for Argonautes.* Nature Reviews Genetics, 2011. **12**(1): p. 19-31.
76. Nelson, P.T., et al., *Specific sequence determinants of miR-15/107 microRNA gene group targets.* Nucleic acids research, 2011. **39**(18): p. 8163-8172.
77. Huntzinger, E. and E. Izaurralde, *Gene silencing by microRNAs: contributions of translational repression and mRNA decay.* Nature Reviews Genetics, 2011. **12**(2): p. 99-110.
78. Forman, J.J., A. Legesse-Miller, and H.A. Coller, *A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence.* Proceedings of the National Academy of Sciences, 2008. **105**(39): p. 14879-14884.
79. !!! INVALID CITATION !!! .
80. Dharap, A., et al., *MicroRNA miR-324-3p induces promoter-mediated expression of RelA gene.* PLoS one, 2013. **8**(11).
81. Jo, M.H., et al., *Human Argonaute 2 has diverse reaction pathways on target RNAs.* Molecular cell, 2015. **59**(1): p. 117-124.
82. Jonas, S. and E. Izaurralde, *Towards a molecular understanding of microRNA-mediated gene silencing.* Nature reviews genetics, 2015. **16**(7): p. 421-433.
83. Tüfekci, K.U., R.L.J. Meuwissen, and Ş. Genç, *The role of microRNAs in biological processes*, in *miRNomics: microRNA biology and computational analysis*. 2014, Springer. p. 15-31.

84. Bernstein, E., et al., *Dicer is essential for mouse development.* Nature genetics, 2003. **35**(3): p. 215-217.
85. Wang, Y., et al., *DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal.* Nature genetics, 2007. **39**(3): p. 380-385.
86. Park, C.Y., Y.S. Choi, and M.T. McManus, *Analysis of microRNA knockouts in mice.* Human molecular genetics, 2010. **19**(R2): p. R169-R175.
87. Chandrasekaran, K., et al., *Role of microRNAs in kidney homeostasis and disease.* Kidney international, 2012. **81**(7): p. 617-627.
88. Shim, J. and J.-W. Nam, *The expression and functional roles of microRNAs in stem cell differentiation.* BMB reports, 2016. **49**(1): p. 3.
89. Bottani, M., G. Banfi, and G. Lombardi, *Circulating miRNAs as Diagnostic and Prognostic Biomarkers in Common Solid Tumors: Focus on Lung, Breast, Prostate Cancers, and Osteosarcoma.* Journal of clinical medicine, 2019. **8**(10): p. 1661.
90. Liu, P., *MicroRNA Expression Analysis: Next-Generation Sequencing*, in *Gene Expression Analysis*. 2018, Springer. p. 171-183.
91. Palanichamy, J.K. and D.S. Rao, *miRNA dysregulation in cancer: towards a mechanistic understanding.* Frontiers in genetics, 2014. **5**: p. 54.
92. Pal, A.S. and A.L. Kasinski, *Animal models to study microRNA function*, in *Advances in cancer research*. 2017, Elsevier. p. 53-118.
93. Svoronos, A.A., D.M. Engelman, and F.J. Slack, *OncomiR or tumor suppressor? The duplicity of microRNAs in cancer.* Cancer research, 2016. **76**(13): p. 3666-3670.
94. Xiao, C., et al., *Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes.* Nature immunology, 2008. **9**(4): p. 405-414.
95. Fulci, V., et al., *Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia.* Blood, The Journal of the American Society of Hematology, 2007. **109**(11): p. 4944-4951.
96. Venturutti, L., et al., *Stat3 regulates ErbB-2 expression and co-opts ErbB-2 nuclear function to induce miR-21 expression, PDCD4 downregulation and breast cancer metastasis.* Oncogene, 2016. **35**(17): p. 2208-2222.
97. Yan, L.-X., et al., *MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis.* Rna, 2008. **14**(11): p. 2348-2360.
98. Zhang, X.F., et al., *Tumor Suppressor PTPRJ Is a Target of miR-155 in Colorectal Cancer.* Journal of cellular biochemistry, 2017. **118**(10): p. 3391-3400.
99. Jiang, S., et al., *MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene.* Cancer research, 2010. **70**(8): p. 3119-3127.
100. Miska, E.A., et al., *Microarray analysis of microRNA expression in the developing mammalian brain.* Genome biology, 2004. **5**(9): p. R68.

101. Thomson, J.M., et al., *A custom microarray platform for analysis of microRNA gene expression.* Nature Methods, 2004. **1**(1): p. 47-53.
102. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.* Proceedings of the National Academy of Sciences, 2004. **101**(9): p. 2999-3004.
103. Xiong, W.-C., et al., *microRNA-9 functions as a tumor suppressor in colorectal cancer by targeting CXCR4.* International Journal of Clinical and Experimental Pathology, 2018. **11**(2): p. 526.
104. Kapora, E., et al., *MicroRNA-505-5p functions as a tumor suppressor by targeting cyclin-dependent kinase 5 in cervical cancer.* Bioscience reports, 2019. **39**(7).
105. Rubin, R., et al., *Insulin receptor substrate (IRS)-1 regulates murine embryonic stem (mES) cells self-renewal.* Journal of cellular physiology, 2007. **213**(2): p. 445-453.
106. Spizzo, R., et al., *miR-145 participates with TP53 in a death-promoting regulatory loop and targets estrogen receptor-α in human breast cancer cells.* Cell Death & Differentiation, 2010. **17**(2): p. 246-254.
107. Wang, S., et al., *miR-145 inhibits breast cancer cell growth through RTKN.* International journal of oncology, 2009. **34**(5): p. 1461-1466.
108. Sachdeva, M. and Y.-Y. Mo, *MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1.* Cancer research, 2010. **70**(1): p. 378-387.
109. Zhao, H., et al., *miR-145 suppresses breast cancer cell migration by targeting FSCN-1 and inhibiting epithelial-mesenchymal transition.* American journal of translational research, 2016. **8**(7): p. 3106.
110. Sun, Y.-M., K.-Y. Lin, and Y.-Q. Chen, *Diverse functions of miR-125 family in different cell contexts.* Journal of hematology & oncology, 2013. **6**(1): p. 6.
111. Etheridge, A., et al., *Extracellular microRNA: a new source of biomarkers.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **717**(1-2): p. 85-90.
112. Lawrie, C.H., et al., *Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma.* British journal of haematology, 2008. **141**(5): p. 672-675.
113. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proceedings of the National Academy of Sciences, 2008. **105**(30): p. 10513-10518.
114. Yu, D.-C., et al., *Circulating microRNAs: potential biomarkers for cancer.* International journal of molecular sciences, 2011. **12**(3): p. 2055-2063.
115. Guo, H., et al., *Plasma miR-1273g-3p acts as a potential biomarker for early Breast Ductal Cancer diagnosis.* Anais da Academia Brasileira de Ciências, 2020. **92**(1).
116. Nassar, F.J., R. Nasr, and R. Talhouk, *MicroRNAs as biomarkers for early breast cancer diagnosis, prognosis and therapy prediction.* Pharmacology & therapeutics, 2017. **172**: p. 34-49.

117. Lyng, M.B., et al., *Prospective validation of a blood-based 9-miRNA profile for early detection of breast cancer in a cohort of women examined by clinical mammography.* Molecular oncology, 2016. **10**(10): p. 1621-1626.
118. Kodahl, A.R., et al., *Novel circulating microRNA signature as a potential non-invasive multi-marker test in ER-positive early-stage breast cancer: A case control study.* Molecular oncology, 2014. **8**(5): p. 874-883.
119. Chen, X., et al., *Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases.* Cell research, 2008. **18**(10): p. 997-1006.
120. Ng, E.K., et al., *Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening.* Gut, 2009. **58**(10): p. 1375-1381.
121. Huang, Z., et al., *Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer.* International journal of cancer, 2010. **127**(1): p. 118-126.
122. Zheng, H., et al., *Comprehensive Review of Web Servers and Bioinformatics Tools for Cancer Prognosis Analysis.* Frontiers in oncology, 2020. **10**: p. 68.
123. Jinling, W., et al., *Prognostic value of circulating microRNA-21 for breast cancer: a systematic review and meta-analysis.* Artificial cells, nanomedicine, and biotechnology, 2017. **45**(6): p. 1216-1221.
124. Sochor, M., et al., *Oncogenic microRNAs: miR-155, miR-19a, miR-181b, and miR-24 enable monitoring of early breast cancer in serum.* BMC cancer, 2014. **14**(1): p. 448.
125. Bašová, P., et al., *Prediction potential of serum miR-155 and miR-24 for relapsing early breast cancer.* International journal of molecular sciences, 2017. **18**(10): p. 2116.
126. Papadaki, C., et al., *Circulating miRNAs as a marker of metastatic disease and prognostic factor in metastatic breast cancer.* Oncotarget, 2019. **10**(9): p. 966.
127. Sahlberg, K.K., et al., *A serum microRNA signature predicts tumor relapse and survival in triple-negative breast cancer patients.* Clinical cancer research, 2015. **21**(5): p. 1207-1214.
128. Yuxia, M., T. Zhennan, and Z. Wei, *Circulating miR-125b is a novel biomarker for screening non-small-cell lung cancer and predicts poor prognosis.* Journal of cancer research and clinical oncology, 2012. **138**(12): p. 2045-2050.
129. Xu, X., et al., *High circulating miR-18a, miR-20a, and miR-92a expression correlates with poor prognosis in patients with non-small cell lung cancer.* Cancer medicine, 2018. **7**(1): p. 21-31.
130. Khandelwal, A., et al., *Circulating micro RNA-590-5p functions as a liquid biopsy marker in non-small cell lung cancer.* Cancer Science, 2020. **111**(3): p. 826-839.
131. Toiyama, Y., et al., *Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer.* Annals of surgery, 2014. **259**(4): p. 735.

132. Yuan, Z., et al., *Dynamic plasma microRNAs are biomarkers for prognosis and early detection of recurrence in colorectal cancer.* British journal of cancer, 2017. **117**(8): p. 1202-1210.
133. Shi, L., et al., *Downregulation of serum miR-194 predicts poor prognosis in osteosarcoma patients.* Annals of Diagnostic Pathology, 2020: p. 151488.
134. Yan, J., et al., *Downregulated miR-217 expression predicts a poor outcome in acute myeloid leukemia.* Cancer Biomarkers, 2018. **22**(1): p. 73-78.
135. Stamatopoulos, B., et al., *Opposite prognostic significance of cellular and serum circulating microRNA-150 in patients with chronic lymphocytic leukemia.* Molecular medicine, 2015. **21**(1): p. 123-133.
136. Moya, L., et al., *Assessment of miR-98-5p, miR-152-3p, miR-326 and miR-4289 expression as biomarker for prostate cancer diagnosis.* International journal of molecular sciences, 2019. **20**(5): p. 1154.
137. Wang, C., et al., *Increased expression of microRNA-93 correlates with progression and prognosis of prostate cancer.* Medicine, 2020. **99**(22): p. e18432.
138. Hua, Y., et al., *Low serum miR-373 predicts poor prognosis in patients with pancreatic cancer.* Cancer Biomarkers, 2017. **20**(1): p. 95-100.
139. Chen, Q., et al., *Circulating microRNA-182 in plasma and its potential diagnostic and prognostic value for pancreatic cancer.* Medical oncology, 2014. **31**(11): p. 225.
140. Oreglia, M., et al., *Early Postoperative Circulating miR-483-5p Is a Prognosis Marker for Adrenocortical Cancer.* Cancers, 2020. **12**(3): p. 724.
141. Zhou, M., et al., *MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression.* Journal of Biological Chemistry, 2010. **285**(28): p. 21496-21507.
142. Wang, H., et al., *Circulating MiR-125b as a marker predicting chemoresistance in breast cancer.* PloS one, 2012. **7**(4).
143. Liu, B., et al., *Serum miR-21 and miR-125b as markers predicting neoadjuvant chemotherapy response and prognosis in stage II/III breast cancer.* Human pathology, 2017. **64**: p. 44-52.
144. Zhu, W., et al., *Dynamics of circulating micro RNA s as a novel indicator of clinical response to neoadjuvant chemotherapy in breast cancer.* Cancer medicine, 2018. **7**(9): p. 4420-4433.
145. Cui, E.-h., et al., *Serum microRNA 125b as a diagnostic or prognostic biomarker for advanced NSCLC patients receiving cisplatin-based chemotherapy.* Acta pharmacologica sinica, 2013. **34**(2): p. 309-313.
146. Chen, X., et al., *Plasma miRNAs in predicting radiosensitivity in non-small cell lung cancer.* Tumor Biology, 2016. **37**(9): p. 11927-11936.
147. Kjersem, J., et al., *Plasma microRNAs predicting clinical outcome in metastatic colorectal cancer patients receiving first-line oxaliplatin-based treatment.* Molecular oncology, 2014. **8**(1): p. 59-67.
148. Weirauch, U., et al., *Functional role and therapeutic potential of the pim-1 kinase in colon carcinoma.* Neoplasia, 2013. **15**(7): p. 783-IN28.

149. He, J., et al., *Overexpression of microRNA-122 re-sensitizes 5-FU-resistant colon cancer cells to 5-FU through the inhibition of PKM2 in vitro and in vivo.* Cell biochemistry and biophysics, 2014. **70**(2): p. 1343-1350.
150. Khoshinani, H.M., et al., *Involvement of miR-155/FOXO3a and miR-222/PTEN in acquired radioresistance of colorectal cancer cell line.* Japanese journal of radiology, 2017. **35**(11): p. 664-672.
151. Iorio, M.V., et al., *microRNA-205 regulates HER3 in human breast cancer.* Cancer research, 2009. **69**(6): p. 2195-2200.
152. Meng, F., et al., *Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines.* Gastroenterology, 2006. **130**(7): p. 2113-2129.
153. Tan, W., et al., *MicroRNAs and cancer: Key paradigms in molecular therapy.* Oncology letters, 2018. **15**(3): p. 2735-2742.
154. Mandujano-Tinoco, E.A., et al., *New emerging roles of microRNAs in breast cancer.* Breast cancer research and treatment, 2018. **171**(2): p. 247-259.
155. Fu, S.W., L. Chen, and Y.-g. Man, *miRNA biomarkers in breast cancer detection and management.* Journal of Cancer, 2011. **2**: p. 116.
156. Gandellini, P., E. Giovannetti, and F. Nicassio, *MicroRNAs in cancer management: Big challenges for small molecules*. 2015, Hindawi.
157. Tsai, H.-P., et al., *Differential microRNA expression in breast cancer with different onset age.* PLoS One, 2018. **13**(1): p. e0191195.
158. Kolesnikov, N., et al., *Expression of micrornas in molecular genetic breast cancer subtypes.* Cancer treatment and research communications, 2019. **20**: p. 100026.
159. Amini, S., et al., *Expression analysis of MicroRNA-222 in breast cancer.* Clin Lab, 2018. **64**(4): p. 491-6.
160. Tfaily, M.A., et al., *miRNA expression in advanced Algerian breast cancer tissues.* Plos one, 2020. **15**(2): p. e0227928.
161. Saleh, A.A., et al., *Potential value of circulatory microRNA122 gene expression as a prognostic and metastatic prediction marker for breast cancer.* Molecular biology reports, 2019. **46**(3): p. 2809-2818.
162. Li, X., et al., *Plasma-based microRNA signatures in early diagnosis of breast cancer.* Molecular genetics & genomic medicine, 2020: p. e1092.
163. El Saghir, N.S., et al., *Effects of young age at presentation on survival in breast cancer.* BMC cancer, 2006. **6**(1): p. 194.
164. Nassar, F.J., et al., *miRNA as potential biomarkers of breast cancer in the Lebanese population and in young women: a pilot study.* PloS one, 2014. **9**(9): p. e107566.
165. Nassar, F.J., et al., *microRNA Expression in Ethnic Specific Early Stage Breast Cancer: An Integration and Comparative Analysis.* Scientific reports, 2017. **7**(1): p. 16829.
166. Müller, V., et al., *Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: a*

translational research project within the Geparquinto trial. Breast cancer research and treatment, 2014. **147**(1): p. 61-68.
167. Wang, F., et al., *Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor.* Gynecologic oncology, 2010. **119**(3): p. 586-593.
168. Zeng, R.-c., et al., *Down-regulation of miRNA-30a in human plasma is a novel marker for breast cancer.* Medical oncology, 2013. **30**(1): p. 477.
169. El Asmar, M., et al., *Knowledge, Attitudes and Practices Regarding Breast Cancer amongst Lebanese Females in Beirut.* Asian Pacific journal of cancer prevention: APJCP, 2018. **19**(3): p. 625.
170. Karimi, A., et al., *Predictive factors of survival time of breast cancer in kurdistan province of Iran between 2006-2014: a cox regression approach.* Asian Pac J Cancer Prev, 2014. **15**(19): p. 8483-8.
171. Asiaf, A., et al., *MicroRNAs in breast cancer: diagnostic and therapeutic potential*, in *MicroRNA and Cancer*. 2018, Springer. p. 23-43.
172. Bertoli, G., C. Cava, and I. Castiglioni, *MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer.* Theranostics, 2015. **5**(10): p. 1122.
173. Cui, C. and Q. Cui, *the relationship of human tissue microRnAs with those from body fluids.* Scientific reports, 2020. **10**(1): p. 1-7.
174. Motamedi, M., et al., *Plasma Level Of miR-21 And miR-451 In Primary And Recurrent Breast Cancer Patients.* Breast Cancer: Targets and Therapy, 2019. **11**: p. 293.
175. Kumar, S., et al., *Overexpression of circulating miRNA-21 and miRNA-146a in plasma samples of breast cancer patients.* 2013.
176. Ng, E.K., et al., *Circulating microRNAs as specific biomarkers for breast cancer detection.* PloS one, 2013. **8**(1): p. e53141.
177. Soleimanpour, E., et al., *Circulating miR-21 and miR-155 as potential noninvasive biomarkers in Iranian Azeri patients with breast carcinoma.* Journal of cancer research and therapeutics, 2019. **15**(5): p. 1092.
178. Matamala, N., et al., *Tumor microRNA expression profiling identifies circulating microRNAs for early breast cancer detection.* Clinical chemistry, 2015. **61**(8): p. 1098-1106.
179. Khalighfard, S., et al., *Plasma miR-21, miR-155, miR-10b, and Let-7a as the potential biomarkers for the monitoring of breast cancer patients.* Scientific reports, 2018. **8**(1): p. 1-11.
180. Dai, X., et al., *miR‐21 is involved in transforming growth factor β1‐induced chemoresistance and invasion by targeting PTEN in breast cancer.* Oncology letters, 2017. **14**(6): p. 6929-6936.
181. Petrovic, N., *miR-21 might be involved in breast cancer promotion and invasion rather than in initial events of breast cancer development.* Molecular diagnosis & therapy, 2016. **20**(2): p. 97.

182. Zhang, W., C. Chen, and G. Guo, *MiR-155 promotes the proliferation and migration of breast cancer cells via targeting SOCS1 and MMP16.* Eur Rev Med Pharmacol Sci, 2018. **22**(21): p. 7323-32.
183. Wang, W., et al., *Involvement of miR-451 in resistance to paclitaxel by regulating YWHAZ in breast cancer.* Cell death & disease, 2017. **8**(10): p. e3071-e3071.
184. Ding, Y., et al., *miR-145 inhibits proliferation and migration of breast cancer cells by directly or indirectly regulating TGF-β1 expression.* International Journal of Oncology, 2017. **50**(5): p. 1701-1710.
185. Gu, X., et al., *Influence of MiR-451 on drug resistances of paclitaxel-resistant breast cancer cell line.* Medical science monitor: international medical journal of experimental and clinical research, 2015. **21**: p. 3291.
186. Mar-Aguilar, F., et al., *Serum circulating microRNA profiling for identification of potential breast cancer biomarkers.* Disease markers, 2013. **34**(3): p. 163-169.
187. Luo, J., et al., *A novel panel of microRNAs provides a sensitive and specific tool for the diagnosis of breast cancer.* Molecular medicine reports, 2014. **10**(2): p. 785-791.
188. Pan, Y., et al., *MicroRNA-130a inhibits cell proliferation, invasion and migration in human breast cancer by targeting the RAB5A.* International journal of clinical and experimental pathology, 2015. **8**(1): p. 384.
189. Chen, X., et al., *MicroRNA-130a suppresses breast cancer cell migration and invasion by targeting FOSL1 and upregulating ZO-1.* Journal of cellular biochemistry, 2018. **119**(6): p. 4945-4956.
190. Eissa, S., M. Matboli, and H.H. Shehata, *Breast tissue–based microRNA panel highlights microRNA-23a and selected target genes as putative biomarkers for breast cancer.* Translational Research, 2015. **165**(3): p. 417-427.
191. Li, X., et al., *Plasma-based microRNA signatures in early diagnosis of breast cancer.* Molecular genetics & genomic medicine, 2020. **8**(5): p. e1092.
192. Stückrath, I., et al., *Aberrant plasma levels of circulating miR-16, miR-107, miR-130a and miR-146a are associated with lymph node metastasis and receptor status of breast cancer patients.* Oncotarget, 2015. **6**(15): p. 13387.
193. Ferracin, M., et al., *Absolute quantification of cell-free microRNAs in cancer patients.* Oncotarget, 2015. **6**(16): p. 14545.
194. Krishnan, K., et al., *miR-139-5p is a regulator of metastatic pathways in breast cancer.* Rna, 2013. **19**(12): p. 1767-1780.
195. Xiao, S., et al., *miR-425-5p is associated with poor prognosis in patients with breast cancer and promotes cancer cell progression by targeting PTEN.* Oncology reports, 2019. **42**(6): p. 2550-2560.
196. Zhao, H., et al., *A pilot study of circulating miRNAs as potential biomarkers of early stage breast cancer.* PloS one, 2010. **5**(10): p. e13735.
197. Nadeem, F., et al., *Clinicopathological features associated to MiRNA-195 expression in patients with breast cancer: Evidence of a potential biomarker.* Pakistan journal of medical sciences, 2017. **33**(5): p. 1242.

198. McAnena, P., et al., *Circulating microRNAs miR-331 and miR-195 differentiate local luminal a from metastatic breast cancer.* BMC cancer, 2019. **19**(1): p. 436.
199. Akoglu, H., *User's guide to correlation coefficients.* Turkish journal of emergency medicine, 2018. **18**(3): p. 91-93.
200. Chan, Y., *Biostatistics 104: correlational analysis.* Singapore Med J, 2003. **44**(12): p. 614-9.
201. Fangyu, L. and H. Hua, *Assessing the accuracy of diagnostic tests.* Shanghai archives of psychiatry, 2018. **30**(3): p. 207.
202. Huggett, J., et al., *Real-time RT-PCR normalisation; strategies and considerations.* Genes & Immunity, 2005. **6**(4): p. 279-284.
203. Hu, Z., et al., *Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls.* Carcinogenesis, 2012. **33**(4): p. 828-834.
204. Chen, H., et al., *Evaluation of plasma miR-21 and miR-152 as diagnostic biomarkers for common types of human cancers.* Journal of cancer, 2016. **7**(5): p. 490.