

# Dysregulation of microRNAs in triple-negative breast cancer

Sylwia Paszek<sup>1, 2\*</sup>, Natalia Gabło<sup>1, 2\*</sup>, Edyta Barnaś<sup>3</sup>, Małgorzata Szybka<sup>4</sup>, Jan Morawiec<sup>5</sup>,  
Agnieszka Kołacińska<sup>5, 6</sup>, Izabela Zawlik<sup>1, 2</sup>

<sup>1</sup>Centre for Innovative Research in Medical and Natural Sciences, Faculty of Medicine, University of Rzeszow, Poland

<sup>2</sup>Department of Genetics, Faculty of Medicine, University of Rzeszow, Poland

<sup>3</sup>Institute of Obstetrics and Emergency Medicine, Faculty of Medicine, University of Rzeszow, Poland

<sup>4</sup>Department of Microbiology and Laboratory Medical Immunology, Faculty of Medicine, Medical University of Lodz, Poland

<sup>5</sup>Department of Head and Neck Cancer Surgery, Medical University of Lodz, Poland

<sup>6</sup>Department of Surgical Oncology, Cancer Center, Copernicus Memorial Hospital, Poland

\*Equal contribution

## ABSTRACT

**Objectives:** Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer with limited treatment options and poor prognosis. TNBC is usually diagnosed at a relatively young age and is characterized by high risk of developing metastases. Some epigenetic regulation of gene expression is associated with TNBC. Expression of microRNAs (miRNAs) can serve as a potential tool for identifying critical biomarkers in TNBC.

The aim of our study is to examine expression of selected miRNAs in TNBC and to assess the relationship between miRNA expression and clinicopathological factors.

**Material and methods:** Expression levels of 19 selected miRNAs were compared between cancerous and normal breast tissues by use of qPCR method. We have evaluated the relationship between the expression level of miRNAs and clinicopathological factors such as: age, tumor size and lymph node status.

**Results:** We found that in TNBC tissues, when compared with normal breast tissues, the expression of miR-190a, miR-136-5p and miR-126-5p was significantly reduced ( $p = 0.0041$ ,  $p = 0.0007$ ,  $p = 0.0007$ , respectively) whereas expression of miR-135b-5p and miR-182-5p was significantly increased ( $p = 0.0194$ ,  $p = 0.0041$ , respectively). We found a linear trend for tumor size and expression of miR-126-5p ( $p = 0.0296$ ) and miR-135b-5p ( $p = 0.0241$ ).

**Conclusions:** Our study confirms that miRNA expression profile is dysregulated in TNBC patients compared to healthy controls. MiR-190a, miR-136-5p, miR-126-5p, miR-135b-5p and miR-182-5p may be associated with development and progression of TNBC.

**Key words:** TNBC, microRNA expression, biomarkers

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## INTRODUCTION

Breast cancer is a heterogeneous disease and a major cause of female mortality [1]. Triple-negative breast cancer (TNBC) is defined by a lack of expression of estrogen receptor (ER), progesterone receptor (PR) and of human epidermal growth factor receptor-2 (HER2) [2]. TNBC accounts for approximately 15–25% of all breast cancer types and is an aggressive histological subtype. There exist following

TNBC subtypes: basal or BRCA-related, apocrine, normal-like and other rare subtypes including a neuroendocrine TNBC [3]. Subtyping of TNBC is necessary to choose an optimal molecular-based therapy [4]. TNBC is mostly diagnosed in patients at a relatively young age and is characterized by frequent metastasis [5, 6]. Patients with TNBC have increased risk of relapse and tend to resist to chemotherapies [7]. Thus, further subtyping with more individual therapy adjustments

### Corresponding author:

Izabela Zawlik

Department of Genetics, Faculty of Medicine, University of Rzeszow

Laboratory of Molecular Biology, Center for Innovative Research in Medical and Natural Sciences University of Rzeszow

Warzywna 1A, 35-959 Rzeszow, Poland

tel: +48 17 851 68 10, e-mail: izazawlik@yahoo.com

could improve patients' survival. One of the new potentially useful biomarkers in oncology is miRNA [8].

miRNAs are short non-coding RNAs of 19–25 nucleotides in length. They regulate gene expression post-transcriptionally by pairing with complementary nucleotide sequences in the 3'-UTRs of specific mRNA targets [9]. Deregulation of specific miRNAs associated with carcinogenesis causes changes in their expression and function. This way some acquire the features of oncogenes while others become tumor suppressors [10]. Since 1993, when the first miRNA *lin-4* was discovered, the knowledge about miRNAs has been greatly expanded. The regulatory role of miRNA was determined in many cellular pathways involved in cell division, differentiation, morphogenesis, metabolism and apoptosis [11]. Studies carried out on various types of cancers made clear that miRNA expression is an important factor for the carcinogenesis. TNBC is a heterogeneous subtype of breast cancer that has limited treatment options [12]. Thus, miRNA studies may be useful for developing new therapeutic strategies. It has been reported that numerous cellular signaling pathway abnormalities occur in TNBC [13]. There has been increasingly more evidence supporting the role of miRNAs in cell signaling pathways of triple-negative breast cancer but its clinical significance is still unclear [14, 15].

## OBJECTIVES

The aim of our study is to examine the expression of selected miRNAs in TNBC tissues in comparison with normal breast tissue and to assess the relationship between miRNA expression and some clinical features such as: age of onset, tumor size and lymph node status.

## MATERIAL AND METHODS

The study was conducted under Institutional Review Board protocol #RNN/226/11/KE/13/12/2011 at Medical University of Lodz. All patients gave written informed consent. We included in this study 11 TNBC female patients and 3 healthy age-matched women controls. All of 11 examined patients exhibit an invasive ductal histology and a third histologic grade. One of the patients had evidence of distant metastases in lungs and liver. Between December 2011 and April 2012 specimens were collected at the Cancer Center with ultrasound-guided 14-gauge core needle biopsy (ultra-automatic biopsy instrument Pro-Mag TM, Angiotech) from 11 female patients with either inoperable locally advanced breast cancer or with large operable tumor suitable for down-staging. Further specimens were obtained from 3 healthy age-matched women controls (breast reduction procedures). Four to five specimens were obtained from each lesion. Half of samples were frozen immediately at  $-80^{\circ}\text{C}$  while the rest was paraffin embedded and reviewed by breast pathology specialist in the Department of Pathol-

ogy. Estrogen receptor (ER) and progesterone receptor (PR) status were determined by immunohistochemistry (IHC) using the Allred score. Human epidermal growth factor receptor 2 (HER2) status was evaluated by immunohistochemistry or by fluorescence in situ hybridization. Samples were considered ER or PR negative if less than 1% of the tumor cells were immunoreactive. Samples were considered HER2 negative with IHC 1+ staining or with a score of 2+ and no *HER2* gene amplification when tested by FISH. TNBC was diagnosed when result was ER, PR and HER2 negative.

## MicroRNA expression analysis by use of qPCR

Selection of 19 miRNAs was performed on the basis of previous reports in the literature and the miRNA database. In the total of 14 breast tissue samples (11 from TNBC and 3 from healthy controls) we analyzed 19 miRNAs: hsa-miR-512-5p, hsa-miR-190a, hsa-miR-200b-3p, hsa-miR-622, hsa-miR-346, hsa-miR-148b-5p, hsa-miR-449a, hsa-miR-1255b-5p, hsa-miR-203a, hsa-miR-577, hsa-miR-93-5p, hsa-miR-126-5p, hsa-miR-423-5p, hsa-miR-129-5p, hsa-miR-193b-5p, hsa-miR-182-5p, hsa-miR-136-5p, hsa-miR-191-5p and hsa-miR-135b-5p. Hsa-miR-103a-3p and hsa-miR-107 were chosen for normalization, which was further validated by NormFinder algorithm. RNA was isolated using miReasy Mini Kit 50 (Qiagen, Valencia, CA, USA). 10 ng RNA sample was reverse transcribed in 10  $\mu\text{L}$  reactions using the miRCURY LNA<sup>TM</sup> Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Copenhagen, Denmark). Each RT was performed in duplicates. cDNA was diluted 100  $\times$  and assayed in 10  $\mu\text{L}$  PCR reactions according to the protocol for miRCURY LNA<sup>TM</sup> Universal RT microRNA PCR; each miRNA was assayed once by qPCR on the microRNA Ready-to-Use PCR, Custom Pick&Mix panel. Negative controls excluding templates from the reverse transcription reaction were processed and profiled in a similar fashion. Amplification was performed in a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche, Basel, Switzerland). The amplification curves were analyzed using the Roche LC software, both for determination of  $C_p$  (by the 2nd derivative method) and for melting curve analysis.

## Data analysis

The assay efficiency was determined by means of analysis of the amplification curves using algorithms similar to the LinReg software. Individual reactions that gave efficiency of  $< 1.6$  were excluded from the dataset. The efficiencies ranged between 1.8 and 2.1. All assays were inspected for distinct melting curves and the  $T_m$  was confirmed to be within known specifications for the assay. It was important that assays with 5  $C_p$ 's less compared to negative control be detected and those with  $C_p < 37$  be included in the data analysis. Data that failed to reach such criteria was

excluded from further analysis. The stability of miRNAs was measured by NormFinder software [16]. For normalization of the data we have used the average of hsa-miR-103a-3p and hsa-miR-107, which proved to be the most stable normalizer (according to E normalization). (Stability value for best combination of such pair equals 0.045). The formula used to calculate the normalized Cp values was:

Normalized delta Cp = average Cp (hsa-miR-103a-3p and hsa-miR-107) — assay Cp (sample).

### Target gene selection

In order to find miRNA target genes we have searched miRTarBase 6.0 — a database that manually collects experimental data about miRs-genes interactions. We selected only those interactions concerning human miRNAs and human gene targets proved by strong experimental evidence method (Reporter assay, Western blot or qPCR technique) [17].

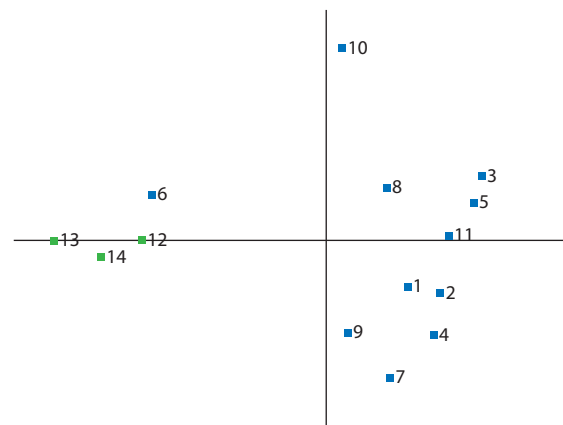
### Statistical analysis

Continuous variables were presented as mean values with respective standard deviations. Selected miRNA expression levels in TNBC tissues and normal tissues were compared using a Student t-test after adjustment for Benjamini-Hochberg multiple comparison (False Discovery Rate). Assessment of correlation between a particular miRNA expression and the patients' age was performed with Pearson correlation. Association between tumor size (T stage) and lymph node status (N stage) was verified by linear trend analysis. For multidimensional data — Principal Component Analysis and Unsupervised Hierarchical Clustering was performed. Statistical analysis was done with MultiExperiment Viewer (MeV) software (Dana Farber, Boston, USA) and STATISTICA 10.0 software (Statsoft, Tulsa, OK, USA). P values lower than 0.05 were considered as statistically significant.

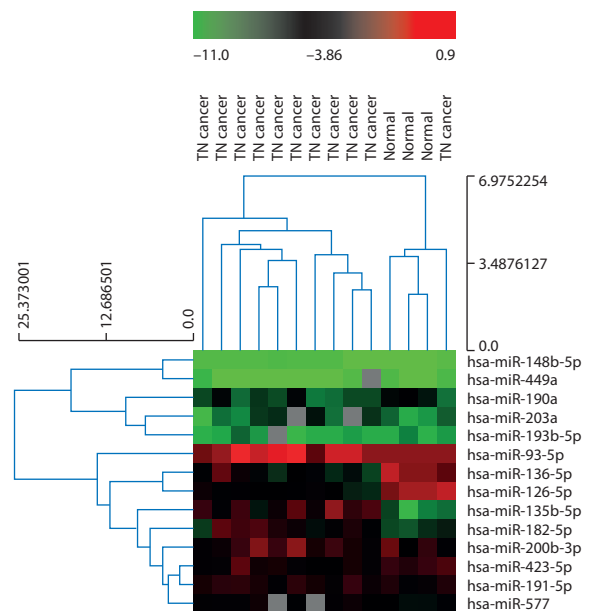
## RESULTS

Ten common miRNAs were identified in all samples with an average of 18 miRNAs detectable per sample. MicroRNAs detected in all control samples and in more than 8 TNBC samples were further analyzed. Thus, 14 miRNAs have been included in the Principal Component Analysis (PCA) (Fig. 1) and in the two-way hierarchical clustering of miRNAs (Fig. 2). Samples seem to cluster based on group information provided; however, sample 9 falls closer to the normal tissue samples than to the TNBC (Fig. 1 and Fig. 2).

The analysis revealed that five miRNAs (miR-136-5p, miR-126-5p, miR-182-5p, miR-190a, miR-135b-5p) have a significantly different expression in TNBC tissue when compared with healthy controls (Table 1). We observed a significant down-regulation of miR-190a, miR-136-5p and miR-126-5p (dCp  $-5.95 \pm 0.91$  vs.  $-4.36 \pm 0.28$ ,



**Figure 1.** The Principal Component Analysis plot. PCA was performed on all samples and on those assays with a count of at least 12 (missing values are substituted by group averages). The normalized (dCp) values have been used for the analysis. The analysis was performed on 3 normal breast tissues (green color) and 11 TNBC tissues (blue color)



**Figure 2.** Heat map and unsupervised hierarchical clustering. The clustering is performed on all samples and on those assays with a count of at least 12. The color scale illustrates the relative expression level of microRNA across all samples: red color represents an expression level above mean, green color represents expression lower than the mean. The normalized (dCp) values have been used for the analysis

$p = 0.0041$ , dCp  $-4.03 \pm 1.21$  vs.  $-0.94 \pm 0.39$ ,  $p = 0.0007$ , dCp  $-3.61 \pm 1.18$  vs.  $-1.06 \pm 0.31$ ,  $p = 0.0007$ , respectively). The following in turn were up-regulated: miR-135b-5p and miR-182-5p (dCp  $-3.13 \pm 1.44$  vs.  $-7.08 \pm 0.91$ ,  $p = 0.0194$ , dCp  $-3.48 \pm 1.13$  vs.  $-5.79 \pm 0.41$ ,  $p = 0.0041$ , respectively) (Table 1). The association between tumor size (T), lymph node status (N) and patient's age and miRNAs expression is presented in Table 2.

**Table 1.** Selected miRNAs expressed in normal breast tissues and in TNBC tissues

miRNA names	Count	Average dCp ± standard deviation		p-value	FDR*
		TNBC patients	Healthy controls		
hsa-miR-136-5p	14	-4.03 ± 1.21	-0.94 ± 0.39	7.34E-05	0.0007
hsa-miR-126-5p	14	-3.61 ± 1.18	-1.06 ± 0.31	8.89E-05	0.0007
hsa-miR-182-5p	14	-3.48 ± 1.13	-5.79 ± 0.41	0.0008	0.0041
hsa-miR-190a	14	-5.95 ± 0.91	-4.36 ± 0.28	0.0010	0.0041
hsa-miR-135b-5p	14	-3.13 ± 1.44	-7.08 ± 0.91	0.0061	0.0194
hsa-miR-577	12	-3.57 ± 0.34	-4.37 ± 0.29	0.0333	0.0888
hsa-miR-423-5p	14	-3.17 ± 0.54	-2.70 ± 0.15	0.0415	0.0948
hsa-miR-93-5p	14	-0.40 ± 1.04	-1.14 ± 0.05	0.0488	0.0977
hsa-miR-203a	12	-6.44 ± 1.38	-7.65 ± 0.78	0.1547	0.2751
hsa-miR-148b-5p	14	-10.65 ± 0.48	-11.09 ± 0.33	0.1878	0.3005
hsa-miR-191-5p	14	-3.00 ± 0.28	-3.23 ± 0.23	0.2984	0.3979
hsa-miR-449a	13	-11.03 ± 0.75	-11.21 ± 0.82	0.7935	0.8521
hsa-miR-200b-3p	14	-2.70 ± 0.76	-2.88 ± 0.85	0.7989	0.8521
hsa-miR-193b-5p	13	-8.09 ± 0.59	-8.08 ± 0.58	0.9832	0.9832

\*False Discovery Rate

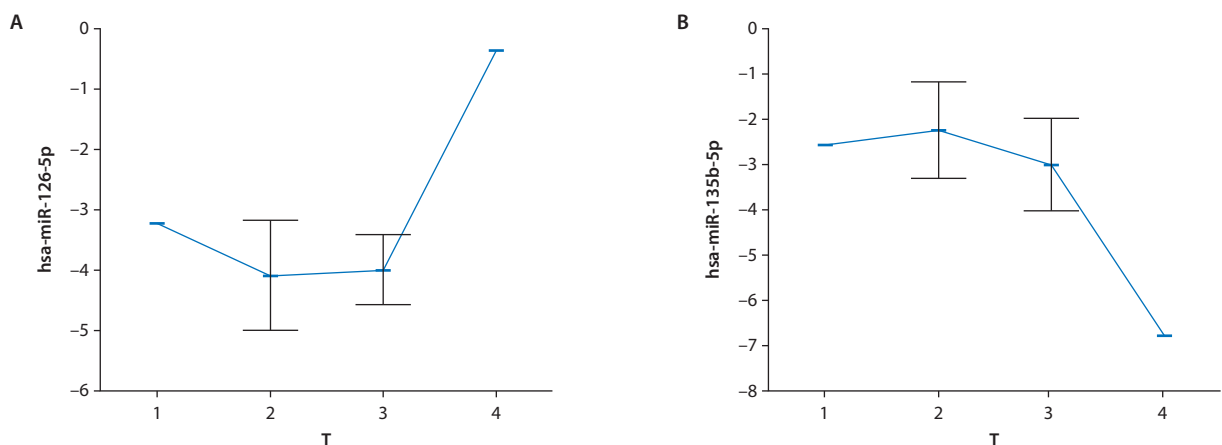
**Table 2.** P values for association of miRNA expression with patient age, tumor size (T stage) and lymph node status (N stage)

miRNA name	T	N	Age
hsa-miR-136-5p	0.0681	0.4447	0.4627
hsa-miR-126-5p	0.0296	0.7999	0.4627
hsa-miR-182-5p	0.1491	0.1186	0.7473
hsa-miR-190a	0.5595	0.8718	0.6667
hsa-miR-135b-5p	0.0241	0.5629	0.7678

[For T and N, p value was calculated with linear trend whereas for Age with Spearman correlation]

We noticed a significant linear trend for T value and expression levels of miR-126-5p ( $p = 0.0296$ ) and miR-135b-5p ( $p = 0.0241$ ) (Figure 3). Moreover, miR-126-5p correlated positively with expression of miR-136-5p ( $r = 0.61$ ,  $p = 0.0444$ ) and negatively with miR-135b-5p ( $r = -0.72$ ,  $p = 0.0124$ ).

Moreover, we searched for 5 selected miRNA validated target genes. MiRTarBase has showed associations with 50 genes with strong evidenced for being targets for one of the 5 miRNAs (Table 3).

**Figure 3.** Association between hsa-miR-126-5p (A), hsa-miR-135b-5p (B) and tumor size (T stage)

**Table 3. Human target genes for significantly dysregulated human microRNAs in a TNBC from miRTarBase, confirmed by strong evidence validation methods (Reporter assay, Western blot, qPCR)**

MicroRNA name	Target gene name
hsa-miR-136-5p	MTDH, BCL2, PPP2R2A
hsa-miR-126-5p	SLC45A3, PTPN7, ADAM9, MMP7, CXCL12, MYC
hsa-miR-182-5p	CDKN1A, FOXO3, MTSS1, FOXO1, RARG, MITF, ADCY6, CLOCK, TSC22D3, CREB1, FGF9, NTM, CYLD, BCL2, CCND2, PDCC4, PFN1, SNAI2, RECK, SMAD4, FOXF2, FLOT1, PTEN, GSK3B, ANUBL1, SATB2, CHL1, CADM1, TP53INP1, ATF1, BARD1, CREB5, RAD17, TP53BP1, CHEK2, CDKN1B, SMARCD3, TCEAL7, FBXW7, LRRCC4, NDRG1, THBS1, ULBP2, BDNF, EP300
hsa-miR-190a-5p	CDKN1B, PHLPP1, KCNQ5, MARK2, IGF1
hsa-miR-135b-5p	APC, KLF4, MAFB, CASR, PPP2R5C, SMAD5, LZTS1, MID1, MTCH2, ACVR1B, BMPR2, TGFBR1, IBSP, BGLAP, RUNX2, SP7

## DISCUSSION

We found that expression of miR-182-5p and miR-135b-5p was significantly increased while that of miR-190a, miR-136-5p and miR-126-5p was significantly reduced in TNBC tissues in comparison with normal breast tissues. We observed a linear trend for T stage and expression of miR-126-5p and miR-135b-5p. In the study we did not find a significant association between the expression level of miRNAs and patients' age of onset or lymph node status.

MiR-182-5p belongs to the miR-183 family and miR-182-5p is encoded on chromosome 7q31-34 [18]. Medimegh et al. (2014) examined expression of miR-182 in the following three groups: TNBC, NTNBC (Non Triple Negative Breast Cancer; Luminal A, Luminal B and HER2 positive patients) and healthy controls. The analysis showed significantly higher expression of miR-182 in TNBC with respect to NTNBC cases. Moreover, the expression of miR-182 in both cancer groups was still higher than in healthy controls [19]. In present study we confirm the increased expression of miR-182-5p in TNBC patients.

It was reported that miR-182-5p is important for the tumor development as miR-182 has been associated with DNA damage repair, cell proliferation and apoptosis [20]. MiR-182 inhibits expression of *BRCA1* [21] that is responsible for repair of double-stranded DNA damage (DSBs) in homologous recombination (HR). Deficiency of *BRCA1* protein leads to activation of other than HR repair mechanisms, the inefficiency of which causes genetic instability [22]. Moreover, silencing of miR-182-5p resulted in increase of *BRCA1* levels and induction of resistance to radiation and to treatment with poly (ADP-ribose) polymerase 1 (PARP-1) inhibitors [21]. This phenomenon could be explained by activation of *BRCA1*-related tumor cell repair mechanism. Therefore, tumor cells do not activate the apoptotic pathway and become more resistant to treatment [23]. Medimegh et al. have noticed a significant association of lymph node metastases with miR-182 overexpression in TNBC of Tunisian population [19]. In present study we did not find any significant association between miR-182-5p expression and lymph node status, which is consistent with results of Krishnan et al. (2013) [24].

In our study we also noticed a significantly higher expression of miR-135-5p. The knowledge about miR-135-5p function in TNBC development is limited. Up-regulated miR-135b was so far identified only in basal like subtype of breast cancer and estrogen-negative patients. In turn, miR-135b expression was correlated with worse survival of breast cancer patients and metastasis status [25]. In the study of Muñoz-Rodríguez et al. (2015) it was showed that miR-135b expression levels are higher in patients with breast cancer in early postpartum period compared to late postpartum period. Moreover, the miR-135b gene expression and its DNA methylation had a significant inverse correlation [26]. MiR-135b has been showed to be up-regulated in breast cancer [27], which is consistent with our study. Moreover miR-135b promotes cellular proliferation and disrupts the cell cycle of breast cancer cells by regulating *LATS2* [27]. In our study we show a linear trend for T stage and expression of miR-135b-5p; we observe lower expression in higher tumor size.

Our study also showed that miR-190a, miR-126-5p and miR-136-5p are down-regulated in TNBC tissues. Hao et al. (2014) brought attention to the role of miR-190a in suppression of tumor metastasis via interaction with VEGF-mediated tumor angiogenesis [28]. In breast cancer, overexpression of miR-190a has inhibited the cell migration and invasiveness. Moreover, miR-190a expression in primary breast carcinomas correlated with overall survival [29]. Nevertheless, the role of miR-190a in breast cancer biology is still unclear.

We also report down-regulated miR-126-5p expression in TNBC. A different study has revealed that miR-126 plays a tumor-suppressor role and impedes the metastasis of non-small cell lung cancer by inhibiting tumor growth and cell invasion [30]. Moreover, miR-126 target genes include a variety of oncogenic factors such as *ADAM9* [31]. The loss of miR-126 expression was associated with poor distal metastasis-free survival of breast cancer patients [32]. In our study there is linear trend for miR-126; we observed higher miR-126 levels in bigger tumor size.

When it comes to miR-136, it has been shown that it is up-regulated in lung cancer [33]. In turn, Zhao et al. ob-

served low expression of miR-136 in patients with chemoresistant epithelial ovarian cancer [34]. Generally, it has been demonstrated that miR-136 was down-regulated in TNBC [35], which is consistent with our study. Yan et al. showed that suppression of tumor invasion and metastasis by miR-136 was mediated through targeting RASAL2, which plays oncogenic role in TNBC. Moreover, expression of miR-136 was negatively correlated with WHO grades in TNBC [35].

In summary, the present study confirms the aberrant expression of miRNAs in TNBC patients when compared to healthy tissues. Such results indicate the influence of epigenetic factors in the development of TNBC. We have demonstrated a dysregulated expression of 5 miRNAs in TNBC tissues. Moreover, we have found a linear trend for tumor size and expression levels of miR-126-5p and miR-135b-5p.

Yet, it is necessary to verify our results on a larger population. The present study provides further experimental data for these candidate miRNAs in order to aid the identification of reliable molecular diagnostic markers of TNBC in the future.

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### Author disclosure statement

None.

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