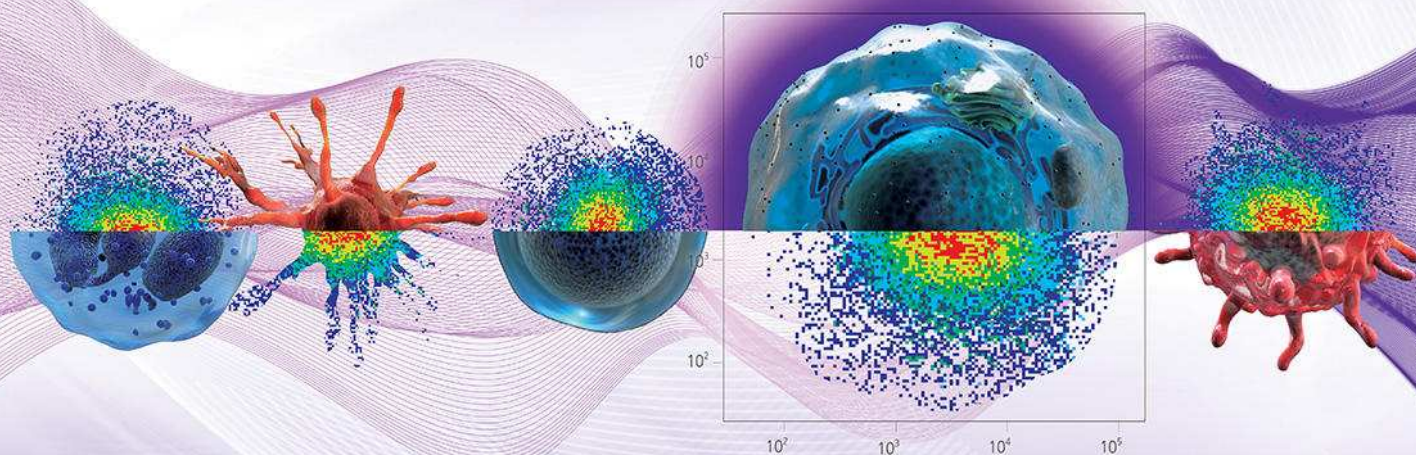




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Neoadjuvant Chemotherapy in Breast Cancer Patients Induces miR-34a and miR-122 Expression

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Circulating microRNAs (miRNAs) have been extensively studied in cancer as biomarkers but little is known regarding the influence of anti-cancer drugs on their expression levels. In this article, we describe the modifications of circulating miRNAs profile after neoadjuvant chemotherapy (NAC) for breast cancer. The expression of 188 circulating miRNAs was assessed in the plasma of 25 patients before and after NAC by RT-qPCR. Two miRNAs, miR-34a and miR-122, that were significantly increased after NAC, were measured in tumor tissue before and after chemotherapy in 7 patients with pathological partial response (pPR) to NAC. These two chemotherapy-induced miRNAs were further studied in the plasma of 22 patients with adjuvant chemotherapy (AC) as well as in 12 patients who did not receive any chemotherapy. Twenty-five plasma miRNAs were modified by NAC. Among these miRNAs, miR-34a and miR-122 were highly upregulated, notably in pPR patients with aggressive breast cancer. Furthermore, miR-34a level was elevated in the remaining tumor tissue after NAC treatment. Studying the kinetics of circulating miR-34a and miR-122 expression during NAC revealed that their levels were especially increased after anthracycline-based chemotherapy. Comparisons of the plasma miRNA profiles after NAC and AC suggested that chemotherapy-induced miRNAs originated from both tumoral and non-tumoral compartments. This study is the first to demonstrate that NAC specifically induces miRNA expression in plasma and tumor tissue, which might be involved in the anti-tumor effects of chemotherapy in breast cancer patients.

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Breast cancer is the most frequently diagnosed cancer worldwide, with 1.38 million new cases recorded in 2008, and it is the leading cause of cancer death in females (Jemal et al., 2011). However, breast cancer death rates have decreased in the past 25 years due to early detection and improved treatments (Bleyer and Welch, 2012).

MicroRNAs (miRNAs) are small, non-coding RNAs that act as negative regulators of gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) (Bartel, 2004; Du, 2005; Eulalia et al., 2008). miRNAs play important roles in cellular processes such as differentiation, proliferation, stress-response and cell death (Ambros, 2004; Bartel, 2004). To date, thousands of human miRNAs have been identified (Kozomara and Griffiths-Jones, 2010), and bioinformatics analyses have estimated that miRNAs may regulate as many as 60% of the human protein-coding genes (Friedman et al., 2009).

Several studies have revealed that miRNAs are deregulated inside tumor tissues, including breast cancer (Lu et al., 2005; Blenkiron et al., 2007). Notably, Lu et al. (2005) analyzed miRNA expression from multiple human cancers and observed differential expression of nearly all miRNAs across tumor types. Moreover, miRNA expression reflects the developmental lineage and differentiation of a specific tumor and is associated with its clinico-pathological features. These findings have strongly suggested that miRNAs play an important role in tumorigenesis.

In the past decade, miRNAs have been demonstrated to be present in the peripheral bloodstream. These circulating miRNAs are protected from degradation by lipoprotein vesicles, called exosomes, and their presence remains highly stable compared with other RNA molecules (Chen et al., 2008; Mathivanan et al., 2010). Tumor cells are assumed to actively secrete specific circulating miRNAs (Pigati et al., 2010) that are

Abbreviations: 3'-UTR, 3'-untranslated region; AC, adjuvant chemotherapy; AJCC-UICC, American Joint Committee on Cancer and the International Union for Cancer Control; AUC, area under the curve; Ct, cycle threshold; DNA, deoxyribonucleic acid; FDR, false discovery rate; FFPE, formalin-fixed paraffin-embedded; gDNA, genomic DNA; HER2, human epidermal growth factor 2; miRNAs, microRNAs; mRNAs, messenger RNAs; NA, not accessed; NAC, neoadjuvant chemotherapy; Ns, nonsignificant; pCR, pathological complete response; pPR, pathological partial response; RNA, ribonucleic acid; ROC, receiver operating curve; SEM, standard error of the mean; TNBC, triple-negative breast cancer.

Conflict of interest: None.

PF performed the molecular genetic studies, participated in the design of the study and statistical analyses, and drafted the manuscript. CJ participated in the design of the study and the writing of the manuscript. MB and NB performed certain molecular studies. IS, VB and GJ conceived the study, participated in its design

and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

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therefore easily accessible and can be used as diagnostic markers for multiple cancers (Mitchell et al., 2008; Kosaka et al., 2010). miRNAs appear to also be passively released by dying cells (Brase et al., 2010), and as a result, circulating miRNA profiling may potentially reflect response to anti-cancer treatments.

Adjuvant chemotherapy (AC) for breast cancer is administered after definitive breast surgery to reduce the risk of cancer recurrence. On the other hand, preoperative or neoadjuvant chemotherapy (NAC) is planned for patients with locally advanced breast cancer or with tumors primarily not suitable for breast-conserving surgery. One of the aims of NAC is to reduce disease burden and render cancers operable. Typically, NAC consists of anthracycline-based chemotherapy followed by tubulin-binding agents. The tumor is then removed, and the response to NAC is assessed by the amount of tumor cells in the surgical specimen. A pathological complete response (pCR) is crucial because it is associated with a better outcome than residual disease (Mieog et al., 2006).

Two studies have already implicated circulating miRNAs as predictive markers of the pathological response to NAC in breast cancer patients (Zhao et al., 2009; Wu et al., 2011). The aim of this work was to examine circulating miRNA profiles during NAC because this condition is the only opportunity to observe the *in vivo* exposition of treatment-naïve tumor cells to the chemotherapy.

In the present study, we demonstrated for the first time that NAC increased circulating miR-34a and miR-122 levels. Comparisons of the plasma miRNA content after different chemotherapies and in the presence/absence of a tumor suggested that miR-34a originated from both tumoral and non-tumoral compartments, but miR-122 only originated from non-tumoral tissues. We also showed that increased miR-34a and miR-122 expression was greater after anthracycline treatments. Whether these miRNAs are markers and/or players in NAC remains to be elucidated.

Materials and Methods

Patients, controls, plasma and tissues collection

Ethics approval was obtained from the Institutional Review Board (Ethical Committee of the Faculty of Medicine of the University of Liège) in compliance with the Declaration of Helsinki. Patients with newly diagnosed primary breast cancer were recruited prospectively at CHU of Liège (Liège, Belgium) from 7/2011 to 7/2013. All patients signed a written informed consent form. This work consisted of a prospective study and did not lead to any changes in the treatment of enrolled patients; 59 patients were included in this study. Control plasma samples were obtained from 20 healthy female volunteers of a similar mean age (51 ± 10 years) and without a history of cancer. Blood samples were collected into 9-ml EDTA tubes. Plasma was prepared within 1 h by retaining the supernatant after double centrifugation at 4 °C (10 min at 815 g and 10 min at 2500 g) and stored undefined at -80°C .

Twenty-five patients with primary locally advanced breast cancer received NAC with 3 or 4 courses of alkylating agents (cyclophosphamide or fluorouracil) and anthracycline-based chemotherapy (epirubicin) followed by 3 or 12 courses of tubulin-binding agents (docetaxel or paclitaxel). Only patients achieving a pCR (ypT0N0 following the AJCC-UICC classification) were considered responders. Blood samples were drawn from NAC-treated patients according to the time course described in Figure 4A.

For 7 NAC-treated patients, 4- μm tumor slices from formalin-fixed paraffin-embedded (FFPE) tissues samples were obtained from diagnostic core-needle biopsies (2 to 3 passes in the primary tumor) and from the corresponding tumorectomy. The histological status of all tissues samples was established by a pathologist using hematoxylin and eosin staining from the FFPE sections.

Approximately 15 days after the initial surgery, 22 primary breast cancer patients were treated with AC containing 3 or 4 courses of alkylating agents (cyclophosphamide or fluorouracil) and anthracycline-based chemotherapy (epirubicin) followed by 3 or 12 courses of tubulin-binding agents (docetaxel or paclitaxel). For these patients, blood samples were collected as shown in Figure 6A.

Twelve primary breast cancer patients did not receive any chemotherapy, and their plasma was collected 8 days before (D-8) and 3 months after (3M) surgery.

Patients who would benefit from breast preservation received post-operative radiotherapy. Endocrine therapy (tamoxifen, goserelin, anastrozole, or letrozole) was administered to hormone receptor-positive patients, and anti-HER2 therapy (trastuzumab, lapatinib, or pertuzumab) was administered to patients with HER2 overexpression.

All patients and tumor characteristics are summarized in Table 1.

Study design

Figures 4A and 6A summarize the plasma withdrawal time points in each patient group.

A pilot study was first conducted and consisted of measuring the expression of 742 plasma miRNAs in 18 patients before and after treatment (12 with NAC, 3 with AC, and 3 without chemotherapy). On the basis of their high expression levels in the pilot experiment, 188 miRNAs were chosen (data not shown).

The first study (*study 1*) consisted of measuring 188 miRNA levels in plasma in 13 additional patients with NAC at NA1 and D-8. Altogether, 188 miRNA profiles were obtained for 25 NAC-treated patients before and after treatment. The mean cycle threshold (Ct) of the 50 most highly expressed miRNAs was used for normalization. The lists of miRNAs and sequences used in *study 1* are provided in the supplementary data (Additional file 1).

In a second study (*study 2*), the levels of 2 miRNAs were measured in the blood of 25 patients with NAC at NA1, NA2, D-8 and 3M, 22 patients with AC at D-8 and 3M, 12 patients without chemotherapy at D-8 and 3M. miR-34a and miR-122 were selected because of their significant deregulation by NAC, as established in *study 1*. The mean Ct of the two most stable miRNAs across NAC-treated patients (miR-484 and miR-652) was used as reference gene in addition to the cel-miR-39 spike-in.

The levels of tumor miR-34a and miR-122 were measured in the initial core-needle biopsies and corresponding tumorectomy for 7 patients with a pathological partial response (pPR) to NAC. The relative expression levels of tumor miR-34a and miR-122 were obtained by normalizing to the mean Ct of cel-miR-39 spike-in, cel-miR-238 spike-in, RNU44 snoRNA, and RNU48 snoRNA.

Confirmation of plasma miR-34a variations was assessed by a second RT-qPCR technique on the same plasma from 17 NAC-treated patients at NA1 and D-8. The relative expression levels of plasma miR-34a were normalized to the mean Ct of cel-miR-39 spike-in and cel-miR-238 spike-in.

RNA extraction and qRT-PCR of miRNAs

Circulating miRNAs were purified from 100 μl of plasma with the miRNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. The standard protocol was adapted on the basis of Kroh's recommendations (Kroh et al., 2010). MS2 (Roche, Belgium) was added to the samples as a carrier, cel-miR-39 and cel-miR-238 were added as spike-ins. RNA was eluted in 50 μl of RNase-free water at the end of the procedure.

Reverse transcription was performed using the miRCURY LNATM Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon, Denmark). Quantitative PCR was performed according to the manufacturer's instructions on custom panels of 188 selected miRNAs in *study 1* and on two selected miRNAs in *study 2* (Pick-&-Mix microRNA PCR Panels, Exiqon). Controls

TABLE I. Patients and tumors characteristics.

Characteristics	Primary breast cancer patients with NAC (n = 25)	Primary breast cancer patients with AC (n = 22)	Primary breast cancer patients without chemotherapy (n = 12)
Age (mean $\pm$ SD) (y)	47 $\pm$ 14	52 $\pm$ 11	67 $\pm$ 9
Estrogen receptor [n (%)]	14 (56)	19 (86)	12 (100)
Progesterone receptor [n (%)]	12 (48)	14 (64)	11 (92)
HER2 [n (%)]	9 (36)	7 (32)	1 (8)
KI-67 (mean $\pm$ SD) (%)	36 $\pm$ 19	28 $\pm$ 19	9 $\pm$ 8
Molecular subtype [n (%)]			
NA	0 (0)	0 (0)	0 (0)
Luminal A	1 (4)	3 (14)	9 (75)
Luminal B	14 (56)	16 (72)	3 (25)
HER2-enriched	3 (12)	0 (0)	0 (0)
ER-negative	8 (32)	3 (14)	0 (0)
Initial T staging [n (%)]			
NA	0 (0)	0 (0)	0 (0)
1	1 (4)	12 (55)	11 (92)
2	14 (56)	8 (36)	1 (8)
3	2 (8)	2 (9)	0 (0)
4	8 (32)	0 (0)	0 (0)
Size (mean $\pm$ SD) (mm)	48 $\pm$ 27	23 $\pm$ 15	13 $\pm$ 6
Posttreatment ypT staging [n (%)]			
NA	0 (0)	22 (100)	12 (100)
0	9 (36)	0 (0)	0 (0)
1	7 (28)	0 (0)	0 (0)
2	8 (32)	0 (0)	0 (0)
3	0 (0)	0 (0)	0 (0)
4	1 (4)	0 (0)	0 (0)
Initial N staging [n (%)]			
NA	0 (0)	0 (0)	0 (0)
0	9 (36)	11 (50)	9 (75)
1	14 (56)	7 (32)	3 (25)
2	0 (0)	3 (14)	0 (0)
3	2 (8)	1 (4)	0 (0)
Posttreatment ypN staging [n (%)]			
NA	0 (0)	22 (100)	12 (100)
0	15 (60)	0 (0)	0 (0)
1	5 (20)	0 (0)	0 (0)
2	4 (16)	0 (0)	0 (0)
3	1 (4)	0 (0)	0 (0)
Scarff-Bloom-Richardson grading system [n (%)]			
NA	0 (0)	0 (0)	0 (0)
1	0 (0)	0 (0)	0 (0)
2	11 (44)	8 (36)	12 (100)
3	14 (56)	12 (54)	0 (0)
Histologic subtype [n (%)]			
NA	0 (0)	0 (0)	0 (0)
IDC	24 (96)	18 (82)	6 (50)
ILC	1 (4)	3 (14)	4 (33)
Others	0 (0)	1 (4)	2 (17)
Lymphovascular invasion [n (%)]	6 (24)	7 (32)	2 (17)
Trastuzumab [n (%)]	10 (40)	7 (32)	0 (0)
Radiotherapy [n (%)]	25 (100)	19 (86)	10 (83)
Hormonotherapy [n (%)]			
SERM	10 (40)	8 (36)	9 (75)
AI	4 (16)	11 (50)	3 (25)

included the reference genes described in the text, inter-plate calibrators in triplicate (Sp3) and negative controls.

Confirmation of plasma miR-34a expression variations was determined using TaqMan assays (Applied Biosystems, USA) with predesigned probes according to the manufacturer's instructions. Total RNA extracted from plasma was reverse transcribed using the TaqMan miRNA RT Kit and stem-loop RT primers (Applied Biosystems).

Tumor miRNAs were extracted from 5 macrodissected sections of 4- μ m FFPE tissues scrubbed from glass slides after tumor status confirmation by a pathologist. Genomic DNA (gDNA) and DNase-treated RNA were isolated using the Allprep DNA/RNA FFPE Kit (Qiagen) according to the manufacturer's protocol. The concentration of the isolated RNA was measured with the Quant-iTTM RiboGreen[®] RNA Assay Kit as recommended (Invitrogen, UK). Reverse transcription was performed on 5 ng of RNA using the TaqMan miRNA RT Kit, and quantitative PCR used stem-loop RT primers (Applied Biosystems).

All PCR reactions were performed on an Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems). miRNAs were considered for analysis with a Ct value <36 .

Data analyses

Analyses were conducted using the $2^{-\Delta Ct}$ method ($\Delta Ct = Ct_{\text{sample}} - Ct_{\text{reference gene}}$) for each sample to obtain a normalized expression value (Schmittgen and Livak, 2007).

The reference genes were chosen as follows:

(i) Circulating miRNAs in the Pick-&-Mix microRNA PCR Panels (Exiqon)

Data were normalized using the ΔCt method as recommended by Mestdagh et al. (2009). In *study 1*, the mean Ct of the 50 most highly expressed miRNAs was used for normalization, as it was the most stable reference gene according to the GeNorm software (results of GeNorm analysis are available in Additional file 2). For *study 2*, the mean Ct of the two most stable miRNAs across NAC-treated patients (miR-484 and miR-652) was used as reference genes in addition to the cel-miR-39 spike-in.

(ii) Circulating and tumoral miRNAs with the TaqMan miRNA RT-PCR Kit (Applied Biosystems)

The relative expression levels of miR-34a were obtained by normalizing to the mean Ct of cel-miR-39/cel-miR-238 spike-ins (for plasma and tumor miRNAs) and RNU44/RNU48 snoRNAs (for tumor miRNAs only).

Statistical analyses were performed with the GraphPad Prism software, version 6.00 (GraphPad Software, USA) (www.graphpad.com/scientific-software/prism/). The normal distribution of values was evaluated with the D'Agostino-Pearson omnibus and Shapiro-Wilk tests. To compare miRNAs levels, Student's t-test (paired or unpaired, two-tailed) and non-parametric tests (Wilcoxon or Mann-Whitney tests, two-sided) were used. Correlations between plasma and tumor miRNA levels, and between circulating miR-34a and troponin T levels were assessed with the Spearman test. Statistical significance was established as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) or $P < 0.0001$ (****).

Results

NAC induces high plasma expression levels of miR-34a and miR-122

Twenty-five patients with locally advanced primary breast cancer treated with NAC were selected, and 188 miRNA profiles were assessed in plasma drawn before and after NAC.

Twenty-five miRNAs were significantly modified by NAC (Fig. 1). The two most significantly upregulated miRNAs were miR-34a and miR-122 (Figs. 1B and C), which were elevated by 2.5 and 1.9-fold, respectively, after NAC (Fig. 1B and Fig. 2A and B).

miR-34a upregulation was confirmed by a second RT-qPCR technique using TaqMan assays on the same plasma samples for 17 patients, and the results remained significant ($P < 0.001$, 3.2-fold change, Additional file 3).

Interestingly, we observed that 7 patients had a particularly high fold change for chemotherapy-induced miR-34a and/or miR-122 expression (Fig. 2, blue points). Moreover, 6 out of these 7 patients had a pPR to NAC. The deregulation of circulating miRNAs in patients who achieved a pCR ($n = 8$) after NAC was so compared with that of patients who did not achieve a pCR ($n = 17$). pPR patients tended to have a greater fold change for miR-34a and miR-122 compared with pCR patients (Fig. 3A and B). Patients who attained a pCR after NAC, which is defined as no residual tumor tissue in the breast

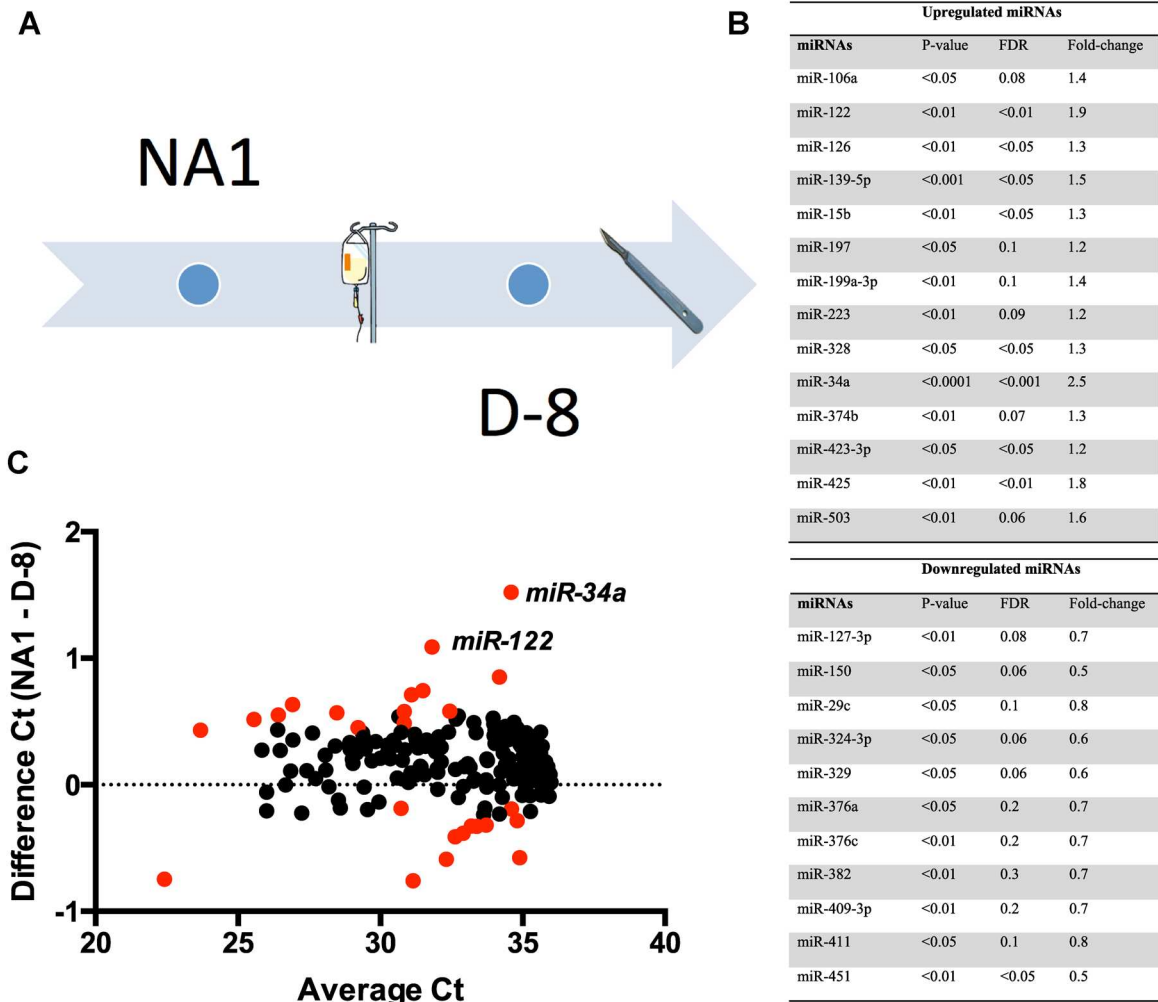


Fig. 1. Variations in circulating miRNAs after neoadjuvant chemotherapy (NAC). Expression of 188 circulating miRNAs was determined at two time points by RT-qPCR in 25 NAC-treated patients. Expression was normalized to the mean Ct of the 50 most highly expressed miRNAs. The Wilcoxon matched-pairs signed rank test was used to compare miRNA profiling before and after NAC. The results were adjusted for multiple testing by controlling for the false discovery rate (FDR) according to the Benjamini-Hochberg method. (A) Blood samples were collected from patients before (NA1) and after (D-8; ~1 week before surgery) NAC. (B) Twenty-five circulating miRNAs were significantly modified after NAC. (C) Bland-Altman plot of 188 circulating miRNAs before (NA1) and after (D-8) NAC. In red, 25 circulating miRNAs significantly modified after NAC. miR-34a and miR-122 were the most upregulated miRNAs at the end of NAC.

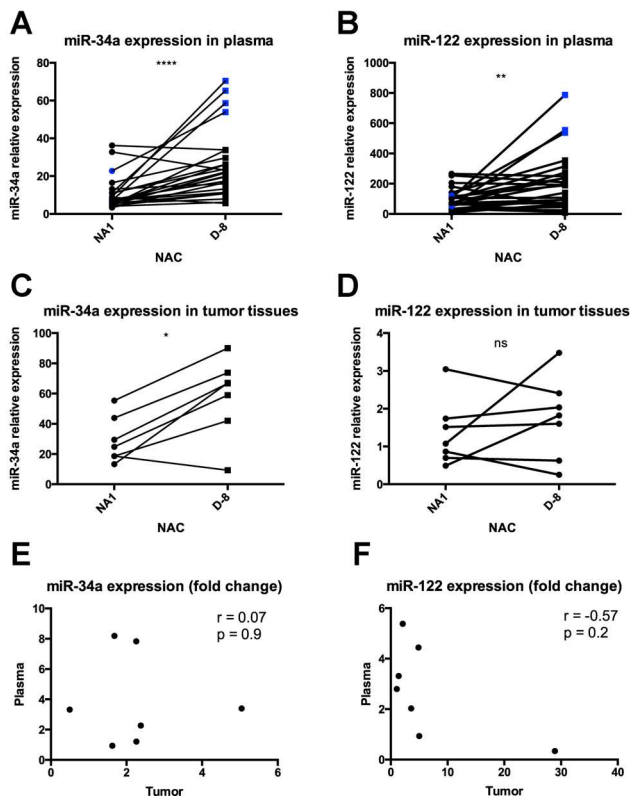


Fig. 2. Upregulation of miR-34a and miR-122 in plasma and tumor tissue at the end of neoadjuvant chemotherapy (NAC). Expression of miR-34a and miR-122 was determined by RT-qPCR in the plasma of 25 NAC-treated patients and in the tumor tissue of 7 patients with partial pathological response (pPR) to NAC. Expression was normalized to the mean Ct of the 50 most highly expressed miRNAs in A and B and to the mean Ct of RNU 44, RNU 48, cel-miR-39, and cel-miR-238 in C-F. Comparisons between the initial and subsequent time points were calculated using the Wilcoxon matched-pairs signed rank test. Plasma miR-34a (A) and miR-122 (B) were upregulated after NAC ($P < 0.0001$ and $P < 0.01$, respectively). (C) Upregulation of miR-34a was observed in tumor tissues from patients with pPR after NAC ($P < 0.05$). (D) miR-122 was not significantly upregulated in tumor tissues after NAC. (E and F) Pearson and Spearman correlations did not show any association in the fold change (NA1 vs. D-8) of plasma and tumor miRNAs.

and lymph nodes, have improved survival. Two meta-analyses have confirmed that pCR is a greatest surrogate marker for predicting long-term clinical outcome in aggressive tumor subtypes, namely HER2-positive/hormone receptor-negative and triple-negative breast cancers (TNBC, i.e., breast tumors negative for hormone receptors and for HER2 gene amplification), compared with hormone receptor-positive tumors (Minckwitz et al., 2012; Cortazar et al., 2014). Analyses were thus conducted only on the HER2-enriched and TNBC subgroups and revealed that the fold change of circulating miR-34a after treatment was significantly greater in pPR patients compared with pCR patients (3.4 vs. 1.8, $P < 0.01$, Fig. 3C). For miR-122, the difference between pPR and pCR patients showed a considerable trend toward significance (Fig. 3D, $P = 0.06$). Interestingly, the initial miR-34a expression levels (NA1) were similar in the pCR and pPR subgroups (Fig. 3E). The fold change of miR-34a effectively distinguished patients with pCR and pPR, as shown in Fig. 3F. To predict the pathological response to NAC, the ROC of the miR-34a upregulation was calculated, and it showed a high performance with a sensitivity of 100% and a specificity of 80% (AUC = 0.97).

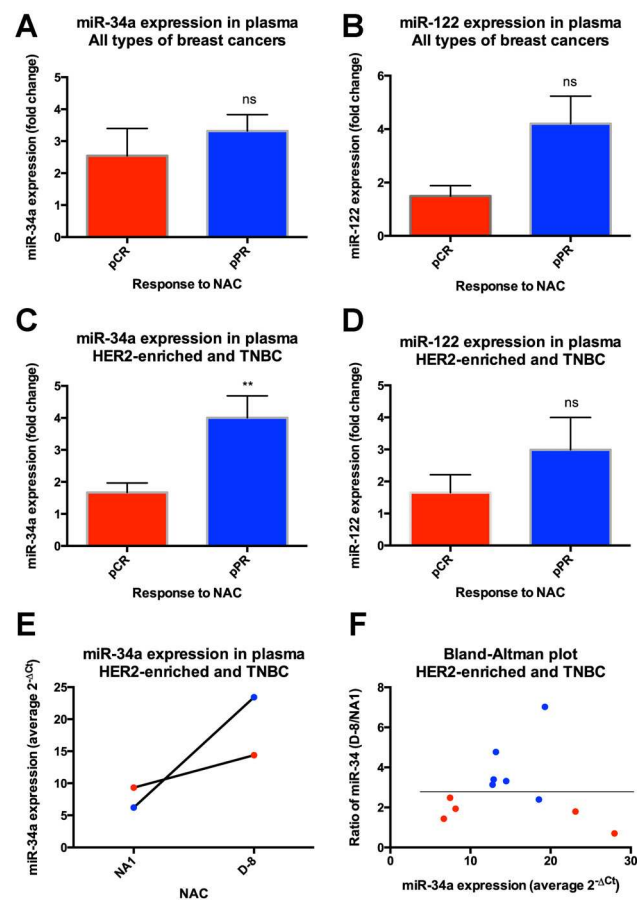


Fig. 3. Comparisons of circulating miR-34a and miR-122 in NAC-treated patients with pathological complete response (pCR, in red) and pathological partial response (pPR, in blue). Expression of miR-34a and miR-122 was determined by RT-qPCR in 25 breast cancer patients before (NA1) and after (D-8) NAC. Expression was normalized to the mean Ct of the 50 most highly expressed miRNAs. Comparisons between pCR and pPR patients were calculated using the Mann-Whitney test. The fold change of circulating miR-34a (A) and miR-122 (B) was elevated in pPR patients ($n = 17$) compared with pCR patients ($n = 8$). The data are expressed as the mean $\pm$ SEM. (C and D) miR-34a expression was significantly increased after NAC in pPR patients ($n = 6$) compared with pCR patients ($n = 5$) with HER2-enriched and triple negative breast cancers (TNBC). The data are expressed as the mean $\pm$ SEM. (E) The initial level of miR-34a expression (NA1) was similar in the pCR and pPR subgroups. (F) Bland-Altman plot of NAC-induced miR-34a upregulation in patients with pCR and pPR.

NAC induces miR-34a expression in tumor tissue

The 2 NAC-induced miRNAs (miR-34a and miR-122) have been described as tumor suppressors in several cancer studies, including studies on breast cancer (Hermeking, 2009; Wang et al., 2011). To determine whether the upregulation of these circulating miRNAs correlated with increased expression in tumor tissue, we collected initial core-needle biopsies and corresponding tumorectomy for 7 patients with a pPR to NAC and studied miRNA expression by RT-qPCR. A significant increase in miR-34a expression was present in residual tumoral tissue after NAC compared with the initial biopsy ($P < 0.05$, 1.9-fold change, Fig. 2C). Interestingly, decreased miR-34a expression (0.5-fold change) was evident for the one patient who had a poor response to NAC (ypT2N2Mx) and an early

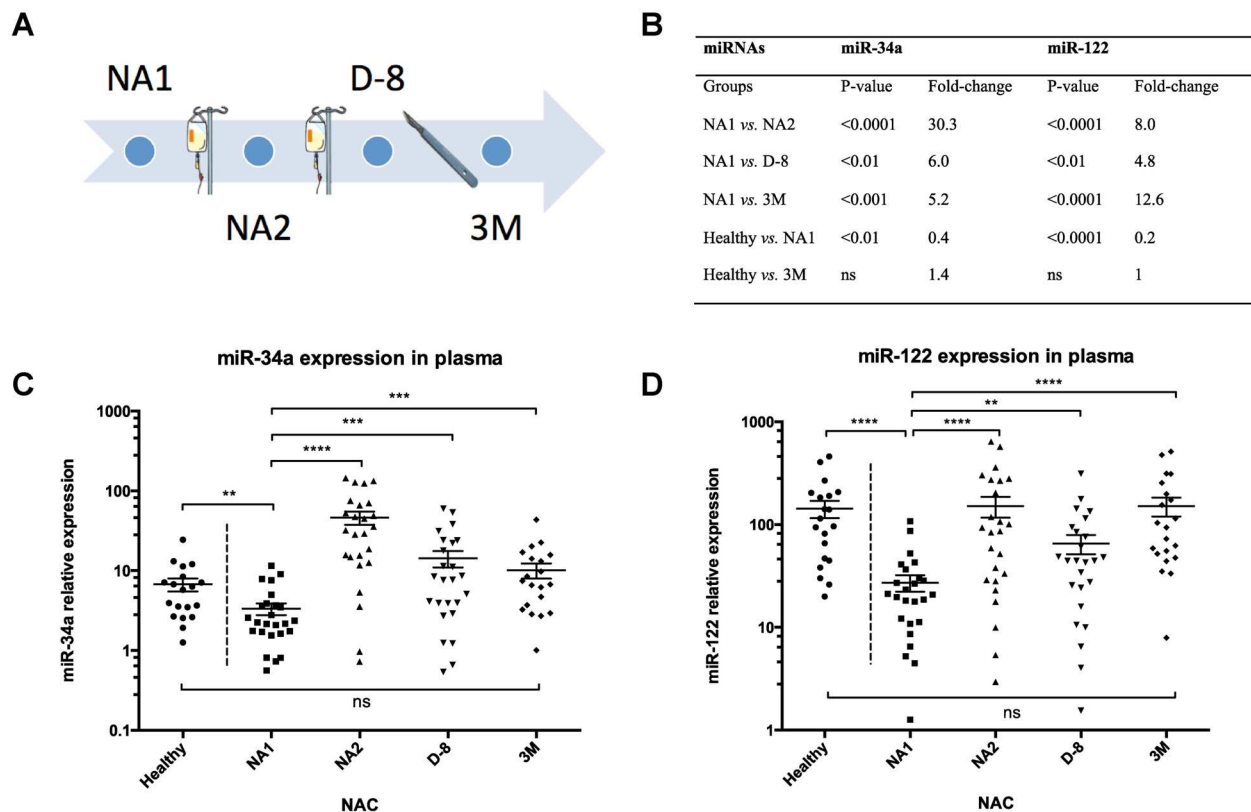


Fig. 4. Kinetics of circulating miR-34a and miR-122 variations during neoadjuvant chemotherapy (NAC). Expression of miR-34a and miR-122 was determined by RT-qPCR in 25 NAC-treated patients at 4 time points and in 20 women without a history of cancer. Expression was normalized to the mean Ct of miR-484, miR-652, and cel-miR-39. Comparisons between the initial and subsequent time points were calculated using the Wilcoxon matched-pairs signed rank and Mann-Whitney tests. (A) Blood samples were drawn from NAC-treated patients as follows: before therapy (NA1); after 1 or 2 cycles of anthracycline-based chemotherapy (NA2); ~1 week before surgery (D-8) and ~3 months after surgery (3M). (B) Results of the Wilcoxon matched-pairs signed rank test. Circulating miR-34a and miR-122 upregulation after anthracycline-based chemotherapy was greater than at the end of NAC. Plasma miR-34a, (C) and miR-122 (D) levels were highly upregulated after anthracycline-based NAC and remained at elevated levels 3 months after surgery. Circulating miR-34a (C) and miR-122 (D) levels were downregulated in breast cancer patients compared with healthy women ($P < 0.01$ and $P < 0.0001$, respectively), and their expression levels were similar between NAC-treated breast cancer patients and healthy women. The data are expressed as the mean $\pm$ SEM.

metastatic relapse. Surprisingly, this patient presented with upregulated circulating miR-34a levels after NAC, with a 3-fold increase. miR-122 was weakly expressed in tumor tissues, nearly 100-fold lower than miR-34a, and any significant upregulation of tumoral miR-122 occurred after NAC (Fig. 2D). No correlation could be established between the fold change of circulating miRNAs and tumor miRNAs (Figs. 2E and F). These data suggest a non-tumoral source of NAC-induced miR-34a and miR-122. As miR-122 is highly expressed in the liver, and as we did not observe increased expression in the tumor after NAC, its plasma levels might reflect chemotherapy-induced liver release.

Circulating miR-34a and miR-122 levels significantly increase after anthracycline-based chemotherapy and up to 3 months post-surgery

Circulating miR-34a and miR-122 expression levels were then assessed at different time points during NAC. The mean expression of miR-484, miR-652, and the spike-in cel-miR-39 was used to normalize expression levels, as this combination was determined to be the most stable by GeNorm analysis (Additional file 2).

The plasma levels of the two selected miRNAs were measured in 25 patients with NAC before therapy (NA1), after 1 or 2 cycles of anthracycline-based chemotherapy (NA2), at the end of NAC (D-8) and 3 months after surgery (3M). The study sequence is shown in Figure 4A, and the results are provided in Figure 4B and D.

The upregulation of selected miRNAs after anthracycline-based chemotherapy was greater than at the end of NAC, particularly for miR-34a, which had a fold change of 30. Three months after surgery, the miRNA expression levels remain elevated compared with the initial levels.

Circulating miR-34a and miR-122 levels are downregulated in breast cancer patients and normalized after treatments

miR-34a and miR-122 levels were measured in 20 age-matched healthy women and were compared with their expression levels in NAC-treated breast cancer patients.

Both circulating miR-34a (Fig. 4C) and miR-122 (Fig. 4D) were initially downregulated in breast cancer patients compared with controls ($P < 0.01$ for miR-34a and $P < 0.0001$ for miR-122). miRNA expression levels in patients returned to

Correlation between upregulation of plasma miR-34a and troponin T

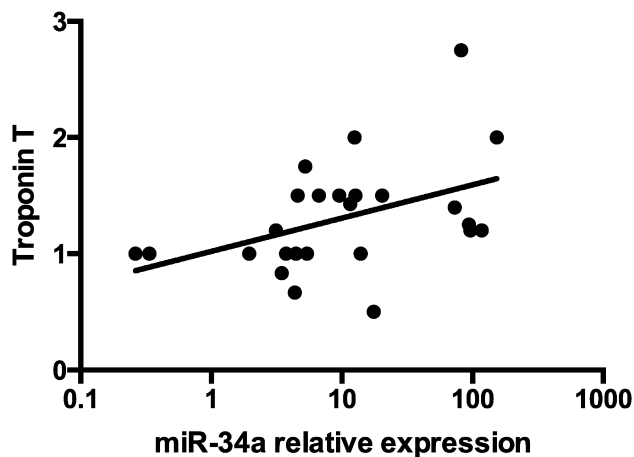


Fig. 5. Correlation between miR-34a and troponin T upregulation during neoadjuvant chemotherapy (NAC). Pearson and Spearman correlations showed a positive association between the fold change (NA1 vs. NA2) of miR-34a and troponin T ($P < 0.05$, $r = 0.4673$, $n = 25$) in the plasma of NAC-treated patients.

levels similar to controls 3 months after chemotherapy and surgery.

Correlation between circulating miR-34a upregulation and anthracycline cardiotoxicity

Recently, Boon et al. (2013) demonstrated that miR-34a plays a role in cardiomyopathy associated with aging and stroke. Because anthracyclines are known to be cardiotoxic drugs (Floyd et al., 2005), we measured plasma troponin T levels in the same patients ($n = 25$, NA1 and NA2) to determine whether there was a correlation between increased miR-34a expression and this cardiotoxicity. No patients had a history of heart or renal failure or myocardial infarction. The Pearson and Spearman correlation test showed a positive association between the fold change of miR-34a expression and troponin T levels ($P < 0.05$, $r = 0.4673$), suggesting that miR-34a may play a role in anthracycline cardiotoxicity (Fig. 5).

Tumor presence is primordial for the increase in circulating miR-34a and miR-122 expression during chemotherapy

The two selected miRNAs were also studied in 22 patients with AC to determine the influence of the tumor presence on miRNA upregulation during chemotherapy.

miRNA expression was determined D-8 before surgery and 3M after surgery, which corresponds to the end of

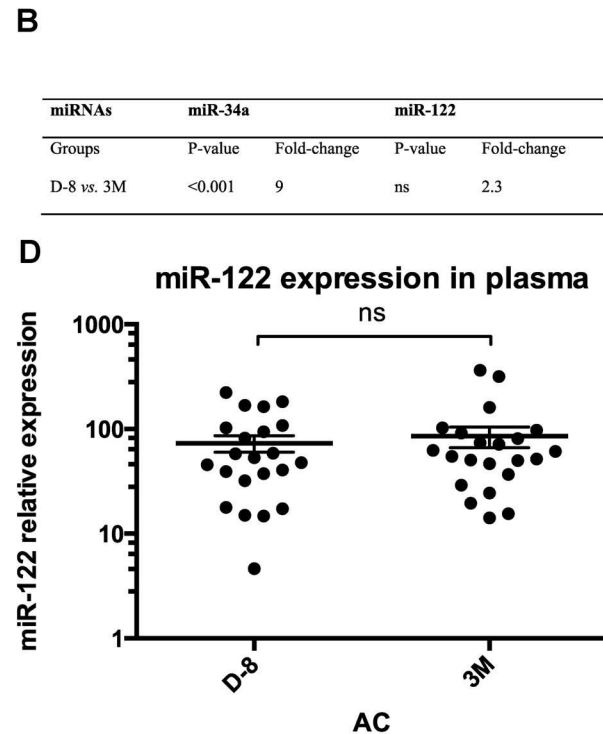
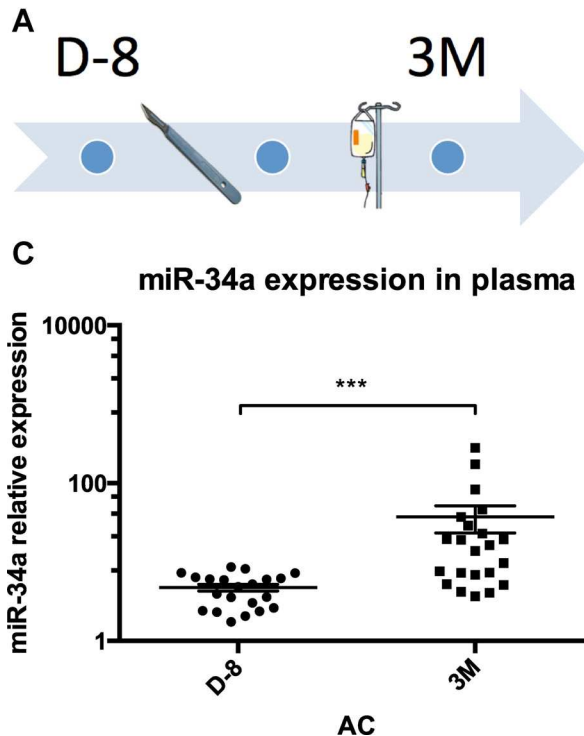


Fig. 6. Kinetics of circulating miR-34a and miR-122 variations during adjuvant chemotherapy (AC). The expression of miR-34a and miR-122 was determined at two time points by RT-qPCR in 22 AC-treated patients. Expression was normalized to the mean Ct of miR-484, miR-652, and cel-miR-39. Comparisons between the initial and subsequent time points were calculated using the Wilcoxon matched-pairs signed rank test. (A) Blood samples were collected from AC-treated patients as follows: ~1 week before surgery (D-8) and ~3 months after surgery (3M), which corresponded to the end of anthracycline-based chemotherapy. (B) Results of the Wilcoxon matched-pairs signed rank test. (C) Plasma miR-34a levels increased after anthracycline-based AC but lesser than with NAC ($P < 0.001$). The data are expressed as the mean $\pm$ SEM. (D) Nonsignificant upregulation of miR-122 expression was found after AC. The data are expressed as the mean $\pm$ SEM.

anthracycline-based treatment. The study sequence is shown in Fig. 6A, and the results are summarized in Figs. 6B–D.

miR-34a expression significantly increased by 9 fold after anthracycline-based AC, which corresponded to approximately one-third of the increase after NAC. miR-122 was not significantly modulated after AC.

These results suggest that the presence of the tumor is important for increasing these two miRNAs during chemotherapy and that miR-34a expression is highly sensitive to the effects of anthracycline-like agents.

The expression of the two selected miRNAs was also measured in the plasma of 12 patients with primary breast cancer treated only by surgery without chemotherapy to determine whether the increased miR-34a levels in patients with AC related to tumor removal. miRNA expression was measured before D-8 and 3M after surgery. No significant variations were evident (Additional file 4).

Discussion

This study is the first reported investigation that describes alterations in circulating miRNA expression after NAC in breast cancer patients. Twenty-five circulating miRNAs were significantly deregulated after NAC. Our results reveal that two miRNAs with anti-oncogenic functions, miR-34a and miR-122, were highly increased in response to NAC.

The source of these chemotherapy-induced miRNAs is not known, and in our experimental settings, they appeared to arise from multiple origins. The tumor itself is a known source of circulating miRNAs. *In vitro* experiments have notably demonstrated that several tumor cell lines exposed to cytotoxic agents or ionizing radiation re-expressed miR-34a (Tazawa et al., 2007; Ghawanmeh et al., 2010; Chiyomaru et al., 2012; John-Aryankalayil et al., 2012; Stankevicius et al., 2013; Wang et al., 2013). Our team also observed miR-34a overexpression in breast tumor tissues after the NAC. However, the tumor cannot be the only source of chemotherapy-induced miRNA expression for the following reasons: (i) miR-122 was not significantly elevated in breast tumor after NAC; (ii) no correlation between plasma and tumor miRNA upregulation was evident; and (iii) circulating miR-34a was upregulated after tumor removal under the effect of AC. The two miRNAs we studied are also broadly expressed in normal tissues (Hsu et al., 2007), which could contribute to the release of miRNAs in response to chemotherapy. The most abundant liver-specific miRNA (Hu et al., 2012a), miR-122, might be induced by NAC because of the hepatotoxicity of chemotherapy; however, we failed to show any correlation between its expression and detectable hepatic damage (data not shown).

The tumor suppressor p53 is known to promote either growth arrest and apoptosis, depending on DNA damage (Boominathan, 2010). A number of miRNAs are involved in the p53 pathway, particularly miR-34a and miR-122, which are directly induced by p53 to exert anti-tumor functions (He et al., 2007; Manfè et al., 2012). This feature might explain the significant upregulation observed after anthracyclines, which cause DNA breaks and p53 activation (Lowe et al., 1994). Indeed, cancer cell lines treated with anthracyclines showed a p53-dependent increase in miR-34a expression (Tazawa et al., 2007; Wang et al., 2013). However, no correlation was observed between miR-34a downregulation in breast cancer and the presence of p53 mutations (Javeri et al., 2013). Moreover, miR-34a is also induced by chemotherapy in a p53-independent manner, e.g., platinum-based chemotherapy induced epigenetic activation of miR-34a by demethylating its promoter in bladder cancer cells (Li et al., 2013).

miR-34a and miR-122 are predominantly tumor suppressors. To slow tumor progression, they target many oncogenes, such as Bcl-2, N-Myc, and E2F3 for miR-34a

(Hermeking, 2009) and the PI3K/Akt pathway for miR-122 (Manfè et al., 2012). These miRNAs are downregulated in tumors compared with normal adjacent tissues (Peurala et al., 2011; Wang et al., 2011; Javeri et al., 2013) and in the plasma of breast cancer patients compared with healthy women. miR-34a downregulation was observed only in the tumor of a patient with a poor response to NAC, and early metastatic relapse correlated with its tumor suppressive role. Moreover, we suggest that the NAC-induced circulating miRNAs might be involved in the anti-tumor effects of chemotherapy. In fact, some studies have proposed a biological function for circulating miRNAs, illustrating their intercellular communication properties (Mathivanan et al., 2010; Turchinovich et al., 2013). Li et al. (2012) have perfused liposomal nanoparticles containing miR-34a in a mouse model of breast cancer and showed significantly decreased tumor growth and prolonged survival, proving the systemic anti-tumor activity of miR-34a. In this context, high levels of circulating tumor suppressor miRNAs might help prevent metastatic relapse.

Boon et al. (2013) demonstrated that miR-34a is involved in fibrosis and cell death following aging and acute myocardial infarction by inhibiting PNUITS (PPP1R10), which normally inhibits telomere shortening and apoptosis in cardiomyocytes. Anthracyclines, which induce high miR-34a expression in neoadjuvant and adjuvant settings, are known to produce short- and long-term cardiotoxicity; however, the exact mechanism of this toxicity has not been elucidated (Floyd et al., 2005). These preliminary results may implicate miR-34a in the cardiotoxicity of anthracyclines. Furthermore, clinical studies are required to determine whether circulating miRNAs could be biomarkers of drug-induced cardiotoxicity.

Finally, the importance of selecting appropriate endogenous controls to obtain reproducible results is of great interest. The GeNorm analysis performed to select the best circulating miRNAs for normalization revealed miR-484 as the most stable miRNA in NAC-treated patients. This result confirms that miR-484 is a candidate endogenous control for plasma miRNA detection in breast cancer, as previously proposed by two different groups (Hu et al., 2012b; Dvinge et al., 2013).

Conclusions

In conclusion, this study is the first to highlight the effects of NAC on circulating miRNAs. NAC significantly increased miR-34a expression, which might play a key role in its anti-tumor effects, as well as the expression of the tumor suppressor miR-122. Our results also revealed a heterogeneous biological response to NAC. In this context, chemotherapy-induced miR-34a and miR-122 may serve useful purposes. First, they may aid in understanding the molecular mechanisms underlying the pathological response to NAC. Secondly, miRNAs could potentially be predictive biomarkers of the response to NAC, which would allow patients to benefit from personalized medicine. Further research is nevertheless required to confirm that circulating miRNAs are diagnostic, prognostic, and predictive biomarkers in breast cancer patients.

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