Let-7a-5p, miR-100-5p, miR-101-3p, and miR-199a-3p Hyperexpression as Potential Predictive Biomarkers in Early Breast Cancer Patients

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Abstract: Background: The aim of this study is to identify miRNAs able to predict the outcomes in breast cancer patients after neoadjuvant chemotherapy (NAC). Patients and methods: We retrospectively analyzed 24 patients receiving NAC and not reaching pathologic complete response (pCR). miRNAs were analyzed using an Illumina Next-Generation-Sequencing (NGS) system. Results: Event-free survival (EFS) and overall survival (OS) were significantly higher in patients with up-regulation of let-7a-5p (EFS \( p = 0.006; \) OS \( p = 0.0001)\), mir-100-5p (EFS \( p = 0.01; \) OS \( p = 0.03)\), mir-101-3p (EFS \( p = 0.05; \) OS \( p = 0.01)\), and mir-199a-3p (EFS \( p = 0.02; \) OS \( p = 0.01)\) in post-NAC samples, independently from breast cancer subtypes. At multivariate analysis, only let-7a-5p was significantly associated with EFS \( (p = 0.099) \) and OS \( (p = 0.0008) \). Conclusion: Up-regulation of the above miRNAs could represent biomarkers in breast cancer.

Keywords: subtypes breast cancer; miRNAs; breast cancer treatment; chemotherapy; integrated therapies; next-generation-sequencing; target therapy; precision medicine; personalized medicine.
1. Introduction

Breast cancer is a heterogeneous disease and many molecular changes occur during the course of the disease; this is the main cause of treatment failure. This characteristic of breast cancer is reflected on the basis of gene expression pattern classification. It falls under five distinct molecular subtypes including luminal A, luminal B, receptor tyrosine-protein kinase erbB-2 (HER2)-enriched, basal-like, and normal-like subtype. Luminal A breast cancer is hormone-receptor positive (estrogen-receptor (ER) and/or progesterone-receptor (PR) positive), HER2-negative, has low levels of the protein Ki-67, and is low-grade. Luminal B breast cancer is hormone-receptor positive (ER and/or PR positive) and either HER2-positive or HER2-negative with high levels of Ki-67. HER2-enriched breast cancer is hormone-receptor negative (ER and PR negative) and HER2-positive. Triple negative breast cancer (TN) is defined as the absence of estrogen receptor, progesterone receptor, and HER2 expression accounting for approximately 15–20% of all breast cancer patients. The majority of TN patients (up to 70%) overlap with the basal-like gene expression subtype.

Tumor evolution is a unique process for each patient and is influenced by intrinsic genetic variability and external factors such as cancer therapy. Neoadjuvant setting is an ideal scenario to understand tumor evolution at a single patient level, because make it possible to identify molecular changes occurring in tumors due to treatment by comparing pre and post-chemotherapy samples [1–3].

Finding the patients most likely to benefit from NAC is a crucial need and increasing experimental and clinical studies are centered on identifying the predictors of long-term benefit. Several surrogate endpoints have been examined in the neoadjuvant setting such as the pCR, which has been identified as a primary endpoint in numerous clinical trials despite the controversies on its power of predicting the outcome [3,4].

It is noteworthy that not all patients with residual disease after NAC relapse, and the prognostic impact of pCR varies among breast cancer-intrinsic subtypes, whereas patients with luminal A-like breast cancer show a low pCR rate, their overall prognosis is favorable, and patients with TN breast cancer show a high pCR rate but may have a poorer outcome; moreover, if all intrinsic subtypes are considered, the prognostic information of pCR is reduced [5–9].

Several studies have been performed to discover molecular breast cancer biomarkers in order to predict response to neoadjuvant therapy.

miRNAs are involved in pathway regulation (one miRNA can target many genes and a single gene can be modulated by several miRNAs), and finally, miRNAs show tissue and cell-specific expression profiles, and their role in the pathophysiology of the disease is supported extensively in the literature [10].

Each miRNA can regulate the expression of several genes; thus, each one can simultaneously modulate multiple cellular signaling pathways. Depending on their modulation (amplification/deletion) and on target gene function (tumor suppressor/oncogene), miRNA can play alternatively an oncosuppressor or oncogene function. MiRNAs expression in tumors can be altered due to epigenetic, genetic, and transcriptional alterations [11,12].

Several studies have demonstrated that many miRNAs are aberrantly expressed in breast cancer, according to breast cancer molecular subtypes and thus potentially play a role of biomarkers for cancer diagnosis and for response to therapy [13].

We hypothesized that miRNA are differently expressed at different steps of the disease, and it could be possible to identify a set of miRNA associated with disease progression or response to therapy and to attribute to them a predictive and prognostic value.

The aim of the present exploratory study was to identify a set of miRNAs able to predict the prognosis of patients who underwent NAC not achieving pCR.
2. Materials and Methods

2.1. Patients’ Characteristics and Tumor Specimen Collection

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study has the approval of the Ethics Committee of Fondazione Poli-clinico A. Gemelli IRCCS of Rome (Italy) (N protocol 27736/16), and all patients gave written informed consent. We analyzed our database that contains clinical and pathological data on ≈200 cases that underwent neoadjuvant treatment from July 1997 to April 2014 at Fondazione Policlinico A. Gemelli. Patients had measurable breast tumors. Patients were staged according to the American Joint Committee on Cancer (AJCC) Eighth edition [14]. A TRU-CUT biopsy was obtained from each patient. Classification of intrinsic subtypes was defined according to 16th St. Gallen and ESMO guidelines. Histological type, tumor grade, Ki67, ER, PR, and HER2 status were evaluated in the pre-NAC biopsy and in post-surgical neoplastic specimens. Treatment of HER2-negative breast cancer patients consisted of a combination of anthracyclines, taxanes, and cyclophosphamide, while patients with HER2-positive tumors received taxanes and carboplatin combined with trastuzumab, the latter continued after surgery to complete one year of treatment. Patients with ER and/or PR positive tumors received adjuvant endocrine treatment for at least 5 years. Adjuvant radiotherapy was offered according to the national guidelines [15]. The pCR was defined as the absence of any residual invasive cancer on resected breast specimen and on all sampled ipsilateral lymph nodes (ypT0/is ypN0) [16,17] (Table 1).

Table 1. Baseline patients’ characteristics (N 200).

| Characteristics          | N   | %   |
|--------------------------|-----|-----|
| Demographic and clinical |     |     |
| Age                      |     |     |
| Mean (SD) (years)        | 49.4±10.4 | |
| ≤40                      | 69  | 34.5|
| >40                      | 131 | 65.5|
| ER status                |     |     |
| Positive                 | 130 | 65.0|
| Negative                 | 55  | 27.5|
| Unknown                  | 15  | 7.5 |
| PR status                |     |     |
| Positive                 | 128 | 64.0|
| Negative                 | 58  | 29.0|
| Unknown                  | 14  | 7.0 |
| HER2 status              |     |     |
| Positive                 | 58  | 29.0|
| Negative                 | 127 | 63.5|
| Unknown                  | 15  | 7.5 |
| Subtype                  |     |     |
| Luminal A                | 46  | 23.0|
| Luminal B/HER2-negative  | 48  | 24.0|
| Luminal B/HER2-positive  | 37  | 18.5|
| HER2-positive (non-luminal) | 21 | 10.5|
Table 1. Cont.

| Characteristics                  | N   | %   |
|----------------------------------|-----|-----|
| Triple negative                  | 33  | 16.5|
| Unknown                          | 15  | 7.5 |
| **Ki 67**                        |     |     |
| ≤20%                             | 59  | 29.5|
| >20%                             | 120 | 60.0|
| Unknown                          | 21  | 10.5|
| **Grade**                        |     |     |
| 1                                | 0   | 0.0 |
| 2                                | 51  | 25.5|
| 3                                | 84  | 42.0|
| Unknown                          | 65  | 32.5|
| **2 Histologic type**            |     |     |
| Lobular                          | 18  | 9.0 |
| Ductal                           | 150 | 75.0|
| Other                            | 28  | 14.0|
| Unknown                          | 4   | 2.0 |
| **3 Tumor characteristics before treatment** |     |     |
| cT stage                         |     |     |
| cTx                              | 1   | 0.5 |
| cT1                              | 11  | 5.5 |
| cT2                              | 71  | 35.5|
| cT3                              | 55  | 27.5|
| cT4                              | 48  | 24.0|
| Unknown                          | 14  | 7.0 |
| cN stage                         |     |     |
| cN0                              | 33  | 16.5|
| cN1                              | 106 | 53.0|
| cN2                              | 36  | 18.0|
| cN3                              | 9   | 4.5 |
| Unknown                          | 16  | 8.0 |
| **Clinical AJCC stage**          |     |     |
| 0                                | 0   | 0.0 |
| I                                | 1   | 0.5 |
| II                               | 76  | 38.0|
| III                              | 105 | 52.5|
| IV                               | 1   | 0.5 |
| Unknown                          | 17  | 8.5 |
| **Treatment**                    |     |     |
| Neoadjuvant                      |     |     |
| TAC                              | 138 | 69.0|
| TCH                              | 54  | 27.0|
| Other                            | 8   | 4.0 |
### Table 1. Cont.

| Characteristics                                      | N  | %  |
|------------------------------------------------------|----|----|
| **Adjuvant hormone**                                 |    |    |
| Yes                                                  | 130| 65.0|
| No                                                   | 54 | 27.0|
| Unknown                                              | 16 | 8.0 |
| **Tumor pathology after neoadjuvant treatment**      |    |    |
| yT stage                                             |    |    |
| yT0/is                                               | 42 | 21.0|
| yT1                                                  | 75 | 37.5|
| yT2                                                  | 37 | 18.5|
| yT3                                                  | 13 | 6.5 |
| yT4                                                  | 7  | 3.5 |
| Unknown                                              | 26 | 13.0|
| yN stage                                             |    |    |
| yN0                                                  | 83 | 41.5|
| yN1                                                  | 62 | 31.0|
| yN2                                                  | 18 | 9.0 |
| yN3                                                  | 12 | 6.0 |
| Unknown                                              | 25 | 12.5|
| **Pathologic yAJCC stage**                           |    |    |
| 0                                                    | 34 | 17.0|
| I                                                    | 46 | 23.0|
| II                                                   | 55 | 27.5|
| III                                                  | 37 | 18.5|
| IV                                                   | 1  | 0.5 |
| Unknown                                              | 27 | 13.0|
| **Treatment outcomes**                               |    |    |
| Response to neoadjuvant treatment                    |    |    |
| Complete response (R0)                               | 44 | 22.0|
| Microscopic residual disease (R1)                    | 53 | 26.5|
| Macroscopic residual disease (R2)                    | 101| 50.5|
| Unknown                                              | 2  | 1.0 |
| Events within 3 years                                |    |    |
| Distant relapse                                      | 37 | 18.5|
| Local recurrence                                     | 11 | 5.5 |
| Death                                                | 12 | 6.0 |
| Unknown                                              | 18 | 9.0 |
| **Median follow-up, months**                         |    | 80 |

*Abbreviations: TAC, taxanes, anthracyclines, and cyclophosphamide-based regimen; TCH, taxanes, carboplatin, and trastuzumab-based regimen; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor; pCR, pathologic complete response; SD standard deviation.*
From the entire database, we selected twenty-four patients homogeneously distributed according to clinical and pathological characteristics not achieving pCR to which the maximum amount of paraffin-embedding samples of both pre- and post-treatment specimen were available (Tables 2 and 3). In particular, we analyzed pre- and post-NAC samples of the three main molecular subtypes, respectively HER2-positive luminal, HER2-positive non-luminal, and TN subtypes, respectively. For each subtype, we selected four patients with good prognosis and four with poor prognosis.

Table 2. Clinicopathological characteristics of breast cancer selected patients (N 1–12).

| Patients | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Age      | 42  | 54  | 46  | 40  | 54  | 46  | 45  | 53  | 41  | 68  | 35  | 72  |
| Hystological type | IC  | DIC | DIC | DIC | IC  | DIC | IC  | DIC | IC  | DIC | IC  | IC  |
| Grade    | 3   | 3   | 3   | 3   | 2   | 3   | 2   | 3   | 2   | 3   | 15  | 15  |
| cKi67    | 65  | 40  | 80  | 45  | 16  | 60  | 30  | 80  | 45  | 15  | 15  | 15  |
| Receptor subtype | B2  | B2  | B2  | B2  | B2  | B2  | B2  | B2  | B2  | TN  | TN  | TN  |
| cTMN     | cT1N1| cT2N1| cT4N1| cT3N1| cT3N1| cT4N1| cT2N1| cT2N1| cT2N1| cT2N1| cT2N1| cT2N1|
| Preoperative staging | IIA | IIB | IIB | IIA | IIA | IIB | IIA | IIB | IIB | IIB | IIA | IIA |
| Pathological response | R1  | R2  | R2  | R2  | R2  | R2  | R2  | R2  | R1  | R1  | R2  | R2  |
| yKi67    | 70  | 70  | 45  | 60  | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   |
| ypTNM    | ypT1N0| ypT1N0| ypT2N1| ypT1N1| ypT2N1| ypT2N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0|
| ySTADINO | IA  | IA  | IIA | IIA | IIA | IIA | IIA | IIA | IIA | IIA | IIA | IIA |
| NAC      | TCH | TCH | TCH | TCH | TCH | TCH | TCH | TCH | TCH | TCH | TAC | TAC |
| Type of surgery | Q + L| M + L| M + L| Q + L| M + L| M + L| Q + L| M + L| M + L| Q + L| M + L| M + L|
| ADJUVANT CHT | H  | H  | H  | H  | H  | H  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| ADJUVANT CHT | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  |

Table 3. Clinicopathological characteristics of breast cancer selected patients (N 13–24).

| Patients | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | 23  | 24  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Age      | 60  | 52  | 57  | 38  | 67  | 44  | 47  | 40  | 36  | 55  | 57  | 57  |
| Hystological type | DIC | DIC | DIC | DIC | DIC | DIC | DIC | DIC | DIC | DIC | DIC | DIC |
| Grade    | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   | 3   |
| cKi67    | 45  | 45  | 90  | 80  | 35  | 35  | 60  | 35  | 40  | 15  | 45  | 45  |
| Receptor subtype | TN  | TN  | TN  | TN  | H  | H  | H  | H  | H  | H  | H  | H  |
| cTMN     | cT2N1| cT2N2| cT2N0| cT2N1| cT2N1| cT4N1| cT4N1| cT3N1| cT3N2| cT4N0| cT4N0| cT2N0|
| Preoperative staging | IIB | IIA | IIB | IIA | IIB | IIB | IIB | IIA | IIA | IIB | IIA | IIA |
| Pathological response | R2  | R1  | R2  | R2  | R1  | R2  | R1  | R2  | R2  | R1  | R1  | R1  |
| yKi67    | 85  | 6   | 80  | 70  | 70  | 4   | 2   | 40  | 45  | 45  | 45  | 45  |
| ypTNM    | ypT2N0| ypT1N0| ypT1N0| ypT1N1| ypT3N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0| ypT1N0|
| Pathological staging | IIA | IA  | IIA | IA  | IIA | IA  | IIA | IA  | IIA | IA  | IA  | IA  |
| NAC      | TAC | TAC | TAC | AC-T| TCH | TCH | TCH | TCH | TCH | TCH | TCH | TCH |
| Type of surgery | M + L| Q + L| M + L| M + L| M + L| M + L| M + L| M + L| M + L| M + L| M + L| M + L|
| ADJUVANT CHT | CMF | AC  | H   | H   | H   | H   | H   | H   | H   | H   | H   | H   |
| ADJUVANT CHT | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  | X  |
2.2. Purification of miRNA from Paraffin-Embedding Tissue Sections

Standard formalin-fixation and paraffin-embedding (FFPE) procedures always resulted in significant fragmentation and crosslinking of nucleic acid. For each of the two samples (pre- and post-NAC) for each patient, the starting material for RNA purification was made by up to 4 sections of paraffin-embedding tissue with a thickness of 5 µm combined in one preparation. After microdissection, the total RNA was extracted using miRNeasy FFPE Kit (Qiagen) following the protocol of the manufacturer. The concentration and purity of the total RNA was isolated from tissues and was determined by measuring the absorbance in a spectrophotometer (Nanodrop). The QIAseq miRNA Library Kit (Qiagen) was used for miRNA libraries. In an unbiased rapid reaction, adapters were ligated sequentially to the 3' and 5' ends of the miRNAs. Subsequently, universal cDNA synthesis with UMI (Unique Molecular Index) assignment, cDNA cleanup, library amplification, and library cleanup were performed following the manufacturer’s recommendation. The integrity and size distribution of the total RNA from the tissue was confirmed using an automated analysis system (Agilent 2100 Bioanalyzer). Successively, the miRNA sequencing libraries was sequenced using MiSeq® Il-lumina NGS system: the molarity of each sample (in nM) was calculated using the following equation: (X ng/µL)(106)/(112450) = Y nM. Individual libraries were diluted to 4 nM using nuclease-free water and then combined in equimolar amounts.

2.3. MiRNA Discovery

2.3.1. Analysis Procedure

The QIAseq miRNA-NGS data analysis software (Qiagen) was used. The results were confirmed manually by aligning the fastqs with the sequences corresponding to all human miRNAs. The miRNA sequences were extracted from the miRBase database [18]. The miRNAs were selected based on the number of reads, and those that differed between pre-NAC and post-NAC were taken into consideration.

2.3.2. MiRNA Target Prediction

To know the potential target site, a computational approach was applied for their validation [19]. The miRNA targets were predicted by the instrument MiRDB [20]. This is an online database for miRNA target prediction and functional annotations with a focus on mature miRNAs. It provides a web interface for target prediction generated by an SVM machine learning algorithm. All gene targets were converted by the Human Gene ID Converter tool into their corresponding NCBI entrez gene ID. Some NCBI-gene ID were searched manually on the HUGO Gene Nomenclature Committee (HGNC) database. Perl language scripts have been made to list the NCBI entrez gene ID for each of the miRNAs to be analyzed. For the mapping of the genes, the KEGG Mapper—Search & Color Pathway tool was used. Only the pathways related to the disease were selected and where the mapped genes were more numerous. The pathways related to the disease were selected in consultation with the bibliographic articles in Pubmed-NCBI.

2.4. Statistical Analysis

The primary endpoint was event-free survival (EFS). The secondary endpoint was overall survival (OS). EFS was considered as the time from diagnosis to any relevant event (progression of disease that precludes surgery, local or distant recurrence, or death due to any cause) and was censored at the last follow-up visit. OS was estimated as the interval from diagnosis to death from any cause, and it was censored at the last follow-up visit for the patients still alive. The Kaplan–Meier method was applied for survival probabilities estimation. For univariate analysis, we used the Fisher exact test. Variables (IHC-based molecular subtypes, histological type, tumor grade, Ki67% value, tumor size, clinical lymph node status, cTNM stage, surgery) were included in the multivariate analysis if the univariate p-value was <0.05. Multivariate analysis was done using the Cox proportional
hazard model. A two-sided \( p \)-value < 0.05 was considered statistically significant. Analyses were performed using SPSS statistical package version 13.0.

3. Results

3.1. Patients Characteristics

Within the entire database, we selected 24 early breast cancer patients, who had undergone neoadjuvant chemotherapy at the IRCCS Fondazione Policlinico A. Gemelli, homogeneously stratified according to clinical and pathological characteristics, and not achieving pCR. In particular, we analyzed pre- and post-NAC samples of eight patients for the following subtypes: HER2-positive luminal, HER2-positive non-luminal, and TN subtypes. Median age at time of study entry was 50.2 years (range, 35 to 72 years). Median follow-up was 80 months, median EFS was 40.7 months, and median OS was 63.3 months.

3.2. Clinicopathological Variables and Outcome

We analyzed the correlation between IHC-based molecular subtypes (luminal B/HER2-positive, HER2-positive/non-luminal and TN breast cancer), histological type (ductal invasive breast cancer and others), tumor grade, Ki67\% value, tumor size, clinical lymph node status, cTNM stage, surgery, and clinical outcome. Variables showing \( p \)-values < 0.05 in univariate analyses were used for multivariate logistic regression. However, none of the selected variables were statistically significant at univariate analysis.

3.3. miRNAs and Outcome

Thanks to the computational algorithms and bioinformatics database, we identified 27 miRNAs that were significantly hypo- or hyper-expressed in pre- versus post-NAC samples: hsa-let-7a-5p, hsa-let-7f-5p, hsa-miR-100-5p, hsa-miR-101-3p, hsa-miR-103a-3p, hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-125a-5p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-143-3p, hsa-miR-191-5p, hsa-miR-196a-5p, hsa-miR-199a-3p, hsa-miR-205-5p, hsa-miR-26a-5p, hsa-miR-26b-5p, hsa-miR-29a-3p, hsa-miR-29c-3p, hsa-miR-30a-5p, hsa-miR-30d-5p, hsa-miR-30e-5p, hsa-miR-510-3p, hsa-miR-92a-3p, hsa-miR-93-5p, hsa-miR-99a-5p, hsa-miR-99b-5p. In Scheme 1, we show the modulation of expression of miRNAs for each subtypes. In Table 4 we presented miRNAs predictive target genes.

![Scheme 1](image.png)

**Scheme 1.** The chart summarizes—for each miRNA and for each subtype—the number of samples that show the same over/under-expression pattern. Bars above the 0 represent overexpression, while bars below represent under-expression.
### Table 4. miRNAs predictive target genes.

| miRNAs | Gene Target Predicted |
|--------|------------------------|
| Let 7a-5p | SMARCAD1 FAM178A LIN28B GATM C8orf58 ADRB2 DNA2 IGDC3 TTL4 NM6e TMPrSS2 HIC2 MAPK6 DMD SCN4B ZFYVE26 Fzd3 LIMD2 SMIM3 TMEM2 PGFC3 COL3A1 ZBTB85 ACVR1C EIF4G2 CLP1 SLC25A27 NPHP3 PTG1 B3GN17 COL1 CCNJ JGF2BP3 FOXP2 TRIM71 PAR6B FRA1 MAPK43 HANDI1 UTRN GN5 NAP1L1 UHR2F LRIG2 ACER2 RICTOR PRF3PB3 NR6A1 BEGAIN NHLRC3 IFIH4E2 IFF1 45 BACH1 PAPP1 STK40 SLC5A9 PDP2 RDX3 HSPR FGN ZBP1 IGFR ERCC6 C8orf58 PBX3 RNF20 TGFBR1 C8orf41 ADAMT51 TSEN4 C14orf28 FIGNL2 ZNF25P2 FRA4B ARHGAP28 EDM1 C10orf39 USP38 EDM6 FNDCC3 ARQ2 SPRYD4 IGDC4 PHiC1 SLC1A7 KCTD11 NDST2 DD2 TRIM41 SLC20A1 DPPE PLXNC1 IIP2 CP4 FXBL12 PALD1 EAA1 HMG1A RAB11FIP3 STX3 CPE135 GDF6 TRIM67 SLC5A6 OBP3L PEKLH6 MM106 DDB2 PLG2 PGRM1 CLDN12 HMG2A TPS1A2 CLASP2 DDX19A KMT2D RFX6 C5orf51 PBX3 RNF20 TGFBR1 C15orf41 ADAMT51 SLC5A9 PDP2 RDX3 HSPR FIGNL2 ZNF25P2 FRA4B ARHGAP28 EDM1 C10orf39 USP38 EDM6 FNDCC3 ARQ2 SPRYD4 IGDC4 PHiC1 SLC1A7 KCTD11 NDST2 DD2 TRIM41 SLC20A1 DPPE PLXNC1 IIP2 CP4 FXBL12 PALD1 EAA1 HMG1A RAB11FIP3 STX3 CPE135 |
| miR-100-5p | KBTBD8 S3ST2 ZEFE1 MTRB1 MBNL1 TRIB2 SMARCAD5 TCT39A ZADH2 REARV2 PPP3CA AP1AR FGFR3 HS5ST3B1 NOX4 BAZ2A AGO2 P53K9 NR6A1 TAOK1 FAS LGF5 DAMD5 CD65 MMS22L OGG1 AMOT RUFY3 BAD2C2 B3GALNT2 SCN8A ARAP2 STAMBP STAU2 KLHDC1 LIN7C ZNF518A PHF20L1 POMP RAB39B ZNF217 SLC38A2 LMBN1 UTS2B RLP2 RAB1A AP3D1 ADAMTS3 GSK3B SLC1A23 |

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**Let 7a-5p**

**miR-100-5p**

**miR-101-5p**
Table 4. Cont.

| miRNAs | miRNAs Gene Target Predicted |
|--------|-----------------------------|
| miR-101-3p | ASCC3 RAB22A BEGAIN ZNF510 RFPL4B CCDC68 TLK2 TAGAP FUCA2 ZNF549 RAB15 OTUD4 CCSER1 ZBED4 RASGRP3 GRIN2A ANXA10 WWC3 HNRNPF KAT6B HAS2 DCUN1D1 CTCF CCDC88A FAM73A MTSS1L BBX FAM60A RNF19A RCN2 PKD2 ATRX POLR3K MAP595 N4BP1 DNM1L MRPL42 KHDHR82 STX6 CANK13 NOTCH1 GABBR2 SPOP GLIPR1L KIF5B C9orf72 DENND2C SCA1ML LRRRC4 MAP3K2 SPG11 DCAF7 ARHGEF10 KL2 ZCCHC2 KPNB1 KIAA1432 CRLS1 BTLA N5D1 MAPK1 TMEM167A PDSSB OG1 KDM6B GCNT1 C11orf70 ANKZ5F1 RNF38 ROBO2 SGM52 EPT1 SLM2 HIVEP3 FAR1 CAT5F2 TMEM231 TKT1 TMEM65 ZNF469 SGPL1 RXRB WDR72 DES12 NACA2 MMTR4 L1G2 CREB5F XPOS PTC1H1 NACA GABBR2 PRR11 CTDSPL DCLR1E1B DD3X MAB21L3 MLEC FAM103A1 GN1B SPAT5L PRRC2C UBR7 FYTTD1 CD86 RPK1 CNIH3 NAIP MON2 ATRNL1 KIAA1462 BCL2L11 RANBP1 FNM3L PHT2F TMF1 LANCL3 ZNF633A TIMM17A PLEK1G1 PBX3 M1XT3 UNKL TE2X RANBP6 AGAPI ZNF235 FAK1 |
| miR-199-3a | ETNK1 CELSR2 ADAMTS1 KLHL3 ACVR2A LRP2 BCAR3 SERPINE2 NOVA1 MAP3K4 FAM110C KIAA0319L RB1 ZH1X KDM5A P3D2 LLIN21 ITGA3 CHMP5 TUBGCP3 FAM60A NKL2 CDAP2 NID2 UTP20 PAK4 C9orf40 KDM6A C7K2 C7orf49 KATNB1 CDK17 PPP2RB2 APLP2 MCFD2 CDNP FRP40A CAXADR PPP2R5E G3BP2 FUBP1 NEDD4 SLC24A2 RASEF SDC2 PGDFRA SCD SUMO3 TTPK1 ARHGFE3 ESP1 ATAD1 MAPK35 APLF ASTN1 ECM1 GGNBP2 CYB5R4 PAWR XPO1 HPS1B1 KIF5B ADU XTRF N2U1 RNRTG MDG2 GORAB PNRC1 VGLL2 FAM199X DEPC1B GNPX2A NF1A DNHD1 RAPH1 TTPP WDRT AR1L15 ADAM10 NLRI1 P1BL RAPGER1 SEMA3A COL12A1 TACCC2 KL13F3 SPP1 ELR1 NMDT3A LOR1 MAPKE1 CD151 FXR1 PLCB1 MMP7 YWHAE EPG5 SMARCC2 EPB41L5 DNM1L1 C2orf91 SIMPMP8 PLB1P1 KIDINS220 GPM6 A3PS3 PON2 TMD5 HNF1B WAPAL DCBLD2 NCIH2 C9orf70 RALGPS2 LAMP3 BEN7 FAM129A ITGB8 ANKRD61 CETN3 KCMF1 FAM76B PDE4B HYPK SLC39A10 NAA25 NTRK2 KDM3A GLT8D2 N2R74 MBLN1 TITOR SOWAC RG54 FGL2 ALX4 YWHAH STARD9 ENOX2 MAP3K1 GALNT7 YWHA2 CREB5F TEND1 TMABI E4L1 RPI1 FNM1 CHRA2 VLMP2 VAMP3 ZCCHC17 TEAD1 SYNJI SLC16A12 PCDH7 ABHD4 DUSP5 KCDN2 SECISBP2L DINTM1 PPR1R9A ATP6V1C2 ME12S ARG2 CHAD SORL1 RNF216 ELAFL2 CAPRIN1 FCGR3A LONRF3 ADD3 RRM2B CN70T SIR1 LL1RI1 ECMV MB21Z2 ADRBI CLDN8 FCGR3B CASSP1 CAB5 VLQNL UBQLN1 EFCB1A TMED6 PTTPRU ABCA1 CABLESI1 S53GLB1 ERO1L ANK2 TMEM218 KIAA0907 ASAP2 ACOX1 SYPL1 BRWD3 DPAG7 PIK3CB NF1 ZNF614 SLC3A7 CLRN9H1 CYP1B1 ZC3H14 LOC10192984 PCDHB12 HECT2 PLECBL1 HUCK2 HNMRT CDC42BPF RFX7 CS6R1 HCTD7 CITED2 CFL2 RHO1T UBXN3 BGF KIAA0141 FXN1W12 GPR160 KCNH2 TRMT61B GNA12 GRHL1 SLC4A5 PHF6 KL2F2 CYP24A1 KDR51 RMRK2 ATPIBP4 CDCDC8C ADAM22 C11orf2 TXNNG CEP85L KAZN PRKCB BAG4 FAM46D CALCRL PRCC1 KIAA1244 SEC16B FKBPI4 CDC14A CTNNA2 NAPL1 UCN45A DDIT4 PAQR3 |

Up-regulation of let-7a-5p, mirR-100-5p, mir-101-3p, and mir-199a-3p in post-NAC specimens was significantly correlated with better EFS and OS compared to those with normal or lower expression, independent from breast cancer subtypes.

At subgroup analysis, the overexpression of mentioned miRNAs in post-NAC samples was linked with an improvement in EFS and OS only in HER2-positive non-luminal subtypes (Table 5). Furthermore, when we stratified patients according to a sort of miRNA signature (let-7a-5p, mirR-100-5p, mir-101-3p, mir-199a-3p), we found that patients who concurrently overexpress all four miRNAs experimented a significantly better prognosis in terms of EFS and OS (Table 5; Figures 1–5). However, at multivariate analysis, EFS (p = 0.009) and OS (p = 0.0008) showed a statistically association exclusively with up-regulation of let-7a-5p.
Table 5. Prognostic impact of miRNA expression profile on EFS and OS in all populations and in HER2 non-luminal subtypes.

| miRNA       | EFS (Months) | p Value | Hazard Ratio (CI 95%) | OS (Months) | p-Value | Hazard Ratio (CI 95%) |
|-------------|--------------|---------|-----------------------|-------------|---------|----------------------|
| Let-7a-5p   | 58 vs. 28    | 0.006   | 0.38 (0.08–0.66)      | 65 vs. 35   | 0.0001  | 0.27 (0.03–0.33)     |
| in HER2     |              |         |                       |             |         |                      |
| non-luminal subtypes | 61 vs. 36    | 0.05    | 0.58 (0.29–6.28)      | 71 vs. 44   | 0.05    | 0.31 (0.02–1.0)      |
| miR-100-5p  | 56 vs. 17    | 0.01    | 0.39 (0.11–0.75)      | 56 vs. 39   | 0.03    | 0.45 (0.15–0.94)     |
| in all populations | 61 vs. 20    | 0.004   | 0.21 (0.01–0.30)      | 70 vs. 35   | 0.004   | 0.19 (0.00–0.30)     |
| miR-101-3p  | 56 vs. 20    | 0.05    | 0.48 (0.16–1.03)      | 58 vs. 35   | 0.01    | 0.38 (0.10–0.75)     |
| in all populations | 61 vs. 24    | 0.02    | 0.28 (0.01–0.77)      | 71 vs. 40   | 0.02    | 0.27 (0.01–0.77)     |
| miR-199a-3p | 61 vs. 20    | 0.02    | 0.41 (0.14–0.85)      | 69 vs. 46   | 0.01    | 0.39 (0.13–0.80)     |
| in all populations | 61 vs. 20    | 0.02    | 0.27 (0.00–0.70)      | 70 vs. 45   | 0.04    | 0.29 (0.01–0.96)     |
| Signature   | 64 vs. 20    | 0.004   | 0.31 (0.4–0.66)       | 71 vs. 46   | 0.005   | 0.31 (0.11–0.68)     |
| in all populations |          |         |                       |             |         |                      |

Figure 1. Prognostic impact of Let7a-5p on EFS (on the left) and on OS (on the right) in all population: blue line refers to patients with overexpression of Let7a; red line refers to patients without overexpression of Let7a-5p.

Figure 2. Prognostic impact of miR100-5p on EFS (on the left) and on OS (on the right) in all population: blue line refers to patients with overexpression of miR100-5p; red line refers to patients without overexpression of miR100-5p.
4. Discussion

Recent suggestions have revealed that the miRNAs can modulate the expression of oncogenes or tumor suppressor genes. Based on this evidence, miRNAs appear as hopeful biomarkers of breast cancer [21].

Bertoli et al. analyzed the role of several miRNAs in breast cancer and showed that some of them could be useful for diagnostic tools (i.e., miR-9, miR-10b, and miR-17-5p); other miRNAs (i.e., miR-148a and miR-335) may have a prognostic role, while still others (i.e., miR-30c, miR-187, and miR-339-5p) may be predictive of treatment response [22].

In our study, we investigated the potential role of miRNAs as predictors of outcome in early breast cancer patients. We found a significantly differential miRNA expression...
among some breast cancer subtypes in pre-NAC and post-NAC paraffin-embedding tissue: in particular, we found that the up-regulation of let-7a-5p, miR-100-5p, miR-101-3p, and miR199a-3p in post-NAC samples was significantly associated with better prognosis in terms of EFS and OS, but at multivariate analysis, only overexpression of let-7 was correlated with survival.

Although miR100, miR101, and miR199 did not maintain a statistically significant correlation with survival outcome in multivariate analysis, there is a strong biological rationale supporting their role in breast cancer prognosis and, in our opinion, they deserve further studies.

Interestingly, all these miRNAs have shown to be normally down-regulated in breast cancer and have a role in cancer pathogenesis affecting cell cycle, proliferation, and metastasis diffusion.

Let-7 employs its antiproliferative activities and its tumor-suppressor role by controlling key checkpoints of several mitogenic pathways and by suppressing different oncogenes, including HMGA2, RAS, and MYC [23,24]. Let-7 expression levels have a role as a prognostic marker in several cancers, and the loss of its expression is a marker for less differentiated cancers [25,26]. It is newsworthy that HMGA2 and H-RAS oncogenes are targeted by an induced expression of let-7 in breast cancer cells, and in a murine model of breast cancer, exogenous let-7 delivery represses mammosphere formation, cell proliferation, and the undifferentiated cell population by downregulating both H-RAS and HMGA2 oncogenes [27]. Barh demonstrated that in silico analysis, apart from repressing HMGA2, RAS, and MYC, let-7 may also target CYP19A1, ESR1, and ESR2, thereby potentially blocking estrogen signaling in ER-positive breast cancers [28]. Moreover, Kim et al. affirmed that let-7a inhibits breast cancer cell migration and invasion through the down-regulation of C-C chemokine receptor type 7 expression (CCR7) [29]. Other authors described a new role of let-7a in regulating energy metabolism in neoplastic cells [30]. To underline the role of Let-7 restoration to prevent tumor progression, our study found that the overexpression of let-7 family members in post-NAC samples is associated with a better prognosis in patients with no pCR. From the therapeutic viewpoint, let-7 is an attractive molecule for preventing tumorigenesis and angiogenesis; thus, it could be a potential therapeutic target in several cancers that lose let-7.

miR-100, miR-99a, and miR-99b belong to the miR-100 family. The miRNA-100 controls several genes playing an important modulatory role. mTOR, PI3K, AKT1, IGF1-R, HS3ST2, HOXA1, RAP1B, and FGFR3 are some of the multiple targets of miR-100. Modulating these important genes, miRNA 100 could block proliferation by promoting cell cycle arrest and apoptosis in tumor cells. Furthermore, recent findings suggest that in breast cancer, the miR-100 may act as a pro-differentiating agent for cancer stem cell modulating Wnt/β-catenin pathway and Polo-like kinase 1 gene. It was found that miR100 overexpression has the capability to inhibit the Wnt pathway. Recent evidence showed that miRNA-100 downregulates Polo-like kinase 1 in basal-like cancer, blocking the maintenance and expansion of breast cancer stem cells (BrCSCs), inducing BrCSC differentiation, thus favoring the transition from undifferentiated tumors into well-differentiated ones [31,32]. Petrelli et al. analyzed 123 early node-negative breast cancer tumor specimens: patients were categorized on the basis of the miR-100 expression status. Patients with low miR-100 levels experienced worst distant metastasis-free survival [32]. According to the literature, the miR-100 family could convert an aggressive tumor into a well differentiated, biologically favorable, phenotype. In support of this potential role, miRNA-100 family members are understudied as targets for differentiation therapy: this therapeutic strategy aims to induce the transformation of aggressive cancer cells into well-differentiated ones, which are more sensitive to therapy [31,32].

miR-101 is known to be involved in many important cancer processes such as inhibition of proliferation, chemoresistance, angiogenesis, invasion, and metastasis [33]. According to this hypothesis, several reports showed that the loss of miR-101 is frequent and is associated to a worse outcome in many types of tumors [34–39]. Several studies
demonstrated that EZH2, a mammalian histone methyltransferase, is emerging as one of the most important targets of miR-101: loss of miR-101 function induces the overexpression of EZH2, which is related to cancer evolution [40,41]. A meta-analysis showed that the down-regulation of miR-101 expression is correlated with a poor prognosis [13]. Liu et al. revealed that a high expression of miR-101 inhibits TNBC progression and increases chemotherapeutic drug-induced apoptosis in TNBC by directly targeting myeloid cell leukemia 1 (MCL-1) [42]. Other authors demonstrated that miR-101 is hypo-expressed in different breast cancer subtypes and stimulates cellular proliferation and invasiveness by targeting Stathmin1 (Stmn1) [43]. According to these findings, our study showed that higher levels of miR-101-3p were correlated with a better EFS and OS, independently from breast cancer subtypes in patients not achieving pCR. Therefore, it is possible to say that miR-101 could be a potential therapeutic target and a novel prognostic factor.

The role in breast cancer progression is unclear regarding miR-199a/b-3p. Some studies showed a loss of miR-199a/b-3p expression in aggressive breast cancer [44]; other evidence demonstrated the ability of miR-199a/b-3p to inhibit proliferation, migration, and multi-drug resistance. miR-199a/b-3p seems to be down-expressed in many types of cancer [45–52]. According to Shou-Qing Li et al., PAK4 could be a possible target of miR-199a/b-3p with an oncospresive role: in human breast cell lines, ectopic expression of miR-199a/b-3p blocks the PAK4/MEK/ERK pathway to inhibit breast cancer progression by inducing G1 phase arrest [52]. Xuelong et al. have shown that the hyper-expression of miR-199a-3p inhibits mitochondrial transcription factor A (TFAM) expression, enhancing sensitivity to cisplatin in breast cancer cells. Hence, miR-199a/b-3p could represent a good prognostic and predictive biomarker [53]. It was found that the overexpression of miR-199a-3p regulates the activation of the G protein coupled receptor (GPER), which is involved in tumorigenesis, and suppresses cells’ proliferation, invasion, and epithelial–mesenchymal transition in TNBC [54].

Taking into consideration all our findings, our hypothesis is that miRNA patterns of expression could help identify, in the group of patients not achieving pCR, a population with better outcome. Moreover, in our opinion, the present study is interesting because it gives further support to the fundamental role of the miRNAs in cancer biology and their potential application as target cancer therapies. Several studies have been conducted in order to modulate cellular miRNA levels as inhibiting the oncogenic miRNAs and as restoring the tumor-suppressive ones, with encouraging results [55–58].

Although larger case series are needed, our findings provide a basis for broader, prospective, and multicenter trials to support the potential role of miRNAs as predictive and prognostic biomarkers not only in early but also in advanced disease. We hope that the identified miRNAs will help in comprehensively understanding their pathway mechanism in breast cancer and improve the therapeutic strategies [59].

5. Conclusions

miRNAs have changed our understanding of cell pathway modulation and opened fields not only for the development of novel cancer target therapies but even for new diagnostic tools. At present, important topics in cancer research are discovering the underlying pathways involved in miRNA expression and secretion and understanding miRNA modulation in different phases of cancer progression. Large cohort studies are still required to analyze and confirm the diagnostic, prognostic, and therapeutic application of miRNA.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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