

Circulating non-coding RNA-biomarker potential in neoadjuvant chemotherapy of triple negative breast cancer?

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Received April 18, 2019; Accepted September 26, 2019

DOI: 10.3892/ijo.2019.4920

Abstract. Due to the positive association between neoadjuvant chemotherapy (NACT) and the promising early response rates of patients with triple negative breast cancer (TNBC), including probabilities of pathological complete response, NACT is increasingly used in TNBC management. Liquid biopsy-based biomarkers with the power to diagnose the early response to NACT may support established monitoring tools, which are to a certain extent imprecise and costly. Simple serum- or urine-based analyses of non-coding RNA (ncRNA) expression may allow for fast, minimally-invasive testing and timely adjustment of the therapy regimen. The present study investigated breast cancer-related ncRNAs [microRNA (miR)-7, -9, -15a, -17, -18a, -19b, -21, -30b, -222 and -320c, PIWI-interacting RNA-36743 and GlyCCC2] in triple positive BT-474 cells and three TNBC cell lines (BT-20, HS-578T and MDA-MB-231) treated with various chemotherapeutic agents using reverse transcription-quantitative PCR. Intracellular and secreted microvesicular ncRNA expression levels were analysed using a multivariable statistical regression analysis. Chemotherapy-driven effects were investigated by analysing

cell cycle determinants at the mRNA and protein levels. Serum and urine specimens from 8 patients with TNBC were compared with 10 healthy females using two-sample t-tests. Samples from the patients with TNBC were compared at two time points. Chemotherapeutic treatments induced distinct changes in ncRNA expression in TNBC cell lines and the BT-474 cell line in intra- and extracellular compartments. Serum and urine-based ncRNA expression analysis was able to discriminate between patients with TNBC and controls. Time point comparisons in the urine samples of patients with TNBC revealed a general rise in the level of ncRNA. Serum data suggested a potential association between piR-36743, miR-17, -19b and -30b expression levels and an NACT-driven complete clinical response. The present study highlighted the potential of ncRNAs as liquid biopsy-based biomarkers in TNBC chemotherapy treatment. The ncRNAs tested in the present study have been previously investigated for their involvement in BC or TNBC chemotherapy responses; however, these previous studies were restricted to patient tissue or *in vitro* models. The data from the present study offer novel insight into ncRNA expression in liquid samples from patients with TNBC, and the study serves as an initial step in the evaluation of ncRNAs as diagnostic biomarkers in the monitoring of TNBC therapy.

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Abbreviations: BC, breast cancer; TNBC, triple negative breast cancer; NACT, neoadjuvant chemotherapy; ACT, adjuvant chemotherapy; pCR, pathological complete response; US, ultrasound; MRI, magnetic resonance imaging; ncRNA, non-coding RNA, miRNA/miR, micro RNA; piRNA/piR, PIWI-interacting RNA; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; Cq, quantification cycle; cCR, complete clinical response; CI, confidence interval; cPR, clinical partial response

Key words: ncRNA, triple negative breast cancer, liquid biopsy, neoadjuvant chemotherapy, therapy response, biomarker

Introduction

As the most frequent malignancy occurring in females, breast cancer (BC) represents a great threat to women's health. In 2018, the World Health Organization registered 2,088,849 BC cases and 626,679 BC-associated deaths globally (1). However, the survival rate for patients with BC has been increasing since the 1990s. One reason for this is the improvement in systemic treatment concepts (2,3). Moreover, molecular subtyping has been implemented in clinical practice as an important tool for risk-adapted therapy in patients with BC (4-7).

Triple negative breast cancer (TNBC) is classified as the most aggressive molecular subtype of BC (8). TNBCs are estimated to comprise ~15% of all cases of BC (9,10), and its occurrence is highest in younger females (9) and those of African-American ancestry (8,11). The prognosis for women

diagnosed with TNBC is less favourable than for those suffering from other intrinsic subtypes (12). Characterised by the absence of hormone receptors and a lack of Her2neu (ErbB2) gene amplification, TNBCs are heterogeneous carcinomas (9) and are often poorly differentiated. Several attempts have been made to further categorise TNBC subtypes according to distinct gene expression patterns. For example, Lehmann *et al* (13) analysed the gene expression profiles of 587 TNBC cases and identified six distinct TNBC subtypes: Two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem-like and a luminal androgen receptor subtype.

TNBC can be further classified into the basal-like subtype (12) or the claudin-low subtype (4). The terms 'basal-like' BC and TNBC are often used interchangeably; however, they do not describe the same condition, as not all basal-like BCs are classified as TNBC and *vice versa*. Most basal-like BC cases present without hormone receptor expression and Her2neu gene amplification, as in TNBC (14); in a study performed by Bertucci *et al* (15), ~71% of TNBCs exhibited basal-like gene expression. Characteristic basal-like markers comprise cytokeratin 5/6 and epidermal growth factor receptor (14). The claudin-low intrinsic subtype, which was described in 2007, is characterised by the high expression of epithelial-mesenchymal transition markers and cancer stem cell-like features, among other traits (4). Unlike the basal-like subtype, the claudin-low intrinsic subtype exhibits lower expression of genes associated with proliferation, such as Ki67 (4). Prat *et al* (4) reported that the prognosis of the claudin-low subtype was poorer than that of luminal A, but improved compared with that of the basal-like subtype.

Due to the absence of hormone receptors and the lack of Her2neu gene amplification, therapy for patients with TNBC is restricted to neoadjuvant chemotherapy (NACT), radiotherapy and surgery (16). In the case of NACT, chemotherapy is administered prior to surgery, while in adjuvant chemotherapy (ACT), surgery precedes the chemotherapy treatment. NACT is considered to be equivalent to ACT in terms of clinical outcome, and has become an established treatment in BC therapy (17-21). Additionally, fewer adverse effects were observed with NACT compared with ACT (17). The primary endpoint of NACT is defined as the pathologically determined response of the tumour in the breast and the axillary lymph nodes. Achieving pathological complete response (pCR) at the time of surgery, defined as no residual invasive or non-invasive tumour tissue in the breast and the axillary lymph nodes, represents an important surrogate marker for beneficial overall survival in these patients (22-27). Further advantages of NACT treatment can be seen in the increasing rate of breast conserving surgery and the evaluation of short-term surrogate markers (pathological, clinical and molecular) for outcome prediction (21,27,28). In this context, it is known that an early tumour response (after 1-2 cycles of NACT) is also a predictive factor for pCR, and offers the opportunity to distinguish responders from non-responders at a very early time point of therapy (27). This early determination of poor response offers the chance to alter treatment regimens and drugs with the goal of achieving an optimized response up to a pCR. Therefore, reliable measures to determine early response to NACT represent an important prerequisite for innovative

personalised treatment concepts for the improvement of the clinical outcome for patients with non-responding tumours. The NACT regime for patients with TNBC includes both an anthracycline and a taxane that should be administered for 18-24 weeks (28). The addition of platinum, irrespective of breast cancer susceptibility protein status, increases pCR rates; however, it is accompanied by enhanced drug-dependent toxicity in patients with TNBC (7). A variety of diagnostic possibilities, including ultrasound (US), palpation, mammography, magnetic resonance imaging (MRI) and positron emission tomography/computed tomography, are available for response monitoring during NACT (28). Although US measurements are used routinely in the clinic to assess tumour response to NACT, few data exist concerning the accuracy of US for the prediction of pCR (29,30). Morphological reactions of solid tumours during therapy (for example, fragmentation and tumour necrosis with or without stromal reaction) may compromise the accuracy of US in discriminating between responding and non-responding tumours. A previous study performed by Chagpar *et al* (31) investigated the accuracy of physical examination, US and mammography in predicting residual tumour size in NACT-treated patients with BC, and found only a moderate correlation. MRI appears to be an accurate method to detect residual disease; however, the false-negative rate is increased in patients receiving chemotherapy. Furthermore, these methods depend on tumour size reduction, which can, in certain cases, take months (32).

As an early response to NACT seems to be associated with achieving pCR and an improved prognosis for patients with TNBC, prediction of therapy response is particularly important in this BC subgroup (24,33-35). A poor prognosis for patients with TNBC improves to a similarly high level if patients achieve a pCR (34). This underlines the need for more specific and sensitive predictive methods. Liquid biopsy-based non-coding RNAs (ncRNAs) in the serum and urine of patients undergoing NACT as innovative biomarkers may be a promising tool to predict early therapy response before the current standard imaging methods are employed.

ncRNAs comprise several classes, according to size, origin and function (36,37). The evidence for the involvement of ncRNAs in carcinogenesis is increasing (38). As ncRNAs are secreted by their cells of origin, they can be detected in body fluids, including serum and urine (39-41). Several previous studies have reported an association between ncRNA expression, chemotherapy response and resistance (42-45). Therefore, ncRNAs may serve as liquid-biopsy-based biomarkers to indicate early response to NACT in TNBC.

To pursue this question, the present study examined the expression of 14 ncRNAs, including 12 microRNAs (miRNA/miRs; let-7a; let-7e; miR-7, -9, -15a, -17, -18a, -19b, -21, -30b, -222 and -320c), one PIWI-interacting RNA (piRNA; piR-36743) and one transfer RNA (GlyCCC2) in a triple positive (BT-474) and three TNBC (BT-20; HS-578T; MDA-MB-231) cell lines treated with the cytostatic drugs carboplatin, epirubicin, gemcitabine and paclitaxel, both intra- and extracellularly. Analysis of cell cycle determinants [cyclin D1, DNA topoisomerase 2 α (TOP2 α) and targeting protein for Xklp2 (TPX2)] and RNA metabolic proteins [DEAD-box polypeptide (DDX)5 and DDX17] were performed at the mRNA and protein level to investigate drug-dependent

effects on the cell cycle. Subsequently, ncRNA expression was analysed in serum and urine specimens from patients with TNBC and healthy controls. Additionally, alterations in ncRNA expression in patients with TNBC prior to NACT (t_0) and immediately prior to the third cycle of therapy (t_1) were investigated.

Materials and methods

Cell culture conditions and treatments. BC cell lines BT-474 and BT-20 (cat. nos. 300130 and 300131; CLS Cell Lines Service GmbH), HS-578T (cat. no. 86082104; Sigma-Aldrich; Merck KGaA) and MDA-MB-231 (cat. no. 92020424; Sigma-Aldrich; Merck KGaA) (46) were incubated in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% HEPES buffer (Gibco; Thermo Fisher Scientific, Inc.) and 1% 100 U/ml penicillin/streptomycin (Sigma-Aldrich; Merck KGaA). Insulin (2.5%; Insuman[®] rapid; Sanofi S.A.) was added to BT-474 cells. Cells were incubated under humidified conditions at 37°C and 5% CO₂. The cell lines were authenticated using PCR-single-locus-technology. Mycoplasma testing is performed regularly.

Cells were treated with 2.0 µg/ml carboplatin (Abmole Biosciences Inc.), 1.0 µg/ml epirubicin (Sigma-Aldrich; Merck KGaA), 40.0 µg/ml gemcitabine (Abmole Bioscience Inc.) or 2.0 µg/ml paclitaxel (Sigma-Aldrich; Merck KGaA) in DMSO-free solution for 18 h. Untreated cells served as a control.

Acquisition of patient samples. Female patients with histologically proven, invasive TNBC as defined by the St. Gallen Consensus 2012 (47), who were subjected to NACT were included in the present study. The inclusion criteria were as follows: i) Primary diagnosis of TNBC; and ii) age of ≥18 years. The exclusion criteria were as follows: i) Distant metastasis at the time of diagnosis; and ii) other previous or coexistent malignancies. The present study was approved by the institutional ethical review board of the University of Freiburg (permit no. 607/16) and written informed consent was obtained from each participant. Patients (aged 40-70 years) of the Department of Gynaecology and Obstetrics at the Medical Center-University of Freiburg were enrolled between February 2017 and September 2017; additionally, 20 healthy controls (aged 23-70 years) were enrolled between March 2016 and August 2016, of whom 10 provided serum samples, and 10 provided urine samples. All patients received NACT according to standard treatment protocols [90 mg/m² epirubicin + 600 mg/m² cyclophosphamide (EC) x4 (once every 3 weeks), followed by 80 mg/m² paclitaxel (P) x12 (weekly)] (28). BC tissue biopsies were collected prior to NACT, and evaluated via haematoxylin and eosin (H&E) and Ki67 staining by the Pathology Department of the Medical Center-University of Freiburg. Histopathological preparations of BC were graded according to the classification published by Elston and Ellis (48) on H&E-stained slides, which includes counting of mitotic cells. Furthermore, the proliferation index was measured in sections immunohistochemically stained with the Ki67 antibody clone MIB-1. Any nuclear positivity (nucleoli excluded) was counted. The percentage of positive nuclei was either calculated from counting at least 200 tumour cells or

estimated semi-quantitatively, especially for percentages <10 and >30%. In total, 9 patients with TNBC were enrolled. Patient characteristics are displayed in Table I. Blood (2-9 ml) was drawn by physicians at two time points (t_0 , prior to NACT; t_1 , immediately prior to third cycle of NACT), according to standard protocols. In order to evaluate the individual response of each patient to NACT, follow-up by US was performed and interpreted, according to the RECIST criteria (30). After coagulation for 20 min, whole blood was centrifuged (10 min, 2,000 x g, 4°C). Cell-free serum specimens were transferred to a fresh test tube. Samples exhibiting haemolytic characteristics were excluded. In total, 20 ml of urine was centrifuged (15 min, 2,000 x g, 4°C) and the cell-free supernatant was transferred to fresh test tubes. Serum and urine samples were stored at -20°C. Of the nine serum and urine samples collected from patients with TNBC, only eight met quality standards and were further analysed.

Isolation of ncRNA. Intracellular ncRNA was isolated using an EURx[®] GeneMATRIX Universal RNA/miRNA Purification kit (EURx Sp. z o.o.) according to the manufacturer's protocol. For the isolation of secreted microvesicular extracellular ncRNA, conditioned cell culture medium from BC cells was decanted into a 15-ml tube and centrifuged at 4°C at 8,000 x g for 5 min. In total, 10 ml supernatant was transferred to a new 15-ml tube. Microvesicles from liquid samples (conditioned cell culture medium, serum and urine) were isolated via filtration. RNA was subsequently isolated using a Norgen Total RNA Purification kit (Norgen Biotek Corp.), according to the manufacturer's protocol. In total, 20 ml urine and 2 ml serum were applied. The RNA concentration was determined using a NanoDrop[™] ND1000 (Thermo Fisher Scientific, Inc.) and RNA was stored at -20°C until further processing.

Reverse transcription (RT). cDNA was generated in a total reaction volume of 20 µl using poly(A) tailing-based RT. The RT reaction mix was as follows: 4 µl RT-buffer (5X), 1 µl 2.5 µM poly A adapter/primer (Integrated DNA Technologies, Inc.), 1 µl 5 mM dNTPs (Jena Bioscience), 0.25 µl Maxima[™] H Minus reverse transcriptase (Thermo Fisher Scientific, Inc.), 0.25 µl SUPERase In[™] RNase inhibitor (Thermo Fisher Scientific, Inc.), 0.5 µl 10 mM ATP (New England BioLabs, Inc.), 0.25 µl poly A polymerase (New England BioLabs, Inc.) and the required amount of RNA sample. Briefly, 1,000 ng of isolated intracellular ncRNA and 50 ng of isolated microvesicular extracellular ncRNA were used for RT. Due to the small amount of ncRNA in serum and urine, a fixed volume of 5 µl was used. RT was performed at 42°C for 30 min and 85°C for 10 min. Processed cDNA was stored at 4°C. Additionally, 2,000 ng of RNA was used for the RT of mRNA. The RT reaction mix contained 5 µl RT-buffer (5X), 1 µl 5 mM dNTPs (Jena Bioscience), 1 µl RT primer (10 µM), 0.25 µl Maxima[™] H Minus reverse transcriptase (Thermo Fisher Scientific, Inc.), 0.25 µl SUPERase In[™] RNase inhibitor (Thermo Fisher Scientific, Inc.) and the RT was carried out in a total volume of 25 µl. The RT temperature protocol was performed as follows: 65°C for 1 min, 25°C for 10 min, 50°C for 45 min and 85°C for 10 min.

Quantitative PCR (qPCR). In total, 1 µl cDNA at a concentration of 5 ng/µl was used for qPCR at a 1:10 dilution with the

Table I. Characteristics of patients with TNBC and the control cohort.

A, Patients with TNBC						
Characteristics	Serum cohort (n=8)			Urine cohort (n=8)		
	N	%	Mean	N	%	Mean
Age	8		55.4	8		53.6
Initial tumour stage (T)						
cT1	6	75.0		6	75.0	
cT2	2	25.0		2	25.0	
Initial nodal status (N)						
cN0	7	87.5		7	87.5	
n.a.	1	12.5		1	12.5	
Initial tumour grade						
G2	4	50.0		3	37.5	
G3	4	50.0		5	62.5	
Lymphovascular invasion (L)						
L0	5	62.5		4	50.0	
L1	1	12.5		1	12.5	
n.a.	2	25.0		3	37.5	
Proliferation rate (MIB-1 reactivity)		64.3			60.3	
Menopausal status						
Premenopausal	1	12.5		2	25.0	
Postmenopausal	4	50.0		4	50.0	
n.a.	3	37.5		2	25.0	
Early response ^a	3	37.5		4	50.0	
cCR ^b	4	50.0		4	50.0	
pCR ^c	3	37.5		5	62.5	
B, Controls						
Control cohort	Serum cohort (n=10)		Urine cohort (n=10)			
	N	Mean	N	Mean		
Age	10	52.0	10	49.5		

^aAccording to RECIST criteria, defined as clinically partial response or cCR immediately prior to the third cycle of NACT, and determined by ultrasonography. ^bAchievement of cCR throughout the course of NACT. ^cAchievement of pCR. All patients displayed a histological subtype of invasive ductal carcinoma. TNBC, triple negative breast cancer; cCR, clinically complete response; pCR, pathological complete response; NACT, neoadjuvant chemotherapy; MIB-1, anti-Ki67 antibody; n.a., no information available.

master mix. Master mix was composed of 1 μ l qPCR buffer (10X), 0.5 μ l 5 mM dNTPs (Jena Bioscience), 0.5 μ l SYBR[®] Green (Jena Bioscience), 6.45 μ l nuclease-free water (Analytik Jena AG), 0.05 μ l HotStart Taq polymerase (Jena Bioscience) and 0.5 μ l primer pair mix. The primers were designed using miRprimer (49). Sequences of specific sense and antisense primers are listed in Table SI. Duplicate analysis was performed on a LightCycler[®] 480 Instrument II (Roche Diagnostics) with pre-incubation at 95°C for 2 min and a two-step amplification of 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Based on an extensive literature review (date, 28/02/2019) employing the academic web resource PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>) with a focus on (TN)BC-related ncRNAs and their involvement in chemotherapy response and resistance, the following panel of ncRNAs was selected and subsequently analysed: Let-7a; let-7e; miR-7, -9, -15a, -17, -18a, -19b, -21, -30b, -222 and -320c; piR-36743; and GlyCCC2. The following key words were used: 'miRNA' or 'piRNA', combined with 'breast cancer' (4,571 and 4,581 hits, respectively) and 'TNBC' (261 and 157 hits, respectively). The literature review on the chosen panel of ncRNAs was extended by searching for each target name individually combined with the key words 'breast cancer', 'TNBC', 'chemotherapy', 'chemoresponse' and 'chemoresistance'. The findings from this literature search are summarised in Table II.

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According to the $\Delta\Delta Cq$ method (50,51), relative quantification of intracellular ncRNA expression was performed by normalising the quantification cycle (Cq) values to the geometric mean of the two endogenous controls, RNU-44 and -48 (51-54), which were determined as stably expressed using the BestKeeper software tool (55). Due to a lack of reliable endogenous controls in conditioned cell culture media, raw data of extracellular microvesicular ncRNA were normalised to the global mean of all assessed ncRNAs in this setting. Although recommended for larger data sets, this approach is commonly accepted when no suitable endogenous control can be established and can lead to more accurate performance than normalising to stable genes (52,56,57). Within the analysed serum samples, BestKeeper identified miR-26b and -191 as the most suitable endogenous controls. Similarly, miR-16 and -26b served as endogenous controls in the urine-based analysis (58,59). Additionally, 5 fmol of exogenous synthetic miRNA from *Arabidopsis thaliana* (ath-miR-159a) and *Caenorhabditis elegans* (cel-miR-39; both from biomers.net GmbH) were mixed with serum and urine samples as spike-in controls to optimise normalisation (60).

The targets of the mRNA analysis included cyclin D1, TOP2 α , TPX2, DDX5 and -17. Cq values were normalised to the housekeeping gene, 5'-aminolevulinic synthase 1 (61).

Protein isolation and western blotting. Proteins from the BC cell lines were isolated from the flow-through of the RNA-binding columns used to purify RNA by the addition of 1 ml of isopropanol and subsequent centrifugation for 30 min at 12,000 x g at 4°C. The protein pellet was washed with 70% ethanol and lysed in protein lysis buffer (1% SDS, 1% triton, 1 mM EDTA, 0.1 M HEPES) for 30 min at 1,000 RPM and 50°C in an Eppendorf ThermoMixer®. Protein concentrations were determined using the bicinchoninic acid method as previously described (62). Western blotting was conducted as described by Schagger and von Jagow (63). Briefly, 10 μ g/lane of protein was separated on a 10% SDS gel. Proteins were transferred onto a 0.45- μ m PVDF membrane (Sigma-Aldrich; Merck KGaA) and membranes were blocked with 3% skimmed milk in PBS-Tween 20 at room temperature for 30 min. Monoclonal mouse anti-human antibodies for immunodetection were purchased from Santa Cruz Biotechnology, Inc. The following dilutions were used: Cyclin D1 (1:500; cat. no. sc-8396); DDX5 (1:5,000; cat. no. sc-365164); DDX17 (1:10,000; cat. no. sc-398168); β -Actin (1:1,000; cat. no. sc-47778); and proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. sc-56). Membranes were incubated with primary antibodies overnight at 4°C. A secondary peroxidase-conjugated anti-mouse antibody was purchased from Jackson ImmunoResearch Europe, Ltd. (cat. no. 115-036-146) and was incubated at room temperature with the membrane for 1 h (1:10,000). Bands were visualised using ECL reagent (Santa Cruz Biotechnology, Inc.). PCNA and β -Actin served as loading controls.

Statistical analysis. All statistical tests were performed in R 3.5.2 (64). Multivariable statistical analysis of *in vitro* results, including three independent experimental repeats, consisted of three factors: Cell line (BT-474, BT-20, HS-578T, MDA-MB-231), treatment (control, carboplatin, epirubicin, gemcitabine, paclitaxel) and compartment (intracellular,

extracellular). The untreated BT-20 cell line was defined as the expected control value (intercept) in the statistical data assessment. A linear model for the log-transformed *in vitro* results was selected with the main effect of the cell line, three two-way interactions of the cell line and treatment, cell line and compartment, and treatment and compartment, and the three-way interaction of all three factors. *In vivo* results were statistically analysed by performing a two-sample t-test comparing patients with TNBC to the healthy control group. For a time point comparison (t_0 vs. t_1) within the TNBC group, a two-tailed paired t-test was applied. Preliminary two-tailed paired t-tests were performed for the serum data in a clinical complete response (cCR) vs. no cCR subgroup analysis, defined by the RECIST criteria (30). $P < 0.05$ indicated a statistically significant difference.

Results

In the present study, the influence of chemotherapeutic treatment on the expression levels of 14 BC-related ncRNAs (let-7a/e, miR-7, -9, -15a, -17, -18a, -19b, -21, -30b, -222 and -320c, piR-36743 and GlyCCC2) was investigated in three TNBC (BT-20, HS-578T and MDA-MB-231) cell lines and one triple positive BC (BT-474) cell line, both intra- and extracellularly.

All examined cell lines expressed all of the 14 investigated ncRNA targets. In addition to intracellular expression, the vast majority of the panel were detectable as secreted molecules in microvesicles in cell culture medium, with the exception of miR-7, -9 and -15a in the medium of MDA-MB-231, and miR-9 in the medium of HS-578T cells. Chemotherapy treatment of cells with carboplatin, epirubicin, gemcitabine and paclitaxel resulted in distinct ncRNA expression alterations depending both on the cytotoxic agent and on the specific BC cell line (Fig. 1).

Epirubicin. Epirubicin treatment (1.0 μ g/ml) influenced intracellular and secreted microvesicular expression levels of all the ncRNAs in the panel in all the cell lines investigated. A uniform downregulation of all analysed targets, except for piR-36743 in BT-474 was observed, intracellularly. The levels of secreted microvesicular ncRNAs did not suggest a distinct regulation pattern in respect of target and cell line (Fig. 1).

Multivariable analysis confirmed the inhibitory effect of epirubicin on ncRNA expression, especially in hormone receptor positive BT-474 and TNBC cell lines BT-20 and MDA-MB-231. miR-7, -15a, -17, -18a, -19b, -21, -30b and -222 were downregulated in BT-474, BT-20 and MDA-MB-231 cell lines compared with the intercept, while reduced expression of miR-9 was restricted to BT-474 and MDA-MB-231 cells. GlyCCC2 expression was downregulated in BT-20 and MDA-MB-231 cells. piR-36743 expression decreased following epirubicin treatment in MDA-MB-231 cells, while an increase in expression was found in BT-474 cells. In HS-578T, the effect of epirubicin was limited to miR-17 and -18a expression. All estimates of multivariable analysis, including confidence intervals (CIs) and P-values are listed in Table SII.

Gemcitabine. Gemcitabine (40.0 μ g/ml) predominantly triggered a decrease in the expression of ncRNAs in the

Table II. Involvement of investigated ncRNAs in (TN)BC and chemoresistance.

ncRNA	Involvement in BC	Involvement in TNBC	Involvement in chemoresistance
let-7a	Tissue ↓ (92,93); serum ↓ (94,95); plasma ↓ (96); pathological features (97); response (98); subtyping (99)	Serum ↑ (100); tissue ↑ (100); cell line ↑ (101)	DDP [c (102,103)]; DOX [m (104), c (104-106)]; EPI [t (107), c (107)]; GEM [c (108)]; PTX [t (109), c line (106,110)]; therapeutic approach (111)
let-7e	Metastasis (112); prognosis (113); response (114); subtyping (115)	Tissue ↓ (113)	5-FU [t (113,115), c (116)]; CAP [t (117)]; DDP [c (118,119)]; DOX [c (114)]; PTX (119); PT [t (120)]
miR-7	Tissue ↑ (85); metastasis (121,122); prognosis (67), recurrence (123); response (124)	Tissue ↑ (85); cell line ↓ (125,126)	DDP [t (127)]; DOX [c (65)]; PTX [c (128)]; TAM [t (67), c (66)]; c (129)
miR-9	Metastasis (130,131); survival (132)	Tissue ↓ (133), ↑ (134); cell line ↑ (125,135); survival (136); outcome (137); response (68); neovascularization (138)	5-FU [t (68,139)]; DDP [t (68,139)]; CPA [t (68,139)]; DOX [t (68,139)]; PTX [t (68,139), c (139)]
miR-15a	Plasma ↑ (140); serum ↑ (141); apoptosis (142,143); Her2neu amplification (144); prognosis (145)	Prognosis (145)	BOR [bm (146)]; DDP [c (147)]; PTX [c (148,149)]; TAM [c (150)]
miR-17	Grading (151); metastasis (152); prognosis (153); recurrence (154,155); survival (156); TRA response (157)	Plasma ↓ (158); tissue, cell line ↓ (159); tissue ↑ (85); apoptosis (160); metastasis (161); progression (162)	5-FU [c (163,164)]; CAP [p (165)]; DDP [c (163,164,166)]; DOX [c (163)]; GEM [c (167-169)]; OXP [p (165)]; PTX [t (170), c (170-174)]
miR-18a	Serum ↑ (141); metastasis (152,175); recurrence (155); BC risk (176,177)	Tissue ↑ (85,134,178)	CAP [p (165)]; DDP [c (179,180)]; OXP [p (165)]; PTX [c (178,181)]; TRA [c (182)]
miR-19b	Serum ↑ (183); metastasis (184,185); prognosis (184); tumorigenesis (186); tumour suppressor (187)	Cell line ↑ (188)	5-FU [c (189)]; CAP [p (165)]; DOX [c (190)]; OXP [p (165), c (191)]; SAR [c (192)]
miR-21	Tissue ↑ (85,193-198); serum ↑ (199-202); plasma ↑ (96,203); urine ↓ (59); diagnosis (195,204,205); invasion (206); prognosis (196,207); recurrence (208,209); response (87)	Serum ↑ (100); plasma ↓ (210); tissue ↑ (100,211,212); cell line ↑ (211)	5-FU [p (213), t (214), c (215)]; CAR [c (216)]; DDP [p (213,217), c (218-220)]; DOX [c (221,222)]; FUL [c (223)]; GEM [c (224-226)]; OXP [c (215)]; PTX [c (215,227-231)]; TOP [c (232)]; TRA [m (233), c (234)]; therapeutic approach (222,235-237)
miR-30b	Serum ↓ (238); blood ↑ (239); metastasis (240); non-IBC (144); recurrence (241)	Cell line ↑ (253,254); tissue ↓ (249)	DDP [c (242)]; GEF [c (243)]; TRA [c (244)]; TKI [t (245)]
miR-222	Tissue ↑ (246,247); serum ↑ (176); nodal status (194); prognosis (248); progression (249); recurrence (250); response (251,252)		DDP [c (255)]; DOX [c (256,257)]; EPI [p (251), t (252,257)]; FUL [c (258)]; GEF [c (243)]; GEM [c (259)]; PTX [p (251), c (260)]; TAM [c (254,261,262)]
miR-320c	Not yet connected to BC.		5-FU [c (263)]; GEM [c (264)]
piR-36743 ^a	Tissue ↑ (86)		
GlyCCCC2	Not yet connected to BC. Content of serum and urine exosomes (265)		

^aDQ598677 is an alternative name for piR-36743. BC, breast cancer; TNBC, triple negative BC; miR, microRNA; piR, PIWI-interacting RNA; ↓, downregulation; ↑, upregulation; c, cell line; m, mouse; t, tissue; bm, bone marrow; p, plasma; DDP, doxorubicin; EPI, epirubicin; GEM, gemcitabine; PTX, paclitaxel; 5-FU, 5-fluorouracil; CAP, capecitabine; PT, platinum; TAM, tamoxifen; CPA, cyclophosphamide; BOR, bortezomib; OXP, oxaliplatin; TRA, trastuzumab; SAR, saracatinib; CAR, carboplatin; FUL, fulvestrant; TOP, topotecan; GEF, gefitinib; TKI, tyrosine kinase inhibitors.

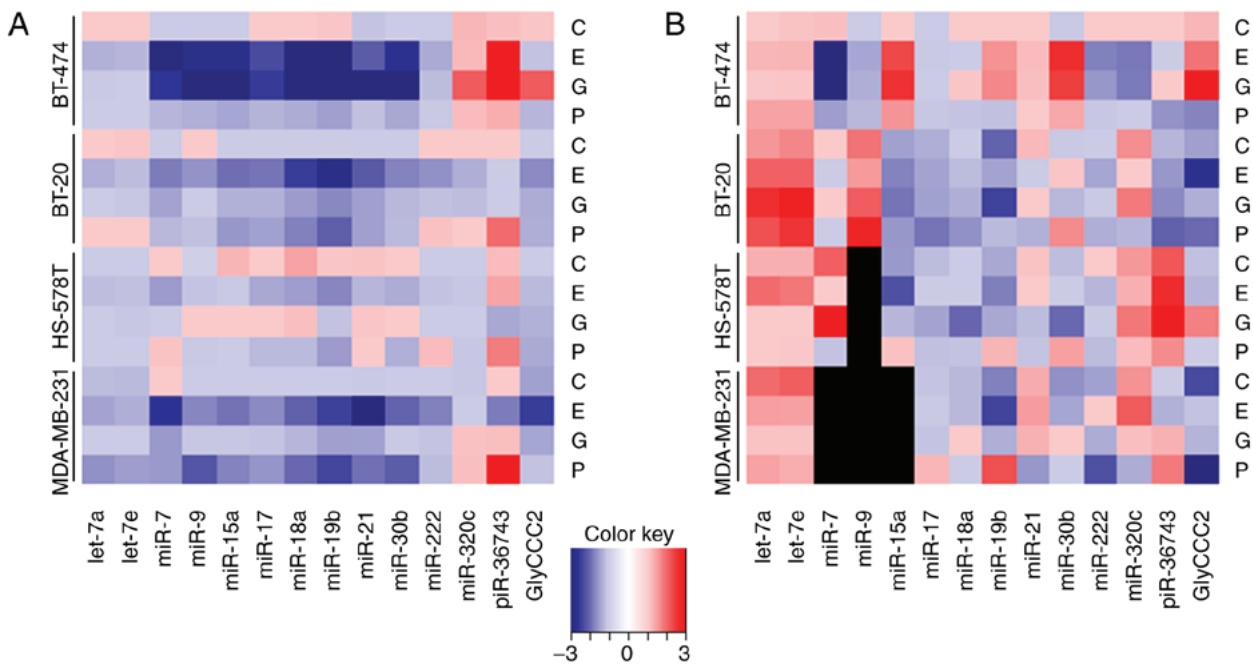


Figure 1. Chemotherapy-driven ncRNA regulations in (triple negative) breast cancer cells. Relative expression levels ($\Delta\Delta Cq$) of ncRNAs in BT-474, BT-20, HS-578T and MDA-MB-231 cells treated with 2.0 $\mu\text{g/ml}$ carboplatin, 1.0 $\mu\text{g/ml}$ epirubicin, 40.0 $\mu\text{g/ml}$ gemcitabine and 2.0 $\mu\text{g/ml}$ paclitaxel for 18 h as determined by reverse transcription-quantitative PCR. Heatmaps demonstrate (A) intracellular expression levels normalised to the geometric mean of RNU-44 and -48, and (B) secreted microvesicular ncRNA levels normalised to the global mean of all investigated targets. Black squares indicate no reliably detectable ncRNA expression. ncRNAs, non-coding RNAs; miR, microRNA; piR, PIWI-interacting RNA; C, carboplatin; E, epirubicin; G, gemcitabine; P, paclitaxel.

intracellular compartment, except in the cases of miR-320c, piR-36743 and GlyCCC2 in BT-474 cells, where an upregulation was observed. The levels of secreted microvesicular ncRNAs were altered bidirectionally (Fig. 1).

In a multivariable analysis, gemcitabine treatment caused major changes in the expression levels of ncRNAs in BT-474 cells, while few detectable alterations were observed in the TNBC cell lines. Specifically, miR-7, -9, -15a, -17, -18a, -19b, -21 and -30b were downregulated compared with the intercept, while miR-320c, piR-36743 and GlyCCC2 were upregulated in BT-474 cells. In the TNBC cell lines, downregulated expression was detected for miR-17, -18a and -21 in BT-20, and miR-7 and -21 in MDA-MB-231 cells. No significant effect was detected in HS-578T cells. All estimates of multivariable analysis, including CIs and P-values are listed in Table SII.

Paclitaxel. Intracellularly, the expression levels of the ncRNAs predominantly decreased following paclitaxel (2.0 $\mu\text{g/ml}$) treatment. However, in the cases of miR-7 (HS-578T), -222 (BT-20 and HS-578T), -320c (BT-474, BT-20 and MDA-MB-231) and piR-36743 (all cell lines), upregulation was detected. The levels of secreted microvesicular ncRNAs were influenced by paclitaxel and differed among the investigated cell lines (Fig. 1).

Multivariable analysis found that paclitaxel triggered ncRNA expression fluctuations predominantly in the TNBC cell lines BT-20 and MDA-MB-231. Compared with the intercept, miR-17, -18a, -19b and -21 were downregulated in BT-20 cells, while piR-36743 was upregulated. In MDA-MB-231 cells, the expression levels of let-7a, let-7e, miR-7, -9, -15a, -17, -18a, 19b, -21, -30b and -222 were decreased, and piR-36743 was upregulated. In the HS-578T cell line, no alteration in the investigated ncRNA profiles was detected, except for the upregulation of

piR-36743. Paclitaxel treatment caused the downregulation of miR-17 in BT-474 cells compared with the intercept. All estimates of multivariable analysis, including CIs and P-value are listed in Table SII.

Carboplatin. Treatment with carboplatin (2.0 $\mu\text{g/ml}$) did not influence ncRNA expression in this model (Table SII).

Intracellular vs. secreted microvesicular ncRNA expression levels. Focusing on the extracellular compartment, no conclusions can be drawn from the observation of an increase in the relative levels of ncRNA expression under control conditions compared with the intracellular expression levels for all targets, which was mirrored by significantly increased estimates in multivariable analysis (Table SIII). This phenomenon cannot be explained by biological variation but is, at least in part, observed due to different normalisation methods in intra- and extracellular analysis.

The applied model of multivariable analysis allowed the estimation of the additional impact on ncRNA expression caused by three-way interactions with the variables 'chemotherapy', 'cell line' and 'extracellular compartment'. Most of these influences were observed in the BT-474 cell line and were less frequent in the TNBC cell lines. Depending on the estimate, the aforementioned regulation of the expression of the ncRNAs will be increased (estimate >1) or alleviated (estimate <1; Table SIII). For the most part, the three-way interactions attenuated the effects presented in Table SII. This was observed for miR-15a, -17, -18a, -19b, -21, -30b, -320c and piR-36743 following gemcitabine treatment, as well as for miR-15a, -17, -30b, -320c and piR-36743 following epirubicin treatment in BT-474 cells. In the TNBC cell line HS-578T,

the three-way interactions resulted in the opposite effects on miR-18a, -21 and piR-36743 following gemcitabine treatment. The same applied to miR-21 and -222 following epirubicin treatment, and for miR-17 and -19b following paclitaxel treatment in MDA-MB-231 cells (Table SIII).

While lacking significance in the aforementioned observations, three-way interaction influences estimated by multivariable analysis added to the effect of piR-36743 upregulation in HS-578T cells following epirubicin treatment (1.310; $P=0.208$) by 2.662 ($P=0.024$). Similarly, let-7a expression following gemcitabine treatment (0.929; $P=0.760$) decreased by 0.357 ($P=0.036$) in this cell line. Let-7e expression (0.887; $P=0.619$) further decreased in the same context by 0.337 ($P=0.027$). The reduced GlyCCC2 expression induced by paclitaxel treatment in MDA-MB-231 cells (0.772; $P=0.372$) was further decreased by a value of 0.277 ($P=0.029$). In BT-474 cells, paclitaxel induced the downregulation of miR-9 (0.752; $P=0.233$), which was increased by 0.332 ($P=0.023$). These effects may indicate that let-7a and -7e, and GlyCCC2 may be promising liquid biomarker candidates depending on the administered chemotherapy (Table SIII). In the case of piR-36743, three-way interaction influences added to already significant findings as aforementioned. While the upregulation of piR-36743 following paclitaxel treatment in HS-578T cells (1.793; $P=0.008$) increased by 3.492 ($P=0.004$), piR-36743 increased in MDA-MB-231 cells (2.922; $P<0.001$) by 2.428 ($P=0.040$; Table SIII).

Chemotherapy-induced ncRNA regulation pattern. Several ncRNAs analysed in the present study showed a similar regulation pattern, especially in the intracellular analysis. This phenomenon was observed for miRNAs of the miR-17~92 cluster (miR-17, -18a and -19b), and for miR-15a and -30b (Figs. 2 and 3).

Markers of proliferation. Cyclin D1 mRNA expression levels reduced after treatment with epirubicin and gemcitabine (Fig. 4A). Multivariable analysis indicated a decrease in cyclin D1 expression levels in BT-474 and HS-578T cells (epirubicin, $P<0.001$ and $P=0.010$, respectively), and in BT-474, HS-578T and MDA-MB-231 cells (gemcitabine, $P=0.002$, $P<0.001$ and $P=0.016$, respectively) compared with the intercept. A reduction was also observed in paclitaxel-treated HS-578T cells ($P=0.007$). Carboplatin did not influence cyclin D1 mRNA expression in this setting. All estimates of multivariable analysis, including CIs and P-values are listed in Table SIV.

TOP2 α mRNA levels were reduced following chemotherapy, except following carboplatin treatment in all the cell lines tested (Fig. 4B). P-values, estimates and CIs are listed in Table SIV.

TPX2 mRNA level alterations were similar to those of cyclin D1, with reductions in expression caused by epirubicin (BT-20, HS-578T and MDA-MB-231; $P<0.001$) and gemcitabine (all cell lines; $P<0.001$) treatment (Fig. 4C). Paclitaxel treatment reduced TPX2 levels in BT-474 and MDA-MB-231 cells ($P<0.001$), but increased TPX2 expression in HS-578T cells ($P=0.001$; Table SIV).

While epirubicin treatment induced differential regulation of DDX5 and -17 mRNA levels, DDX17 was regulated

in a similar manner as the miR-17~92 cluster (Figs. 2 and 5). Specifically, DDX5 mRNA was upregulated by epirubicin compared with the intercept (BT-20 and MDA-MB-231, $P<0.001$) and downregulated in BT-474 by gemcitabine ($P=0.034$). By contrast, DDX17 mRNA was downregulated following epirubicin treatment in all the cell lines tested (all $P<0.001$). Downregulation was also observed following gemcitabine treatment in BT-20, BT-474, HS-578T ($P<0.001$) and MDA-MB-231 cells ($P=0.049$), as well as by paclitaxel in MDA-MB-231 cells ($P=0.006$; Table SIV).

At the protein level, cyclin D1 was detected at a molecular weight of ~65 kDa. This immunoprecipitated protein may represent a ubiquitinated form of cyclin D1 and was detected in all investigated cell lines. The expression of cyclin D1 in HS-578T cells, however, was present at modest levels. Compared with the control conditions, no regulation of cyclin D1 was observed, except for a modest reduction caused by epirubicin treatment in MDA-MB-231 cells (Fig. 6A). Western blotting revealed differential DDX5 regulation following epirubicin treatment at the protein level compared with mRNA regulation; DDX5 was downregulated at the protein level. Following gemcitabine treatment, DDX5 protein levels also declined in BT-474 and HS-578T cells, while they declined in MDA-MB-231 cells following paclitaxel treatment. A reduced DDX17 protein level caused by epirubicin is consistent with reduced DDX17 mRNA. In BT-474 and BT-20 cells, gemcitabine led to lower DDX17 protein expression; paclitaxel had the same effect in MDA-MB-231 cells (Fig. 6B and C).

In vivo pilot study. In serum, untreated patients with TNBC (t_0) could be clearly distinguished from healthy controls by the expression levels of the vast majority of ncRNAs investigated, with the exception of miR-222, -320c and piR-36743 (Fig. 7). A two-sample t-test confirmed upregulated let-7a ($P=0.008$, SE=0.662), -7e ($P=0.005$, SE=0.179) and miR-21 ($P=0.039$, SE=4.090) expression levels in serum from patients with TNBC compared with the control group. Decreased expression was observed for miR-15a ($P=0.008$, SE=0.154), -17 ($P=0.023$, SE=0.087), -18a ($P=0.015$, SE=0.015), -19b ($P=0.002$, SE=0.209), -30b ($P<0.001$, SE=0.031) and GlyCCC2 ($P<0.001$, SE=0.623) in TNBC. In urine, untreated patients with TNBC could be distinguished from healthy women by decreased levels of miR-18a ($P=0.021$, SE=0.006), -19b ($P=0.006$, SE=0.159), -30b ($P=0.002$, SE=0.484), -222 ($P=0.001$, SE=0.071), -320c ($P<0.001$, SE=0.087) and GlyCCC2 expression ($P=0.001$, SE=5.803; Fig. 8).

In the present study, no clear pattern was observed when dividing the TNBC group into early responder and non-responder [early response as defined by the achievement of a clinical partial response (cPR) according to RECIST criteria (30) immediately prior to the third cycle of NACT]. Also, no association was observed regarding pCR as an outcome (data not shown). However, a trend was discovered in the serum of patients receiving a cCR during NACT (Fig. 9). In this respect, those patients ($n=4$) showed a different ncRNA regulation pattern immediately prior to the third cycle of NACT (t_1) compared with patients ($n=4$) who did not achieve a cCR during NACT. A two-tailed paired t-test estimated a difference from t_0 to t_1 between the two groups in terms of serum expression of miR-17 ($P=0.029$, SE=0.173),

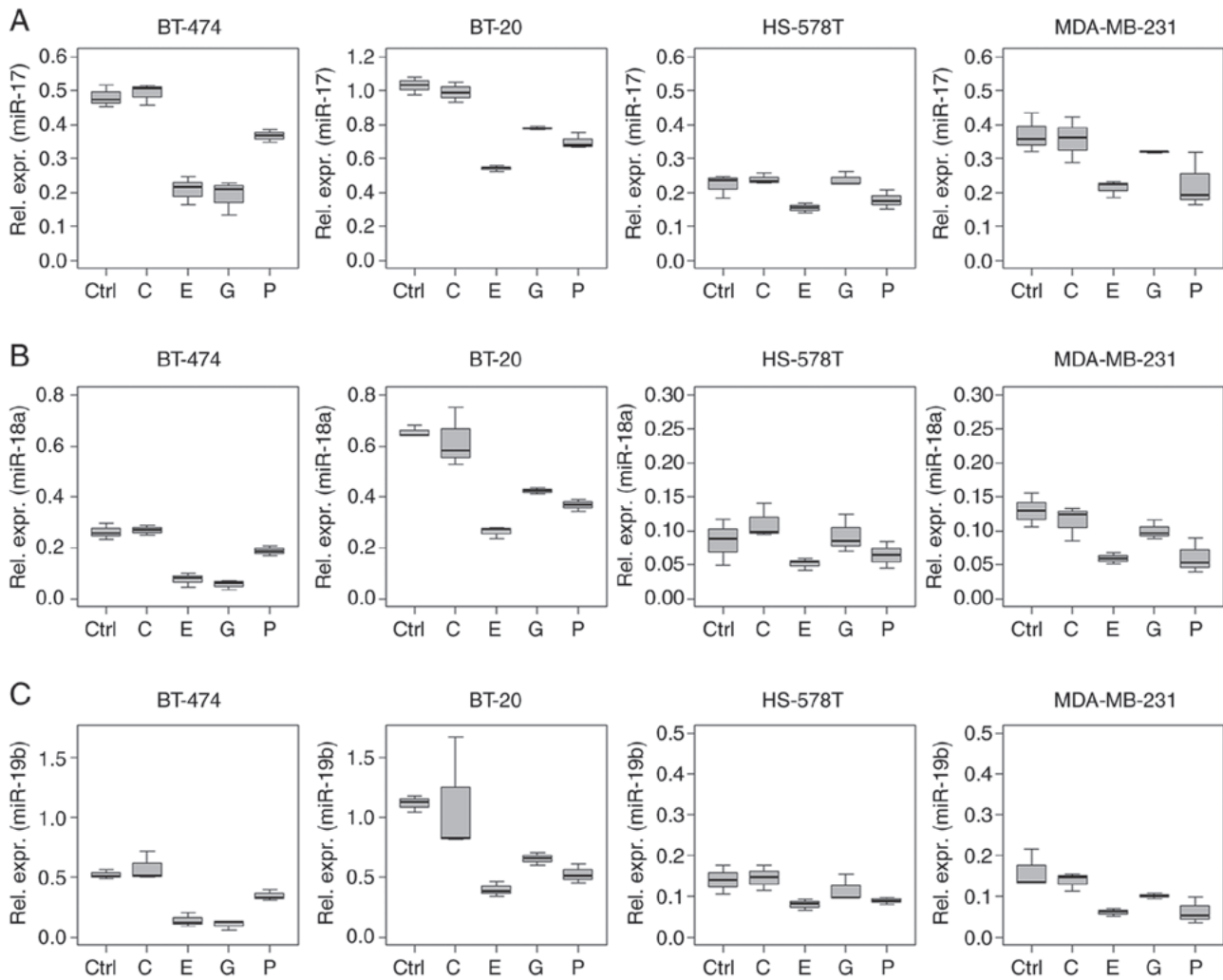


Figure 2. Expression profile of miR-17~92 cluster miRNAs. Relative expression levels (ΔCq) of the miR-17~92 cluster miRNAs (A) miR-17, (B) -18a and (C) -19b in BT-474, BT-20, HS-578T and MDA-MB-231 breast cancer cells treated with 2.0 $\mu g/ml$ carboplatin, 1.0 $\mu g/ml$ epirubicin, 40.0 $\mu g/ml$ gemcitabine and 2.0 $\mu g/ml$ paclitaxel for 18 h as determined by reverse transcription-quantitative PCR. Data are normalised to the geometric mean of RNU-44 and -48. Untreated cells served as the control. Boxplots indicate median (thick line), first and third quartile (box lines) and maximal/minimal value (upper and lower line). Y-axis scaling can deviate to improve readability. miR/miRNA, microRNA; C, carboplatin; E, epirubicin; G, gemcitabine; P, paclitaxel; Ctrl, control.

-19b ($P=0.030$, $SE=0.282$), -30b ($P=0.011$, $SE=0.048$) and piR-36743 ($P=0.028$, $SE=0.072$). However, these results remain preliminary due to the small sample size. By contrast, no specific pattern was detected within the TNBC group using the urine specimens. A general rise of ncRNA expression from t_0 to t_1 was evident (Fig. 10).

Discussion

The present study demonstrated that dysregulation of ncRNAs in TNBC cell lines, serum and urine can be induced by chemotherapy treatment. *In vitro*, the influences of chemotherapeutic treatment with epirubicin, gemcitabine and paclitaxel on the expression levels of a (TN)BC-related ncRNA panel were evaluated. Even in the extracellular compartment, influences were detected, indicating the effects of chemotherapy-driven ncRNA secretion. The expression profiles differed depending on cell line and cytostatic drug. In this *in vitro* model, epirubicin caused the strongest effect on ncRNA regulation.

Except for miR-7 and -9, the present study reliably detected all targets in microvesicles from liquid samples (conditioned

cell culture media, serum and urine). As intracellularly, miR-7 and -9 resulted in distinct expression alterations caused by chemotherapy treatment, these ncRNAs may still be involved in therapy response mechanisms. Previous studies have demonstrated associations between BC therapy response, and the dysregulation of miR-7 and -9 in cell lines and tissue (65-68). However, these two miRNAs were not detectable in microvesicles of liquid samples in the present study, and cannot be recommended as suitable liquid biopsy marker candidates in this context.

ncRNA expression alterations occurred along with altered expression of cell cycle markers and, therefore, may be associated with it. While epirubicin exhibited the most potent effects on ncRNA regulation, carboplatin treatment did not significantly influence ncRNA expression in the present study. This observation is consistent with the present findings on the influence of chemotherapeutic treatment on cell cycle determinants. Cyclin D1, TOP2 α and TPX2 were not influenced by carboplatin, indicating limitations of this *in vitro* model. All cells were treated with the chemotherapeutic agent for 18 h. Carboplatin may cause an inhibitory effect on cell proliferation,

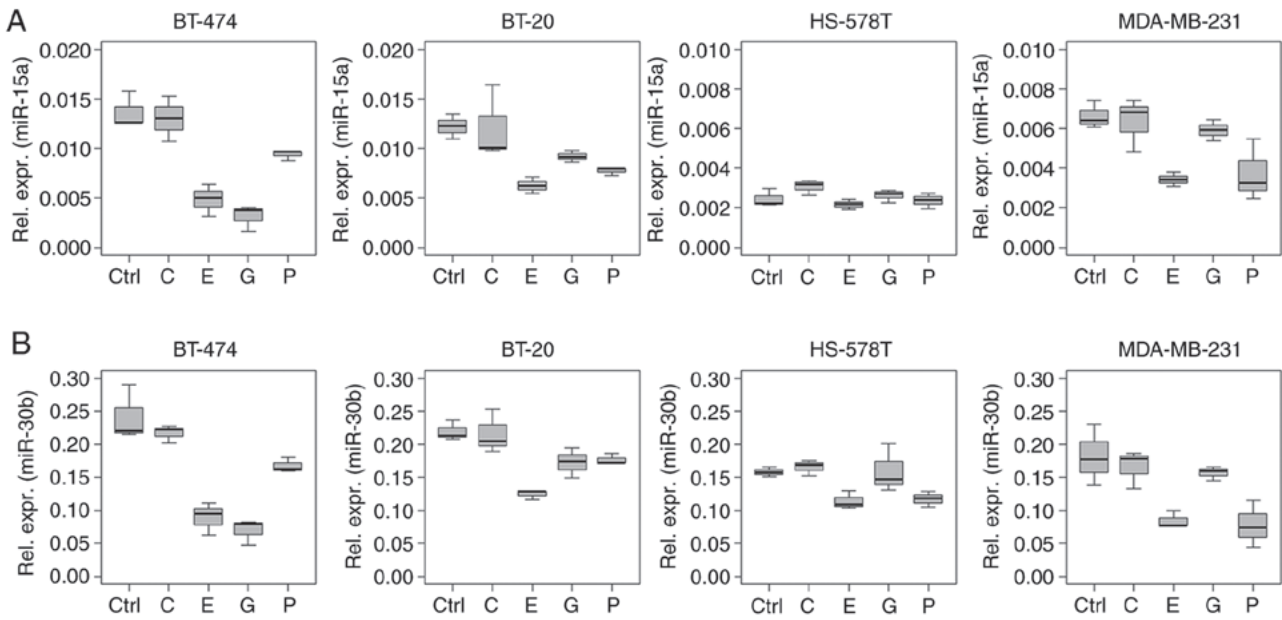


Figure 3. Chemotherapy-driven regulation of miR-15a and -30b in (triple negative) breast cancer cells. Relative expression levels (ΔCq) of (A) miR-15a and (B) -30b in BT-474, BT-20, HS-578T and MDA-MB-231 cells treated with 2.0 $\mu\text{g/ml}$ carboplatin, 1.0 $\mu\text{g/ml}$ epirubicin, 40.0 $\mu\text{g/ml}$ gemcitabine and 2.0 $\mu\text{g/ml}$ paclitaxel for 18 h, normalised to the geometric mean of RNU-44 and -48, as determined by reverse transcription-quantitative PCR. Untreated cells served as the control. Boxplots indicate median (thick line), first and third quartile (box lines) and maximal/minimal value (upper and lower line). Y-axis scaling can deviate to improve readability. miR, microRNA; C, carboplatin; E, epirubicin; G, gemcitabine; P, paclitaxel; Ctrl, control.

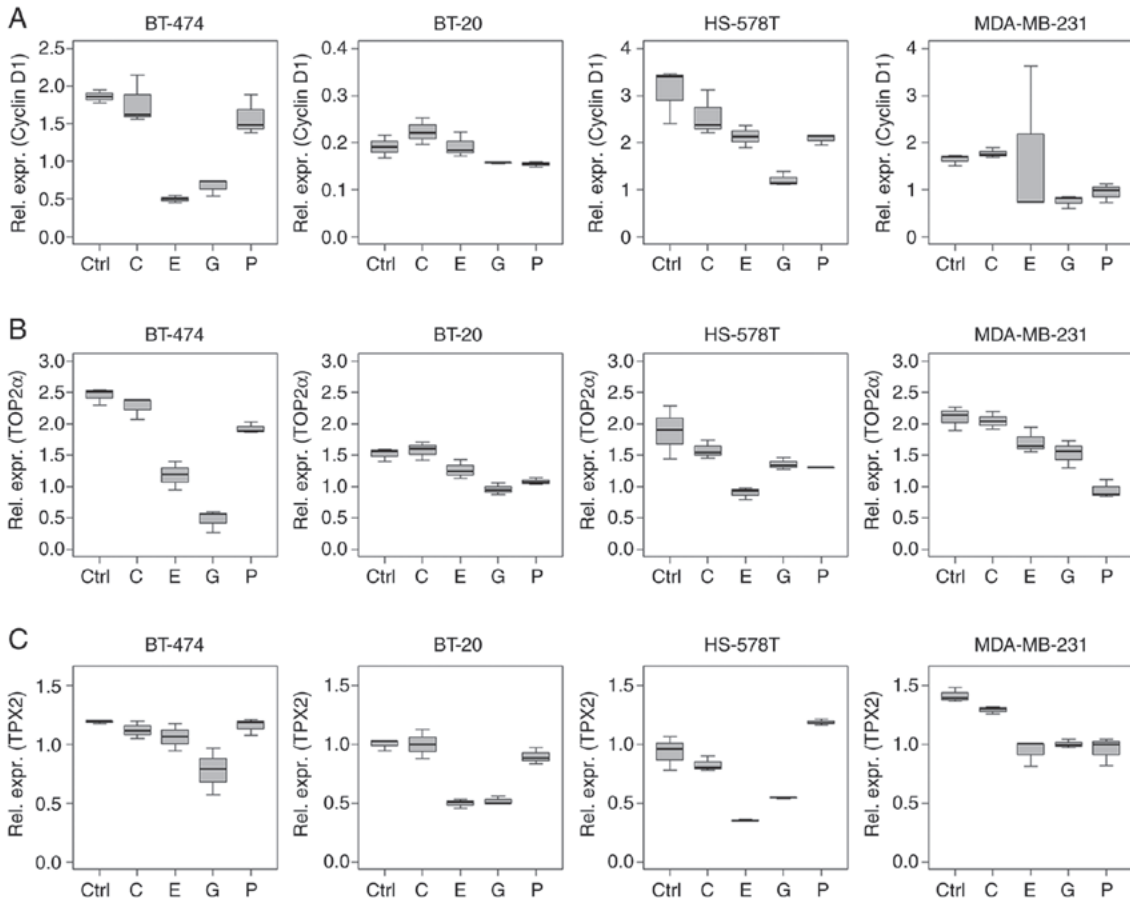


Figure 4. Influence of chemotherapy treatment on cell cycle determinants in (triple negative) breast cancer cell lines. Relative mRNA expression levels (ΔCq) of (A) cyclin D1, (B) TOP2 α and (C) TPX2 in BT-474, BT-20, HS-578T and MDA-MB-231 cells under control conditions, and treated with 2.0 $\mu\text{g/ml}$ carboplatin, 1.0 $\mu\text{g/ml}$ epirubicin, 40.0 $\mu\text{g/ml}$ gemcitabine and 2.0 $\mu\text{g/ml}$ paclitaxel for 18 h. Data are normalised to 5'-aminolevulinatase 1 and values were determined by reverse transcription-quantitative PCR. Boxplots indicate median (thick line), first and third quartile (box lines) and maximal/minimal value (upper and lower line). Y-axis scaling can deviate to improve readability. TPX2, targeting protein for Xklp2; TOP2 α , DNA topoisomerase 2 α ; C, carboplatin; E, epirubicin; G, gemcitabine; P, paclitaxel; Ctrl, control.

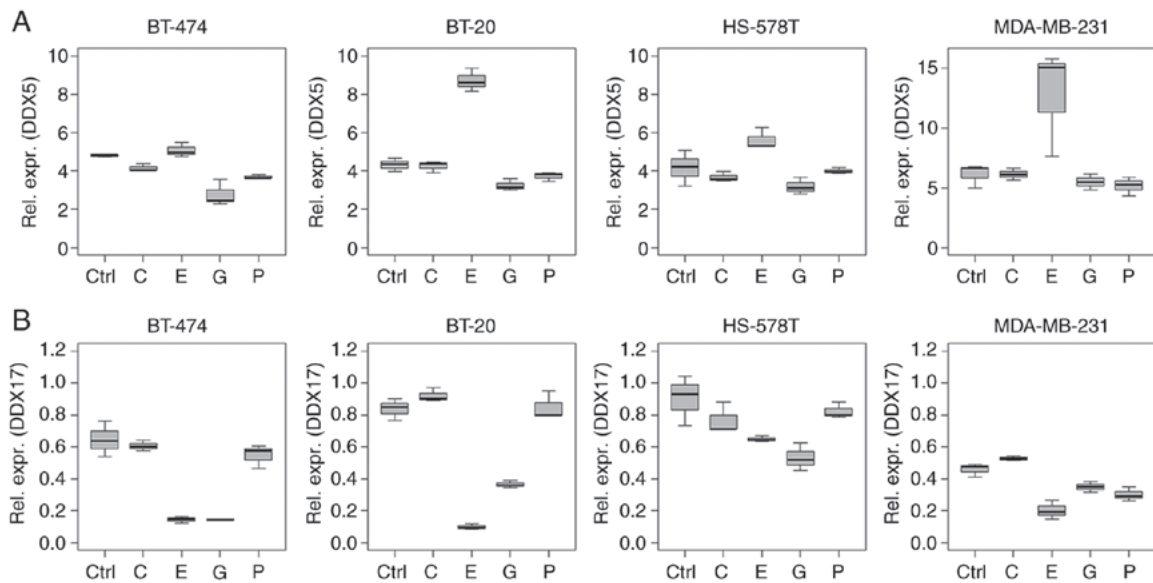


Figure 5. Influence of chemotherapy treatment on DDX proteins in (triple negative) breast cancer cell lines. Relative mRNA expression levels (ΔCq) of (A) DDX5 and (B) DDX17 in BT-474, BT-20, HS-578T and MDA-MB-231 cells under control conditions, and treated with 2.0 $\mu\text{g/ml}$ carboplatin, 1.0 $\mu\text{g/ml}$ epirubicin, 40.0 $\mu\text{g/ml}$ gemcitabine and 2.0 $\mu\text{g/ml}$ paclitaxel for 18 h. Data are normalised to 5'-aminolevulinate synthase 1. Values were determined using reverse transcription-quantitative PCR. Boxplots indicate median (thick line), first and third quartile (box lines) and maximal/minimal value (upper and lower line). Y-axis scaling can deviate to improve readability. DDX, DEAD-box polypeptide; C, carboplatin; E, epirubicin; G, gemcitabine; P, paclitaxel; Ctrl, control.

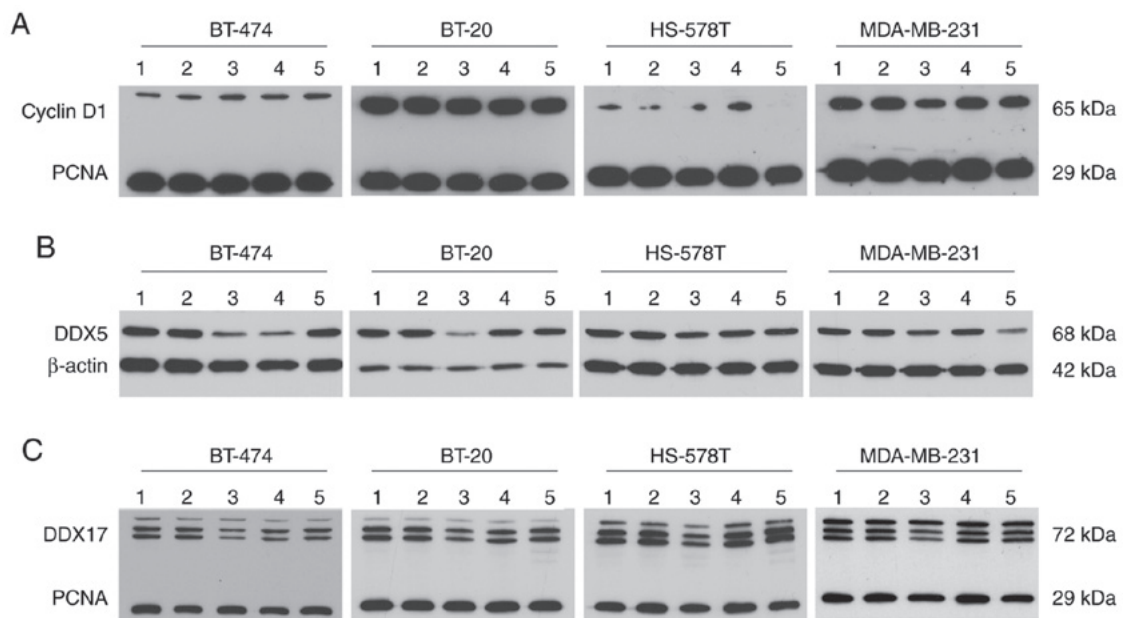


Figure 6. Chemotherapy treatment influences DDX5 and -17 protein levels in (triple negative) breast cancer cells. Protein expression of (A) DDX5, (B) DDX17 and (C) cyclin D1 in BT-474, BT-20, HS-578T and MDA-MB-231 cells treated with 2.0 $\mu\text{g/ml}$ carboplatin (lane 2), 1.0 $\mu\text{g/ml}$ epirubicin (lane 3), 40.0 $\mu\text{g/ml}$ gemcitabine (lane 4) and 2.0 $\mu\text{g/ml}$ paclitaxel (lane 5) for 18 h, as determined by western blotting compared with untreated cells (lane 1). β -actin and PCNA served as loading control. DDX, DEAD-box polypeptide; PCNA, proliferating cell nuclear antigen.

and possibly affect ncRNA expression, when treatment time is prolonged (69-72). In western blotting, cyclin D1 could not be detected in its native conformation, with a molecular weight of 37 kDa. However, the product detected was of 65 kDa, and may represent a dimer or a ubiquitylated form of cyclin D1. Cyclin D1 is degraded by ubiquitylation and the native protein possesses a short half-life of <30 min (73,74).

Apart from carboplatin-treated cells, mRNA expression of cell cycle determinants was reduced in most of the other

samples as aforementioned. Epirubicin and gemcitabine treatment impacted proliferation and led to ncRNA expression alterations.

DDX proteins, a family of RNA helicases, are dysregulated in several types of cancer, including BC (75). Their cellular functions are highly context-dependent and influenced by posttranslational regulation (75-77). DDX5 and -17 are closely related and part of multiprotein complexes. One function of these two proteins is oestrogen receptor- α

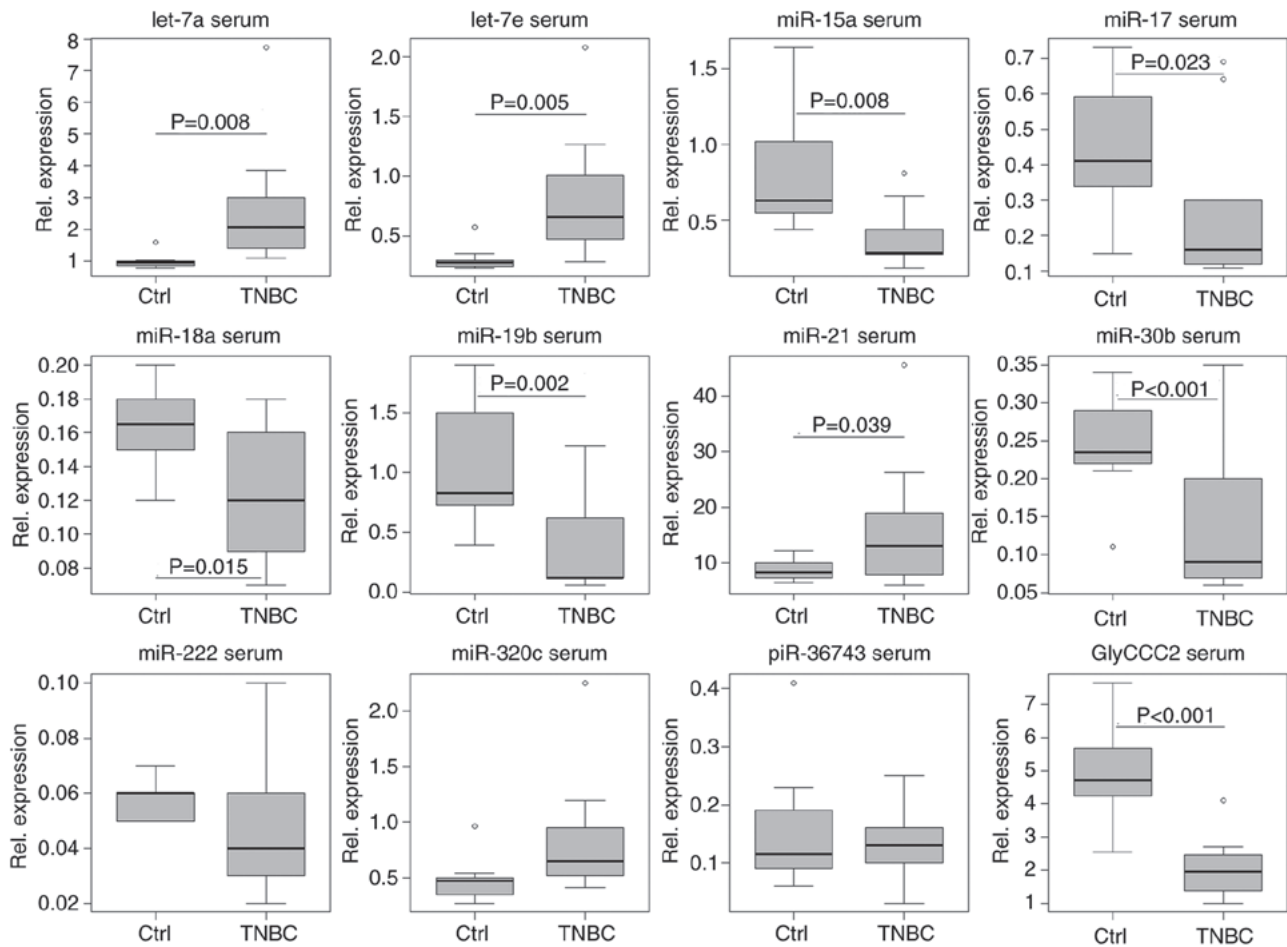


Figure 7. ncRNA expression in serum distinguishes patients with TNBC from controls. Relative expression levels (ΔCq) of microvesicular ncRNAs (let-7a/e, miR-15a, -17, -18a, -19b, -21, -30b, -222 and -320c, piR-36743 and GlyCCC2) in the serum of 8 patients with TNBC and 10 healthy controls, normalised to the geometric mean of miR-26b and -191, as determined by reverse transcription-quantitative PCR. Let-7a/e and miR-21 expression levels were increased in patients with TNBC compared to controls (two-tailed t-test; $P < 0.05$). miR-15a, -17, -18a, -19b, -30b and GlyCCC2 were decreased. Boxplots indicate median (thick line), first and third quartile (box lines), maximal/minimal value (upper and lower line) and ° (moderate outlier). Y-axis scaling can deviate to improve readability. ncRNAs, non-coding RNAs; miR, microRNA; TNBC, triple negative breast cancer; piR, PIWI-interacting RNA; Ctrl, control.

co-activation, which may not play an important role in TNBC; however, the co-activation of p53, RNA metabolism and processing may be of importance (75,78,79). Additionally, DDX proteins are involved in the processing of miRNAs (75). The interplay between DDX proteins and miRNA dysregulation may be an important feature of TNBC. In the present study, it was found that DDX5 and -17 mRNA expression in TNBC cells was influenced by chemotherapy treatment. The mRNA and protein downregulation observed supports results from previous studies that described an inhibition of cancer cell proliferation when simultaneously depleting DDX5 and -17 (80,81). The findings of the present study suggested an oncogenic function for these DDX proteins in TNBC cells. However, the mRNA upregulation of DDX5 triggered by epirubicin is of interest. As protein levels of DDX5 were reduced after epirubicin treatment, a feedback loop may be involved.

The widely studied miR-17~92 cluster exerts oncogenic functions, with its members upregulated in several types of cancer, including TNBC (82-84). Furthermore, the expression of miR-17~92 varies in different BC subtypes (85). In the present study, it was observed that miRNAs of the miR-17~92

cluster (miR-17, -18a and -19b), as well as miR-15a and -30b, were regulated in a similar manner; DDX17 mRNA displayed a similar expression profile to the miR-17~92 cluster. The present study demonstrates that the expression levels of miR-17~92 cluster miRNAs, and miR-15a and -30b, were reduced by epirubicin, gemcitabine and paclitaxel treatment in the *in vitro* cell models. It was also found that the miR-17~92 cluster miRNA expression levels were reduced in serum and urine samples from patients with TNBC compared with healthy controls.

piR-36743 was of interest due to the chemotherapy-driven expression alterations found in the *in vitro* models. piR-36743 was upregulated, while most of the other investigated ncRNAs experienced chemotherapy-dependent downregulation. The functional background of piR-36743 remains poorly understood. Hashim *et al.* (86) found that piR-36743 (also known as DQ598677) was upregulated in BC biopsies compared with healthy breast tissue.

The present *in vivo* study found a clear liquid biopsy biomarker-based distinction between patients with TNBC and healthy controls. Serum and urine samples allowed patients with TNBC to be distinguished from healthy women. ncRNA

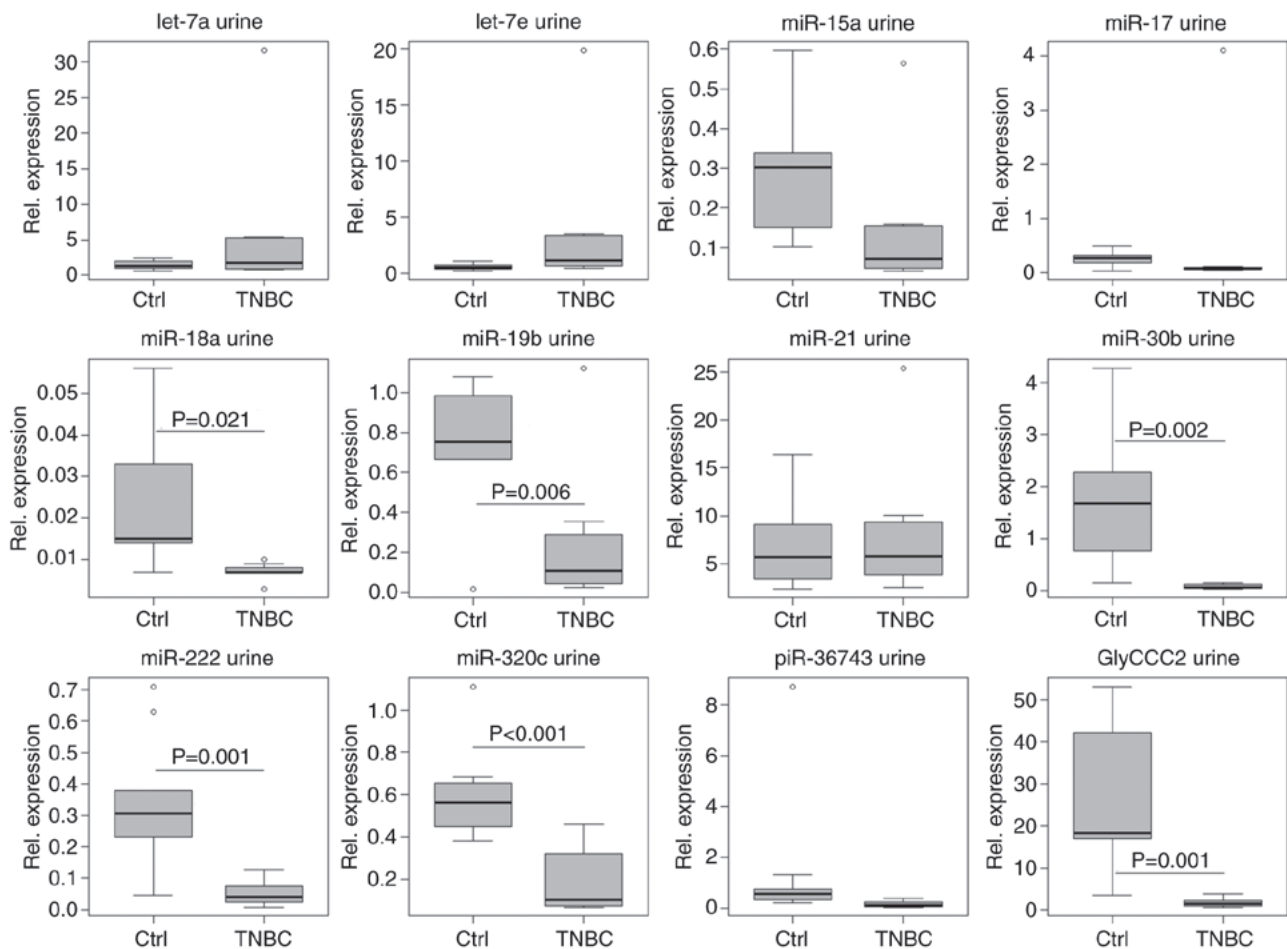


Figure 8. ncRNA expression in urine can distinguish patients with TNBC from controls. Relative expression levels (ΔCq) of microvesicular ncRNAs (let-7a/e, miR-15a, -17, -18a, -19b, -21, -30b, -222 and -320c, piR-36743 and GlyCCC2) in the urine of 8 patients with TNBC and 10 healthy controls, normalised to the geometric mean of miR-16 and -26b, as determined by reverse transcription-quantitative PCR. miR-18a, -19b, -30b, -222 and -320c, and GlyCCC2 expression levels were decreased in patients with TNBC compared to controls (two-tailed t-test; $P < 0.05$). Boxplots indicate median (thick line), first and third quartile (box lines), maximal/minimal value (upper and lower line) and ° (moderate outlier). Y-axis scaling can deviate to improve readability. ncRNA, non-coding RNA; TNBC, triple negative breast cancer; miR, microRNA; piR, PIWI-interacting RNA; Ctrl, control.

dysregulation in TNBC was directed almost uniformly in both serum and urine specimens.

The comparison of microvesicular ncRNA expression levels in the urine and serum of patients with TNBC prior to NACT and immediately prior to the third cycle of therapy provided preliminary results. Urinary ncRNA expression showed a general increase from t_0 to t_1 , except for let-7a/e and miR-17. This observation may be explained by the general effects of treatment or other factors. In serum, a different picture was observed. To evaluate the ncRNA panel for their liquid biopsy biomarker potential in TNBC therapy response evaluation, patients undergoing NACT were divided into a (early) responder and non-responder group. However, it is emphasised that due to a small sample size (8 patients with TNBC), the present study only provided a preliminary result in this respect. As aforementioned, in an attempt to visualize ncRNA differences in the serum of patients with TNBC from t_0 to t_1 of NACT, no clear pattern could be distinguished between the early responders and non-responders, and no association with the achievement of a pCR could be drawn. However, larger cohorts may shed further light on this issue and should be analysed to further investigate this question. It is hoped that

the investigated ncRNA types of the present study may act as potential minimally-invasive biomarkers to indicate therapy response in TNBC. This hypothesis is supported as different ncRNA regulation was identified in the serum of patients with TNBC who achieved a cCR during NACT compared with those who did not. Therefore, the involvement of these ncRNAs in TNBC is likely. However, the small patient number in this pilot study is a limitation. Only studies using bigger cohorts can clarify if this ncRNA panel may serve as a liquid biopsy tool in predicting therapy response.

To date, few studies have been published connecting serum-based ncRNAs to the response of patients with BC to NACT. Liu *et al* (87) reported reduced miR-125b expression levels in responding vs. non-responding patients with BC before, during and after NACT. Additionally, miR-21 expression decreased throughout NACT in responding patients. Furthermore, Gu *et al* (88) reported that lower miR-451 expression in serum was associated with the resistance of patients with BC to NACT. Supporting this, Al-Khanbashi *et al* (89) reported an association between elevated serum levels of miR-451, and improved clinical and pathological response to NACT of locally advanced BC.

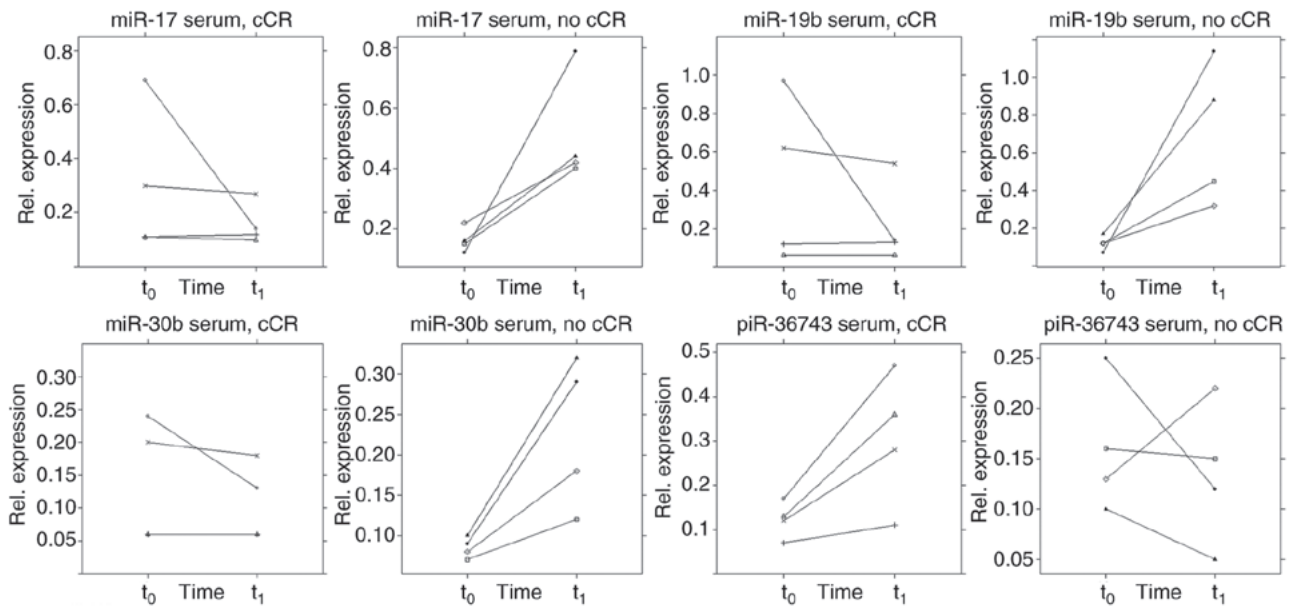


Figure 9. NACT influences ncRNA expression in the serum of patients with TNBC. Relative expression levels (ΔCq) of microvesicular ncRNAs (miR-17, -19b, -30b and piR-36743) in the serum of 4 patients with TNBC who achieved a cCR during NACT and 4 patients with TNBC who did not (no cCR), normalised to the geometric mean of miR-26b and -191, as determined by reverse transcription-quantitative PCR. Samples were taken at two time points: t_0 and t_1 . Y-axis scaling can deviate to improve readability. ncRNA, non-coding RNA; TNBC, triple negative breast cancer; miR, microRNA; cCR, clinical complete response; NACT, neoadjuvant chemotherapy; piR, PIWI-interacting RNA; t_0 , prior to NACT; t_1 , immediately prior to the third cycle of therapy.

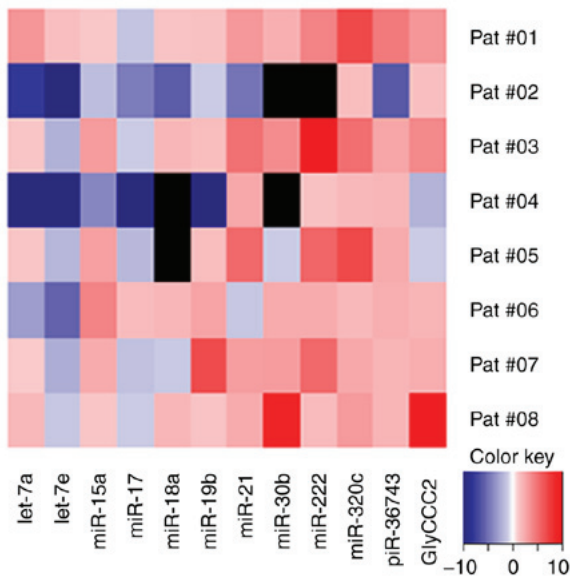


Figure 10. NACT influences ncRNA expression in the urine of patients with TNBC. Relative expression levels (ΔCq) of microvesicular ncRNAs in the urine of 8 patients with TNBC during NACT, normalised to the geometric mean of miR-16 and -26b, as determined by reverse transcription-quantitative PCR. Heatmap demonstrates fold change of ncRNA expression levels from t_0 to t_1 . Black squares indicate no reliably detectable ncRNA expression. ncRNA, non-coding RNA; TNBC, triple negative breast cancer; miR, microRNA; cCR, clinical complete response; NACT, neoadjuvant chemotherapy; piR, PIWI-interacting RNA; t_0 , prior to NACT; t_1 , immediately prior to third cycle of therapy.

In the present study, expression analysis was conducted using relative quantification. As each experimental setup requires the evaluation of the most suitable reference genes, an individual normalisation for each matrix was conducted. As aforementioned, intracellular ncRNA expression was

normalised to the geometric mean of RNU44 and -48, while microvesicular ncRNA in conditioned cell culture media was normalised to the global mean. In serum, miR-26b and -191 served as endogenous controls and in urine, miR-16 and -26b were used as endogenous controls. The most suitable endogenous controls were assessed using the BestKeeper software tool (55). However, different normalisation strategies can complicate the comparability of results. Nevertheless, it is essential to choose an appropriate normalisation approach according to each experiment.

Different sample matrices can represent different physiological and pathophysiological processes. Precise TNBC-related ncRNA expression profiles can be obtained from tumour tissue; however, sample acquisition remains invasive. TNBC cells can serve as a model for tumour tissue. In the case of liquid biopsies, specimens derived from the blood (for example, serum), an altered ncRNA profile could result from tumour-derived and secreted ncRNAs. However, dysregulated ncRNAs may originate from other malignant or non-malignant cells, or may originate from an immune response to the tumour. The latter may indirectly indicate the existence of a malignancy. Immune cells constitutively release exosomes, which can carry ncRNAs (90). Urine-based ncRNAs are further away still from the tumour site. It is assumed that urine-based altered ncRNA levels originate directly from tumour tissue and undergo glomerular filtration by the kidneys after secretion into the circulation. In this manner, ncRNAs may be found in urine. Similarly to serum specimens, urine-based ncRNAs may also be secreted by immune cells. Furthermore, ncRNAs can enter urine from the cells of the urinary tract (91).

The present study detected chemotherapy-driven expression alterations of a (TN)BC-related panel of ncRNAs *in vitro*. Furthermore, the vast majority of this panel was detectable in

serum and urine specimens, and could be used to discriminate between patients with TNBC and healthy controls. Results regarding the eligibility of this panel in predicting therapy response *in vivo* remain preliminary due to the small sample size. The liquid biopsy potential of the investigated ncRNAs in the evaluation of the response of patients with TNBC to NACT should be further investigated in larger cohorts.

Acknowledgements

Not applicable.

Funding

The article processing charge was funded by the open access publication fund of the Albert Ludwigs University Freiburg. No funding was received for the study.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AR, TE, MH and MJ substantially developed the study design and experimental setup. AR, MJ, TE, KB, DW, JA, MM and SG contributed to sample collection and processing. AR, MJ, CN, and SG performed miRNA quantification analysis and data collection. GR conducted statistical analyses *in extenso*. AR, MH, KB, GR, MJ, DW, JA, MM and TE drafted the manuscript or revised it critically. All authors have read and approved the final manuscript.

Ethics approval and consent for publication

The present study was approved by the institutional ethical review board of the University of Freiburg (permit no. 607/16) and written informed consent was obtained from each participant.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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