Supplementary Tables

Table S1: Primer sequences used in this study to amplify miR-1271-5P, miR-646 and miR-135a-5p in RT-PCR.

miR-646	F: AAGCAGCTGCCTCTGA R: TCCAGTTTTTTTTTTTTTTGCCT
miR-1271-5p	F: TGGCACCTAGCAAGCA R: GGTCCAGTTTTTTTTTTTTTTGAGT
miR-135a-5p	F: CGCAGTATGGCTTTTTATTCCT R: GGTCCAGTTTTTTTTTTTTTTCACA

Table S2: The demographic features and the laboratory assessment of the enrolled subjects.

	Control	Benign	Breast cancer cases	Test of Sig.	P-Value
Age (years) Mean ±SD.	52.92± 11.31	44.67± 11.06	53.64± 12.84	ANOVA	**P= 0.007216***
				Post hoc test (Tukey) between BC and benign	P=**0.005110***
White cell count (10^3/cmm)	6.172± 2.790	7.450± 2.374	7.040± 2.215	ANOVA	P=0.2107
Lymphocyte count (10^3/cmm)	1789± 650.1	2536± 868.5	2390± 835.3	ANOVA	P=**0.0090***
				Post hoc test (Tukey) between BC and Control	**P=0.013656***
				Post hoc test (Tukey) between benign and control	**P=0.013124***
Lymphocytes %	31.83± 12.28	32.82± 10.63	34.32± 8.409	ANOVA	P=0.5110

A sample of 160 women, 111 BC patients (the mean age ± SD = 53.64±12.84), 25 benign cases (the mean age ± SD = 44.67±11.06), and 24 healthy controls (the mean age ± SD = 52.92±11.31) participated in this work. Using one-way ANOVA to compare the demographic and laboratory characteristics among the study participants, a major age difference between the groups (P=0.0072) was observed, and post hoc Tukey analysis showed that the BC patients were significantly older than the benign group (P=0.0051). White cell count didn't differ significantly between the groups (P = 0.2107). Conversely, lymphocyte count varied significantly across groups (P = 0.0090), with post hoc analysis having significantly higher values in breast cancer (P=0.0137) and benign groups (P=0.0131) relative to controls. Percentage lymphocytes did not differ significantly across groups (P=0.5110) as shown in **Table S.**

Supplementary Tables

Table S3: Presents a comparison of benign and breast cancer cases based on clinical and demographic characteristics.

SD : Standard deviation, t: Student t-test, $\chi 2$: Chi square test, MC: Monte Carlo, FE: Fisher Exact, p: p value for comparing between the studied groups, * : Statistically significant at $p \leq 0.05$, BMI: body mass index.

	Benign	Breast cancer cases	Test of Sig.	P-Value
Parity				
Nullipara	8 (32%)	10 (9%)	$\chi 2$ =10.740539	^{MC}**p =0.013400***
Primipara (1)	0 (0%)	9 (8.1%)		
Multipara (2-4)	14(56%)	76 (68.5%)		
Grand multipara ≥ 5	3(12%)	16(14.4%)		
Family history				
Negative	20(80%)	62 (55.9%)	$\chi 2$ =4.968416	^{FE}**p=0.040147***
Positive	5(20%)	49 (44.1%)		
Oral contraceptive				
Positive	3(12%)	29(26.1%)	$\chi 2$ =2.262897	^{FE}p=0.191928
Negative	22(88%)	82(73.9%)		
Menstrual Status				
Premenopausal	17 (68%)	57 (51.4%)	$\chi 2$ =2.280008	^{FE}p= 0.182205
Postmenopausal	8 (32%)	54 (48.6%)		
Performance status (ECOG)				
0	17 (68%)	44 (39.6%)	$\chi 2$ =8.301971	^{MC}**p=0.040166***
1	8 (32%)	50 (45%)		
2	0 (0%)	12 (10.8%)		
3	0 (0%)	5 (4.5%)		
BMI (Kg/m2) Mean ±SD	32.73± 7.938	33.42± 7.259	T test	P=0.6853

A comparison of clinical and demographic characteristics between breast cancer and benign groups showed significant differences. The parity distribution was significantly different (χ^2 = 10.74, MCp = 0.013), with a greater proportion of nulliparous women in the benign group (32%) compared to the breast cancer group (9%). Conversely, multiparity (2–4 children) was higher among the breast cancer patients (68.5%) compared to benign cases (56%). In addition, a positive family history for breast cancer was more prevalent in the cancer group (44.1%) than in the benign group (20%) (χ^2 = 4.97, FEp = 0.040). The performance status, based on the ECOG scale, was also significantly poorer in the breast cancer group (χ^2 = 8.30, MCp = 0.040), and the 2–3 scores were seen only in such patients. Body mass index, oral contraceptive, and Menstrual status did not differ significantly between the benign and breast cancer groups, with a P-value > 0.05. These findings are presented in **Supplementary Tables Table S.**

Supplementary Tables

Table S4: Presents the allocation of breast cancer patients included (n = 111) based on various clinical and pathological parameters.

Parameter	No. (%)
Performance status ECOG	
0	44 (39.6%)
≥1	67 (60.4%)
Tumor Side	
Right	50 (45%)
Left	61 (55%)
Pathological Subtype	
DCIS	15(13.5%)
IDC	75(67.6%)
ILC	9 (8.1%)
Mixed	9(8.1%)
Other Types	3 (2.7%)
Stages	
Stage Zero	15 (13.5%)
Stage I (IA, IB)	30(27%)
Stage II (IIA, IIB)	23 (20.7%)
Stage III (IIIA, IIIB, IIIC)	28 (25.2%)
Stage IV	15 (13.5%)
Metastasis status	
No	96 (86.5%)
Yes	15 (13.5%)
Grade	
Grade I	7 (6.3%)
Grade II	68 (61.3%)
Grade III	36 (32.4%)
T status	
Tis	15 (13.5%)
T1	12 (10.8%)
T2	37 (33.3%)
T3	24 (21.6%)
T4	23 (20.7%)
N status	
N0	39 (35.1%)
N1	38 (34.2%)
N2	18 (16.2%)
N3	16(14.4%)
ER Status	
ER+	85 (76.6%)
ER-	26 (23.4%)
PR Status	
PR+	83 (74.8%)
PR-	28 (25.2%)
HER2 Status	
HER2 +	5 (4.5%)
HER2-	106 (95.5%)
Molecular subtype	
Luminal A	69 (62.2%)
Luminal B	16 (14.4%)
HER2 overexpressed	4 (3.6%)
Basal (triple negative)	22 (19.8%)
Relapses or progression status	
No relapse	76 (68.5%)
Relapse	35 (31.5%)

The allocation of breast cancer patients according to different criteria is shown in
. Notably, most patients had an ECOG ≥1 (60.4%), with slightly more left-sided tumors than right-sided (55%). The most predominant pathological subtype was Invasive ductal carcinoma (IDC=67.6%) followed by ductal carcinoma in situ (DCIS =13.5%). The Early stages of breast cancer (Stage zero-II) accounted for 61.2% and the late stages (III-IV)

accounted for 38.7%. the metastasis was observed in 13.5% of the patients. Most of the tumors were Grade II (61.3%), with the T2 tumor stage being the most common (33.3%), and nodal involvement occurring in two-thirds of the cases. Most tumors are estrogen and progesterone receptor-positive ER+(76.6%), PR+(74.8%), and human epidermal growth factor receptor 2 negative HER2- (95.5%). Luminal A was the most common molecular subtype (62.2%), followed by the triple-negative subtype (19.8%) and luminal B (14.4%). Moreover, upon following up relapse occurred in 31.5% of patients **(Table S3)**.

Supplementary Tables

Table S5: Univariate and multivariate logistic regression analysis of plasma miR-1271-5p, miR-646, and miR-135a-5p as predictive biomarkers for breast cancer progression.

Univariate Logistic Regression Analysis of miR-1271-5p, miR-646, and miR-135a-5p as Independent Predictors for Breast Cancer Progression: Comparison Against Controls, Benign Cases, and Between Breast Cancer Stages						
	B = Coefficient (Log-odds)	S.E. = Standard Error	p-value	Wald	Exp(B) = Odds Ratio	Odds ratio (95% CI)
miR-1271-5P						
Benign Vs Control	-3.281001	1.367651	0.01644	5.755219	0.037591	0.002576-0.548563
Benign Vs Stage I	-1.871732	0.910742	0.039862	4.223732	0.153857	0.025816-0.916943
Benign Vs Stage II	-2.062306	0.945841	0.029228	4.754117	0.12716	0.019918-0.811808
Stage III Vs Stage II	-0.275986	0.113142	0.014716	5.950186	0.758823	0.607903-0.947211
Total breast cancer cases vs Benign	1.518258	0.660198	0.021465	5.28862	4.564266	1.251438-16.646870
miR-646						
Benign Vs Control	-5.639166	2.029005	0.005448	7.724381	0.003556	0.000067-0.189685
Stage I Vs Control	-4.314371	1.438666	0.00271	8.99322	0.013375	0.000797-0.224330
Stage III vs. Stage I	1.633553	0.643388	0.011117	6.44645	5.122042	1.451410-18.075737
Benign vs. Stage III	-1.980064	0.766923	0.009828	6.665844	0.13806	0.030709-0.620690
miR-135a-5p						
Benign Vs Stage I	-0.111958	0.053148	0.035158	4.437508	0.894082	0.805634-0.992240
breast tumors (benign and malignant tumors) vs control	0.383214	0.187737	0.041228	4.16662	1.466991	1.015374-2.119480

Univariate and multivariate logistic regression analysis to determine the discriminative ability of the researched miRNAs in discriminating initial-stage (stages 0–II) and advanced-stage (stages III–IV) breast cancer.							
	parameter	B-Coefficient	S.E. = Standard Error	p-value	Wald	Exp(B) = Odds Ratio	Odds ratio (95% CI)
Univariate	miR-646	0.6961	0.259108	0.00722	7.217402	2.005915	1.207144-3.333235
	miR-1271-5p	-0.215139	0.081279	0.008123	7.006186	0.806429	0.687672-0.945695
	miR-135a-5p	-0.073284	0.031613	0.020441	5.373873	0.929336	0.873502-0.988740
Multivariate	miR-646	0.989206	0.377483	0.008779	6.867172	2.689098	1.283194-5.635351
	miR-1271-5p	-0.197420	0.099811	0.047935	3.912252	0.820846	0.674998-0.998207
	miR-135a-5p	-0.086605	0.040682	0.033270	4.531813	0.917040	0.846758-0.993155
	Constant	-0.026668					

Univariate and multivariate logistic regression analysis between the miRNAs researched within non-malignant (control and benign) and malignant (stages 0–IV) breast cancer groups.							
	parameter	B-Coefficient	S.E. = Standard Error	p-value	Wald	Exp(B) = Odds Ratio	Odds ratio (95% CI)
Univariate	miR-646	0.261559	0.234426	0.264533	1.244879	1.298954	0.820445-2.056543
	miR-1271-5p	1.041568	0.370393	0.004923	7.907674	2.833656	1.371096-5.856343
	miR-135a-5p	0.108699	0.036504	0.002904	8.866698	1.114827	1.037851-1.197513
Multivariate	miR-1271-5p	1.183937	0.400570	0.003120	8.735765	3.267212	1.490087-7.163790
	miR-135a-5p	0.126491	0.043920	0.003976	8.294563	1.134840	1.041237-1.236857
	Constant	-1.826191					

Univariate and multivariate logistic regression tests revealed significant predictor miRNAs for breast diagnosis and staging. Univariate logistic regression with plasma expression data indicated highly significant differences in miRNA expression levels by disease states. For miR-1271-5p, plasma expression was highly reduced in benign cases compared to controls, with an odds ratio (OR) of 0.0376(p=0.016, 95% CI: 0.0026–0.5486), representing downregulation in benign samples. Similarly, benign cases expressed lower miR-1271-5p than Stage I and Stage II BC cases, with ORs of 0.1539 (p = 0.0399, 95% CI: 0.0258–0.9169) and 0.1272 (p = 0.0292, 95% CI: 0.0199–0.8118), respectively. Furthermore, Stage III patients expressed significantly lower levels of miR-1271-5p than Stage II (OR =

0.7588,p=0.0147, 95% CI: 0.6079–0.9472), showing a decline in expression of miR-1271-5p at later stages of cancer. In comparison with benign cases to all BC cases (stages combined), plasma miR-1271-5p was markedly elevated in cancer (OR = 4.5643,p=0.0215, 95% CI: 1.2514–16.6469). For miR-646, plasma levels were significantly reduced in benign and Stage I patients relative to controls, at ORs of 0.0036 (p = 0.0054, 95% CI: 0.00007–0.1897) and 0.0134 (p=0.0027, 95% CI: 0.0008–0.2243), respectively, indicating extreme downregulation very early in the disease process. Stage III cases were notably associated with much higher plasma miR-646 levels than Stage I (OR = 5.1220, p = 0.0111, 95% CI: 1.4514–18.0757), which is consistent with upregulation with increasing disease. Stage III again had higher expression than benign (OR = 0.1381, p = 0.0098, 95% CI: 0.0307–0.6207), also in line with its association with disease. For miR-135a-5p, plasma levels were significantly lower in benign cases relative to Stage I patients, indicated by an OR of 0.8941 (p = 0.0352, 95% CI: 0.8056–0.9922).

Additionally, when controls were compared to the tumor group, which included all tumor-bearing individuals (benign and malignant), miR-135a-5p was modestly but significantly increased in the tumor group (OR = 1.4670, p = 0.0412, 95% CI: 1.0154–2.1195). These findings are illustrated in **Errore. L'origine riferimento non è stata trovata.** In stepwise multivariate analysis, a three-miRNA-panel (miR-1271-5p, miR-646, and miR-135a-5p) was significant in discriminating early stages (0, I, II) from late stages (III, IV) of BC **(**Errore. L'origine riferimento non è stata trovata.

Supplementary Tables

Table S6: Diagnostic accuracy of two panels of miRNAs. Panel 1 (miR-1271-5p, miR-646, miR-135a-5p) to distinguish between early and late stages of breast cancer. Panel 2 (miR-1271-5p, miR-135a-5p) to differentiate between malignant and non-malignant cases.

miRNA panels	P value	AUC	Sensitivity %	Specificity%	Std. Error	95% confidence interval	best cutoff
miRNA panel (1)							
Early stages vs late stages	**0.0001**	**0.7625**	**75.86**	**63.27**	**0.05461**	**0.6555 to 0.8695**	**> -0.2659**
miRNA panel (2)							
non-malignant vs malignant	**0.0006**	**0.7005**	**62.82**	**94.44**	**0.04855**	**0.6054 to 0.7956**	**> 0.3816**

The logit equation was used to describe the expected probability of distinguishing between early and late-stage BC using the three-miRNA panel (miR-1271-5p, miR-646, and miR-135a-5p): Logit(P) = –0.0267 + 0.9892 miR-646 – 0.1974 miR-1271-5p – 0.0866 miR-135a-5p. The ROC curve generated by this model showed strong diagnostic performance, with an AUC of 0.7625 (95% CI = 0.6555 to 0.8695), sensitivity 75.86%, and specificity 63.27% **(**Errore. L'origine riferimento non è stata trovata.**).** In the multivariate analysis of malignant vs. non-malignant breast diseases, co-inclusion of miR-1271-5p and miR-135a-5p demonstrated significant diagnostic value. miR-1271-5p was highly upregulated in malignancy (OR = 3.2672, p = 0.0031; 95% CI: 1.4901–7.1638), while miR-135a-5p showed moderate but significant increase (OR = 1.1348, p = 0.0040; 95% CI: 1.0412–1.2369). Together, the miRNA pair achieved an AUC of 0.7005 (95% CI: 0.6054–0.7956) , sensitivity of 62.82%, and specificity of 94.44% in distinguishing between malignant and non-malignant breast disease **(**Errore. L'origine riferimento non è stata trovata.**)**. Comparison of the AUCs for individual miRNAs and their combinations revealed that the miRNA panel outperformed individual miRNAs in differentiating between early and late-stage BC.

Supplementary Tables

Supplementary Table S7: Age-adjusted binary logistic regression analysis of circulating miRNAs for the principal diagnostic comparisons.

Groups	Parameter	B = Coefficient (Log-odds)	*P*value	Exp(B) = Odds Ratio	Odds ratio (95% CI)
Healthy VS Breast cancer after age adjustment	AGE	-0.005186	0.826222	0.994827	0.949816-1.041971
	miR-1271-5p	0.738269	0.037577	2.092311	1.043328-4.195963
	AGE	0.00083	0.969446	1.00083	0.959212-1.044254
	miR-646	-0.046763	0.830364	0.954314	0.622146-1.463829
	AGE	0.008838	0.707021	1.008123	0.966478-1.051563
	miR-135a-5p	0.344552	0.048988	1.406346	1.001514-1.974820
Benign vs Breast cancer after age adjustment	AGE	0.018713	0.488328	1.018889	0.966365-1.074268
	miR-1271-5p	1.451814	0.029929	4.270855	1.151594-15.839096
	AGE	0.046275	0.097427	1.043974	0.992176-1.098476
	miR-646	1.022097	0.041735	3.656244	1.049703-12.735142
	AGE	0.048471	0.057306	1.049665	1.049665-1.103455
	miR-135a-5p	0.045613	0.217904	1.046669	0.973415-1.125435

Binary logistic regression analyses adjusted for age were performed to assess the independent association between circulating miRNA expression and breast cancer diagnosis. Results are presented as regression coefficients (B), odds ratios (Exp(B)), 95% confidence intervals (CI), and corresponding P-values. Comparisons were performed between healthy controls and breast cancer patients and between benign breast disease and breast cancer patients. Statistically significant associations ($P < 0.05$) are highlighted in bold.

Supplementary Tables

Table S8: Univariate logistic regression analysis to identify the variables affecting recurrence in breast cancer patients

	Relapse	
Variable	**P-value**	**OR (95% CI)**
MiR-1271-5p	**0.032021***	**0.843499** (0.721968-0.985487)
MiR-646	**0.009922***	**1.880201** (1.163536-3.038287)
MiR-135a-5p	0.773962	1.007426 (0.957821-1.059600)
Age (years)	0.093274	0.971616 (0.939488-1.004843)
Parity (Para)	0.417746	0.515152 (0.103560-2.562584)
Menstrual status (Postmenopausal)	0.408220	0.711539 (0.317634-1.593934)
Family history	0.158124	1.818376 (0.792632-4.171532)
BMI (Kg/m²)	0.349189	1.026721 (0.971573-1.085001)
Performance status ECOG	0.193640	1.713992 (0.760626-3.862302)
Pathological Subtype (IDC)	**0.035379***	**9.333333** (1.165213-74.759804)
Pathological Stage (≥3)	**0.004594***	**21.411764** (2.573022-178.181003)
T3 (PT_Status (3))	**0.026379***	**11.846153** (1.336602-104.991147)
T4 (PT_Status (4))	**0.033405***	**10.769230** (1.205425-96.211998)
PN status (≥3)	0.381839	1.740000 (0.502839-6.021009)
ER	**0.000351***	**0.179924** (0.070245-0.460853)
PR	**0.001169***	**0.222656** (0.089905-0)551423)
HER2 neu	0.575997	0.529412 (0.056987-4.918229)

OR: Odds ratio, C.I.: Confidence interval, *: Statistically significant at p ≤0.05, IDC: Invasive ductal carcinoma, BMI: body mass index, PT_Status: pathological tumor status, PN status: pathological nodal status, ER: estrogen receptor, PR: progesterone receptor, HER2 neu: human epidermal growth factor receptor 2.

Based on the univariate logistic model, several variables were statistically significant with recurrence in patients with breast cancer. Among microRNAs studied, miR-1271-5p demonstrated a statistically significant negative relation with recurrence(P=0.032), which suggests a potential protective role, where higher levels have less risk of relapse. On the other hand, miR-646 was significantly correlated with higher recurrence risk odds (P = 0.0099), suggesting that it may have an oncogenic role to play. Certain clinicopathological characteristics were also significantly associated with recurrence risk. These were the IDC type(P=0.035), high pathology stage (Stage ≥ III)(P=0.0046), and the larger tumor size groups, such as T3 (P = 0.026) and T4 (P = 0.033), all of which increased relapse risk significantly. Moreover, hormone receptor status was protective, and both estrogen receptor (ER) and progesterone receptor (PR) positivity reduced recurrence risk significantly(P=0.00035 and P=0.00117, respectively).

In contrast, several variables were not statistically significant and were not considered to be independent predictors of recurrence. They included miR-135a-5p, age, parity, menstrual status, family history, body mass index (BMI), ECOG performance status, PN status, and HER2 neu expression. While several of these still may take some part in the development or prognosis of breast cancer, they were not independent predictors of recurrence in this research. These findings are shown in Errore. L'origine riferimento non è stata trovata..

Supplementary Tables

Table S9: Shows the relation between miR-1271-5p, miR-646, and miR-135a-5p expression levels and clinicopathological characteristics in breast cancer patients (n = 111).

Category	N	MiR-1271-5p (Mean ± SD)	Test of Sig. (MiR-1271-5p)	P (MiR-1271-5p)	MiR-646 (Mean ± SD)	Test of Sig. (MiR-646)	P (MiR-646)	MiR-135a-5p (Mean ± SD)	Test of Sig. (MiR-135a-5p)	P (MiR-135a-5p)
Parity										
Nullipara	10	5.333± 4.370	H=3.850	P=0.2781	1.253± 1.528	H=0.1370	P=0.9871	10.88± 14.37	H=1.994	P=0.5736
Primipara (1)	9	0.8910± 0.3106			0.6200± 0.6433			4.000± 7.969		
Multipara (2-4)	76	4.646± 4.596			1.001± 1.425			10.29± 10.94		
Grand multipara ≥ 5	16	2.992± 2.963			0.7087± 0.7218			10.39± 9.900		
Family history										
Negative	62	4.098± 4.184	U=515	P=0.8867	1.001± 1.094	U=595	P=0.1242	11.43± 10.63	U=335	P=0.1025
Positive	49	4.429± 4.575			0.8500± 1.498			7.892± 11.08		
Oral contraceptive										
Positive	29	4.146± 3.438	U=406	P=0.5741	0.8868± 1.109	U=550.5	P=0.5933	5.995± 6.433	U=205	P=0.0725
Negative	82	4.269± 4.658			0.9533± 1.345			10.90± 11.66		
Performance status (ECOG)										
0	44	4.563± 4.583	H=0.4884	P=0.9214	0.8982± 1.177	H=2.757	P=0.4306	11.61± 11.11	H=0.7744	P=0.8556
1	50	3.859± 4.157			1.070± 1.435			8.892± 10.79		
2	12	4.122± 4.851			0.5639± 0.8389			8.775± 9.036		
3	5	4.911± 3.739			0.8748± 1.657			9.712± 17.05		
Pathological Subtype										
DCIS	15	4.986 ± 5.335	H=1.105	P=0.8934	0.6712± 0.9085	H=6.009	P=0.1985	14.97±16.23	H=2.738	P=0.6025
IDC	75	4.082 ± 4.265			1.182± 1.473			8.586±10.40		
ILC	9	3.932 ± 3.979			0.5367±0.7090			7.927±8.614		
Mixed	9	5.272 ±5.565			0.3863±0.3668			15.86±4.5		
Other Types	3	3.155 ±0.006364			0.076±0.005657			11.17±15.40		
Metastasis status										
No	96	4.559±4.427	U=118	P=0.0661	0.9616±1.325	U=282.5	P=0.6692	11.65±11.05	U=49	**P<0.0001***
Yes	15	1.489 ±1.612			0.7348±0.8850			0.78±1.638		
Grade										
Grade I	7	5.752 ±5.625	H=1.349	P=0.5093	0.3281±0.3935	H=8.678	**P=0.0130***	13.83±22.77	H=3.426	P=0.1803
Grade II	68	4.595±4.668			0.7266±0.9887			11.26±10.38		

Grade III	36	3.354±3.430			1.641±1.784			5.776±9.178		
T status										
Tis	15	4.986±5.335	H=2.468	P=0.6504	0.6712±0.9085	H=5.047	P=0.2825	14.97±16.23	H=7.359	P=0.1181
T1	12	6.638±7.323			0.3711±0.4714			14.57±10.84		
T2	37	4.264±4.162			0.8348±0.9591			11.11±9.576		
T3	24	3.821±3.609			1.315±2.110			9±10.23		
T4	23	3.005±3.260			1.258±1.212			5.401±9.771		
N status										
N0	39	4.567±4.373	H=7.080	P=0.0694	0.6422±0.9214	H=5.584	P=0.1337	15.84±12.78	H=12.12	**P=0.0070***
N1	38	5.533±4.859			1.101±1.767			9.235±9.363		
N2	18	2.5±3.211			1.091±1.297			4.132±5.975		
N3	16	2.518±3.133			1.069±0.6759			4.076±6.740		
ER Status										
ER+	85	4.774±4.510	U=305	P=0.1040	0.8757±1.279	U=367	P=0.1839	10.52±11.40	U=223	P=0.3847
ER-	26	2.675±3.349			1.186±1.293			6.79±7.956		
PR Status										
PR+	83	4.841±4.533	U=316	P=0.0966	0.8772±1.297	U=402	P=0.1608	10.41±11.31	U=259	P=0.4137
PR-	28	2.613±3.260			1.144±1.227			7.767±9.302		
HER2 Status										
HER2 +	5	4.07±2.776	U=119	P=0.9071	2.181±2.160	U=91	P=0.2077	1.45±1.741	U=34	P=0.3661
HER2-	106	4.244±4.413			0.8681±1.202			10.13±10.96		
Molecular subtype										
Luminal A	69	4.581±4.676	H=3.785	P=0.2857	0.6494±0.8223	H=7.216	P=0.0653	11.13±11.33	H=2.489	P=0.4773
Luminal B	16	5.634±3.796			2.233±2.404			7.373±11.97		
HER2 overexpressed	4	3.057±2.325			1.788±2.465			0.8641±0.9123		
Basal (triple negative)	22	2.593±3.596			1.036±0.9478			7.447±8.066		
Menstrual status										
Premenopausal	57	4.216±4.187	U=512	P=0.7581	0.9188±1.056	U=737.5	P=0.8557	8.045±11.20	U=342	P=0.1266
Postmenopausal	54	4.256±4.552			0.9548±1.514			11.3±10.57		
Progression status										
No relapse	76	5.106± 4.831	U=349	P=0.0505	0.6351± 0.8220	U=418	**P=0.0057***	9.574± 10.89	U=340	P=0.5486
Relapse	35	2.603± 2.495			1.536± 1.760			10.45± 11.18		

SD: Standard deviation, U: Mann-Whitney test, H: H for Kruskal Wallis test, p: p value for comparing between different categories, *: Statistically significant at $p \leq 0.05$

The correlation between circulating miRNA levels and clinicopathological characteristics revealed that miR-646 expression was notably related to tumor grade($p=0.0130$) and increased progressively from low to high grade. Furthermore, it was significantly elevated in patients with relapse of the disease ($p = 0.0057$). For miR-135a-5p, its expression was significantly decreased when lymph node involvement was more ($p = 0.0070$) and was significantly decreased in the presence of distant metastasis($p<0.0001$). miR-1271-5p was borderline significant when associated with progression status, with higher expression observed among non-relapsing patients ($p = 0.0505$). No statistically meaningful associations were identified for the degree of expression of miR-1271-5p, miR-646, or miR-135a-5p with parity, family history, oral contraceptive use, ECOG performance status, pathological

subtype, molecular subtype, ER status, PR status, HER2 status, or menstrual status($p>0.05$ for all) as shown in Errore. L'origine riferimento non è stata trovata. **S7**.

Supplementary Tables

Table S10: Summarizes target prediction analyses of the three studied miRNAs.

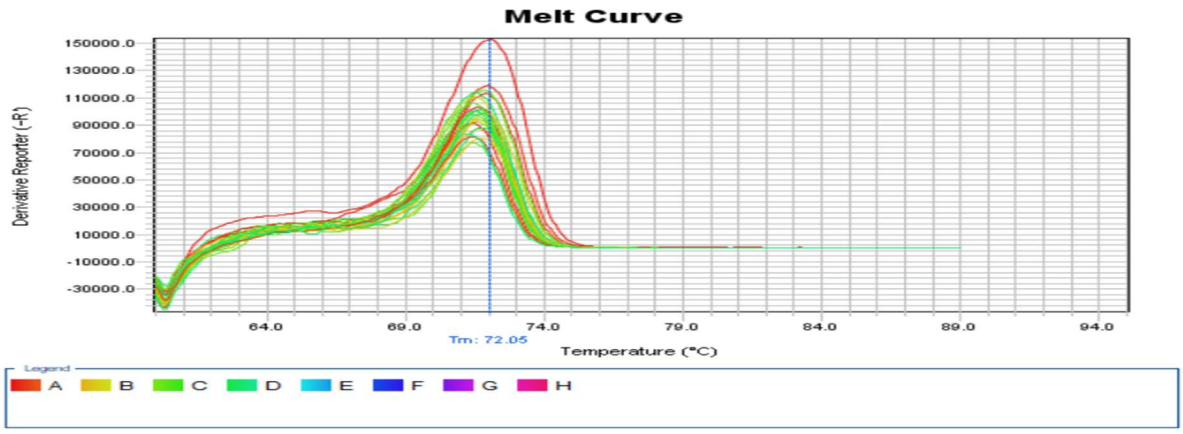
miRNAs	Targets	Expression in benign tumors	Expression in Breast Cancer (Stages 0–IV)
miR-1271-5p	**SPIN1**(spindlin 1)	not reported	Upregulated in Stage IV (X. Chen et al., 2016a)
	HAS2 (hyaluronan synthase 2)	Mildly expressed in benign (Lien et al., 2014).	absent in Stage 0, moderately upregulated in Stage I–II IDC (triple-negative, basal-like), and strongly upregulated in Stage III-IV, especially in triple-negative, basal-like, and metaplastic carcinomas. (Bernert et al., 2011; Lien et al., 2014; Okuda et al., 2012).
	ITPR1(inositol 1,4,5-trisphosphate receptor type 1)	not reported	Its expression was notably lower in breast cancer relative to normal tissue. However, its expression was relatively higher in initial stages (I-II) and significantly downregulated in late stages (III-IV) (B. Han et al., 2022) .
	MTSS1 (MTSS1, I-BAR domain containing)	not reported	Strongly downregulated in Stage III-IV (Parr & Jiang, 2009; Vadakekolathu et al., 2018) .
	CTTN(cortactin)	not reported	Upregulated in Stage III-IV(W. Li et al., 2022; Mazloomi et al., 2021).
	ZEB1(zinc finger E-box binding homeobox 1)	(downregulated) Low ZEB1 Expression (Ang et al., 2017) .	Strongly Upregulated in advanced stages III- IV (Ang et al., 2017).
miR-646	**EphB2** (EPH receptor B2)	Moderately expressed (Chukkapalli et al., 2014; Ebrahim et al., 2021) .	Upregulated in DCIS (higher than benign). It continues to be upregulated in the early stages (I-II) and becomes highly upregulated in Stages III and IV(Chukkapalli et al., 2014; Ebrahim et al., 2021).
	NKD1 (NKD1, WNT signaling pathway inhibitor)	not reported	Progressively downregulated from Stage I to Stage IV, with a more pronounced decrease in Stage III and a significant reduction in Stage IV(Lv et al., 2015).
	CCND2 (cyclin D2)	not reported	Strongly Upregulated in Stages III-IV (H. Zhang et al., 2020) .
	INSR (insulin receptor)	not reported	Generally upregulated in breast cancer, but expression decreases as cancer progresses from Stage I to

			Stage IV (G. Huang et al., 2020) .
miR-135a-5p	**FOXN3**(forkhead box N3)	not reported	Strongly Upregulated in Stages III-IV (W. Li et al., 2017) .
	DIP2C (disco interacting protein 2 homolog C)	Upregulated (Higher Expression) (J. Li et al., 2017).	DIP2C expression exhibits progressive downregulation from initial-stage (Stage I) to late-stage (Stage IV) breast cancer, with an increasing proportion of weak/moderate DIP2C expression in later stages (J. Li et al., 2017) .
	RUNX2 (runt related transcription factor 2)	not reported	Upregulated in Stage IV (supporting metastatic spread to the bone) (Yin et al., 2022) .
	YWHAG (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma)	not reported	Strongly Downregulated in Stage IV (Yoo et al., 2016) .
	RASAL2 (RAS protein activator like 2)	not reported	downregulated in metastatic breast cancer (Stage IV) (McLaughlin et al., 2013) .

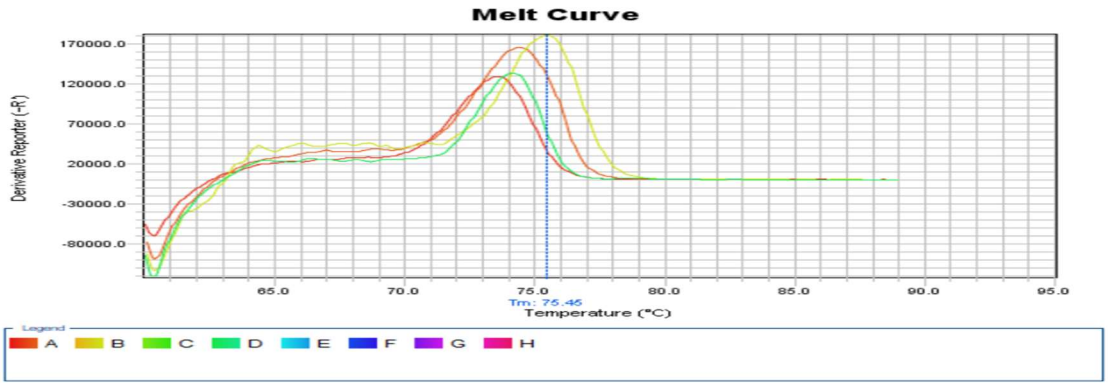
Supplementary Figures

Figure S1: Melt curves of the studied miRNAs. **(A) Melt curve for miR-135a-5p; (B) melt curve for miR-1271-5p; and (C) melt curve for miR-646. Each melt curve exhibits a single distinct peak, confirming specific amplification of the respective miRNA target.**

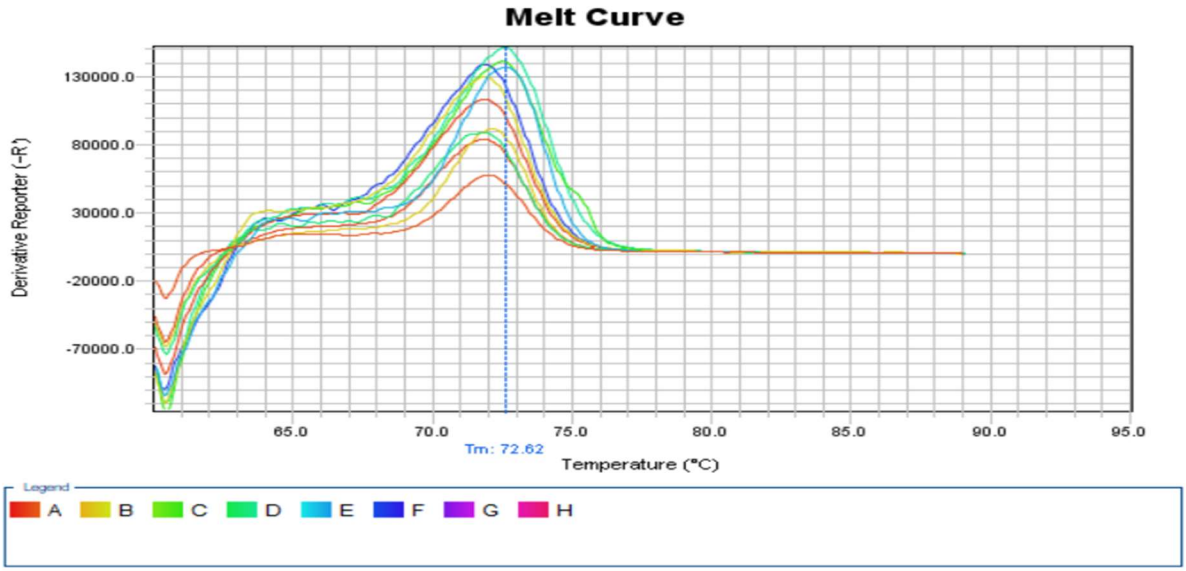
(A)



(B)

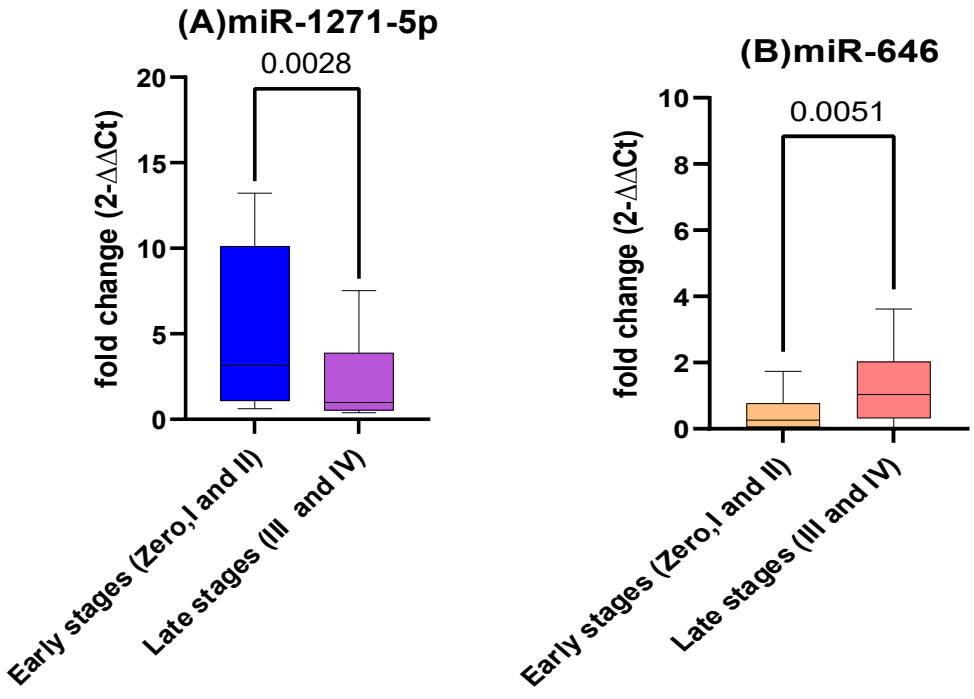


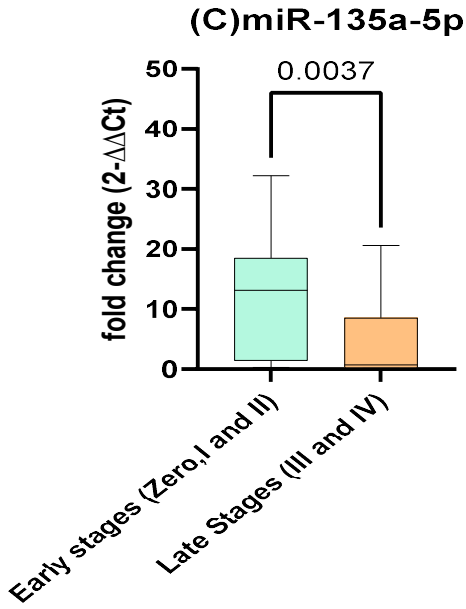
(c)



Supplementary Figures

Figure S2: shows the comparison of plasma miRNA expression in early-stage (stages 0, I, II) and advanced-stage (stages III and IV) breast cancer. Box plots show the interquartile range, with a line for the median, and whiskers for the 10th and 90th percentiles. The Mann-Whitney U-test was used to determine statistical significance(p ≤0.05). (A)miR-1271-5p, (B)miR-646, (C)miR-135a-5p.

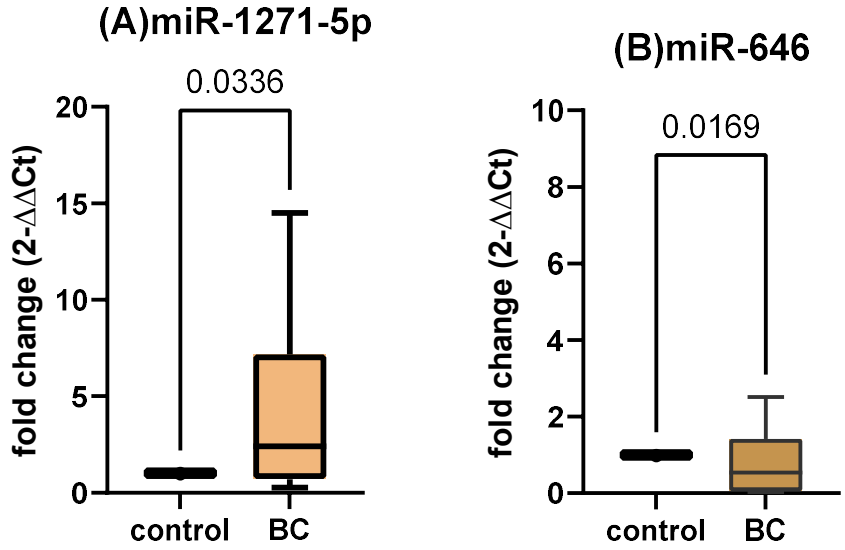


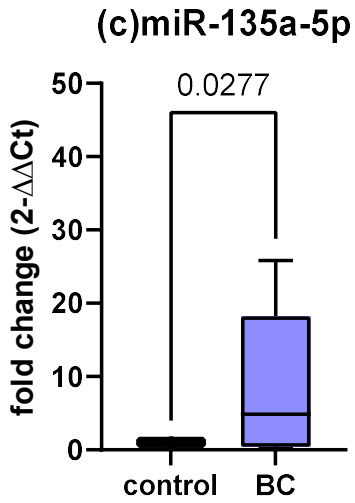


Comparing Early stages of breast cancer (0, I and II) against Late Stages of breast cancer (III and IV) revealed that miR-1271-5p and miR-135a-5p were notably upregulated in early stages of breast cancer (P = 0.0028, P = 0.0037 respectively), meanwhile, miR-646 was significantly downregulated in early stages (P = 0.0051) (**Errore. L'origine riferimento non è stata trovata.**S1).

Supplementary Figures

Figure S3: Differential expression of plasma miRNA in healthy controls and breast cancer patients. Box plots represent the distribution of fold change with interquartile ranges, median, and whiskers for the 10th and 90th percentiles. Mann-Whitney U-test was used for statistical analysis, and significance was determined at p≤0.05. (A)miR-1271-5p, (B)miR-646, (C)miR-135a-5p.

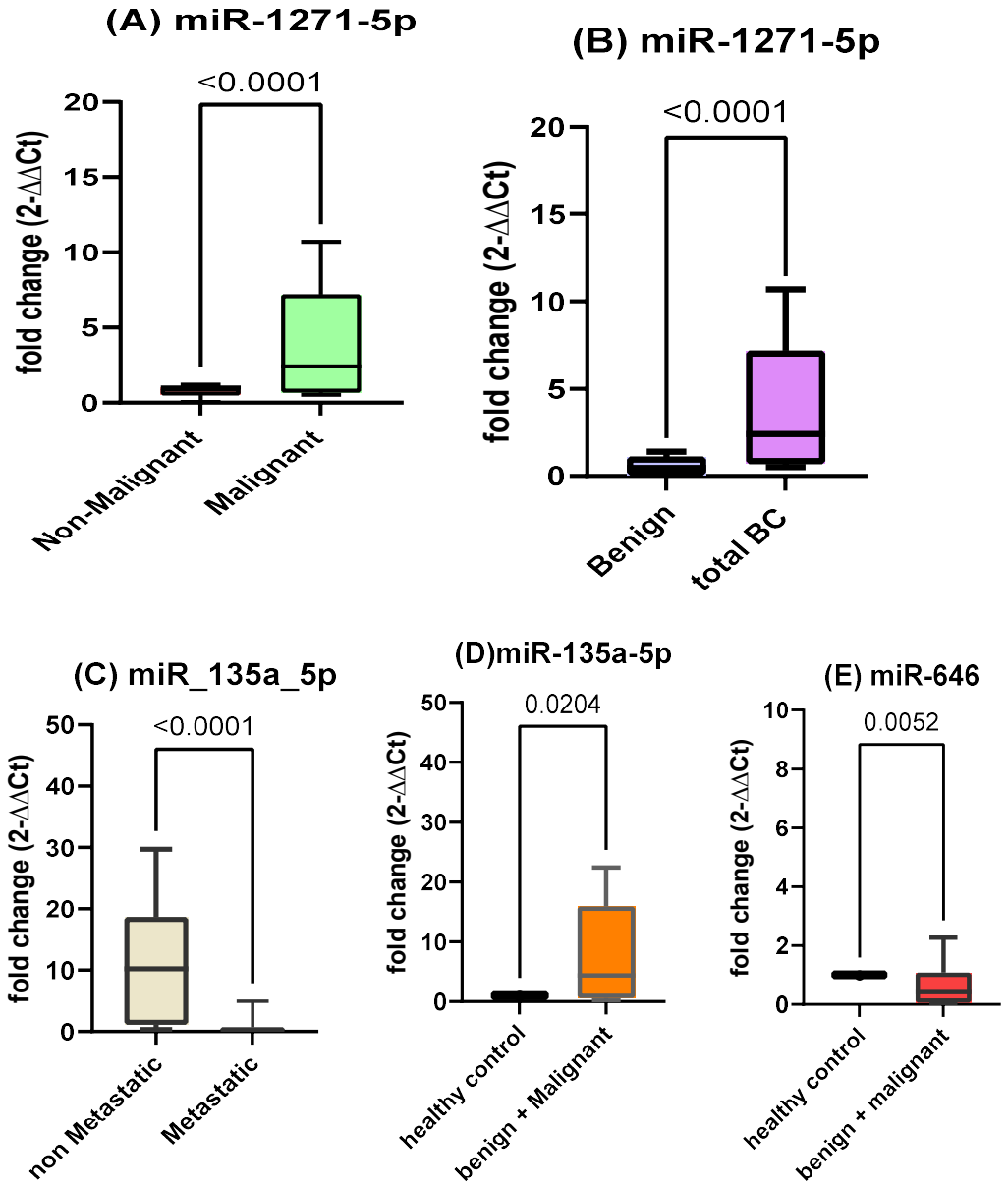




Additionally, when all breast cancer stages (0-IV) were combined and compared against healthy controls, miR-1271-5p, and miR-135a-5p were significantly upregulated (P = 0.0336, P = 0.0277, respectively), whereas miR-646 was significantly downregulated (P = 0.0169) (**Errore. L'origine riferimento non è stata trovata.**).

Supplementary Figures

Figure S4: Plasma miRNA expression profiles in breast cancer progression.
(A) miR-1271-5p expression in combined malignant cases (stages 0–IV) vs. non-malignant cases (benign and healthy).
(B) miR-1271-5p expression in benign conditions vs. all stages of breast cancer.
(C) miR-135a-5p expression in non-metastatic (stages 0–III) vs. metastatic (stage IV).
(D) miR-135a-5p expression in healthy subjects vs. all cases of breast tumors (benign and malignant).
(E) miR-646 expression in healthy controls vs. all breast tumors.
Statistical analysis was conducted with the Mann-Whitney U-test and a p-value≤0.05 level of significance.



Comparing healthy controls against combined breast tumors represented by benign and malignant cases revealed that miR-646 was markedly suppressed in breast tumors (P=0.0052), while miR-135a-5p was dramatically upregulated in breast tumors(P=0.0204) (**Errore. L'origine riferimento non è stata trovata.** d & e).

When comparing non-malignant cases represented by healthy control & benign cases against malignant breast cancer stages (0-IV), miR-1271-5p presented significant downregulation in the non-malignant cases ($P < 0.0001$). Similarly, miR-1271-5p was significantly reduced in benign cases relative to combined breast cancer stages (0- IV) ($P < 0.0001$) (S3 a-c).