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# Novel circulating microRNA signature as a potential non-invasive multi-marker test in ER-positive early-stage breast cancer: A case control study



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## ABSTRACT

**Introduction:** There are currently no highly sensitive and specific minimally invasive biomarkers for detection of early-stage breast cancer. MicroRNAs (miRNAs) are present in the circulation and may be unique biomarkers for early diagnosis of human cancers. The aim of this study was to investigate the differential expression of miRNAs in the serum of breast cancer patients and healthy controls.

**Methods:** Global miRNA analysis was performed on serum from 48 patients with ER-positive early-stage breast cancer obtained at diagnosis (24 lymph node-positive and 24 lymph node-negative) and 24 age-matched healthy controls using LNA-based quantitative real-time PCR (qRT-PCR). A signature of miRNAs was subsequently validated in an independent set of 111 serum samples from 60 patients with early-stage breast cancer and 51 healthy controls and further tested for reproducibility in 3 independent data sets from the GEO Database.

**Results:** A multivariable signature consisting of 9 miRNAs (miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, miR-425) was identified that provided considerable discrimination between breast cancer patients and healthy controls. Further, the ability of the 9 miRNA signature to stratify samples from breast cancer patients and healthy controls was confirmed in the validation set ( $p = 0.012$ ) with a corresponding  $AUC = 0.665$  in the ROC-curve analysis. No association between miRNA expression and tumor grade, tumor size, menopausal- or lymph node status was observed. The signature was also successfully validated in a previously published independent data set of circulating miRNAs in early-stage breast cancer ( $p = 0.024$ ).

**Abbreviations:** miRNA, microRNA; mRNA, messenger RNA; ER, Estrogen Receptor; LNA, Locked Nucleic Acid; PCA, Principal Component Analysis; ROC, Receiver Operating Characteristics; AUC, Area Under the Curve.

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**Conclusions:** We present herein a 9 miRNA signature capable of discriminating between ER-positive breast cancer and healthy controls. Using a specific algorithm based on the 9 miRNA signature, the risk for future individuals can be predicted. Since microRNAs are highly stable in blood components, this signature might be useful in the development of a blood-based multi-marker test to improve early detection of breast cancer. Such a test could potentially be used as a screening tool to identify individuals who would benefit from further diagnostic assessment.

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## 1. Introduction

The incidence of breast cancer, the most frequent cancer among women and the second leading cause of cancer death in women in Europe and North America, is steadily increasing. Currently, mammography is the standard screening tool worldwide, but this technique is not without limitations and is associated with substantial over diagnosis as well as a high false-positive rate (Taplin et al., 2008). About 3% of the population screened annually with mammography will show abnormalities, but about 65% of these will be false positives (Langagergaard et al., 2013). In addition, small cancers are easily missed, especially in younger women due to denser breast tissue (Carney et al., 2003). Therefore, there is a need for highly sensitive and specific minimally invasive biomarker-based assays for early detection of breast cancer, alone or in combination with mammography.

MicroRNAs (miRNAs) are small, 18–25 nucleotide long, non-coding RNA molecules that down-regulate the translation of messenger RNA (mRNA). MicroRNAs control a wide array of physiological and pathological processes, including development, differentiation, cellular proliferation, programmed cell death, oncogenesis and metastasis, by modulating the expression of their target genes through cleaving mRNA molecules or inhibiting their translation. A tumor suppressor miRNA typically blocks the expression of a “true” oncogene mRNA. Conversely, miRNA oncogenes block expression of “true” tumor suppressor genes, leading to increased risk of tumor formation (Di Leva and Croce, 2010; Li et al., 2009; Wijnhoven et al., 2007; Zhang et al., 2007). MicroRNAs represent a promising new class of diagnostic biomarkers that has been shown to be superior to corresponding mRNA signatures based on over 20,000 genes when classifying poorly-differentiated tumors (Lu et al., 2005). Circulating miRNAs have been proposed as promising novel biomarkers for cancer and several other diseases (Weiland et al., 2012). One of the most important advantages of using circulating miRNAs as biomarkers, apart from being easily measured in blood samples, is their remarkable stability in plasma and serum, where they are most likely protected from RNase degradation due to their binding to Argonaut proteins (Arroyo et al., 2011; Mitchell et al., 2008). However, it remains unclear whether a particular combination of circulating miRNAs is superior to single miRNAs as a diagnostic test in early breast cancer. While previous studies on circulating miRNAs in breast cancer have primarily focused on a few preselected miRNAs (Asaga

et al., 2011; Heneghan et al., 2010; Roth et al., 2010; van Schooneveld et al., 2012; Wu et al., 2011), we focused on identifying a multi-miRNA signature optimal for distinguishing between early breast cancer and healthy controls.

Here, we report our study of prospectively collected serum samples from 108 women with early-stage breast cancer and 75 concurrently collected healthy controls using a global Locked Nucleic Acid (LNA™)-based miRNA qPCR platform (Blondal et al., 2013) to assess differences in miRNA expression.

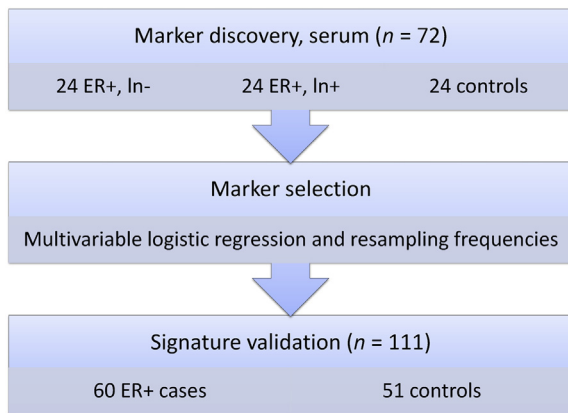
## 2. Materials and methods

### 2.1. Breast cancer patients and healthy controls

Serum samples (total 183, 108 cases and 75 healthy controls) were selected from a larger cross sectional cohort collected at the Department of Oncology, Odense University Hospital, Denmark, in the period 1994–1997 (Espelund et al., 2008). All patients were part of the Danish Breast Cancer Cooperative Group (DBCG) treatment program (Blichert-Toft et al., 2008). Blood samples were obtained from patients the morning prior to surgery. Serum was prepared within 1 h of sample collection and immediately stored at  $-80^{\circ}\text{C}$ . Histopathological diagnosis was confirmed after surgical resection of the tumors. No patients had received any treatment, such as neo-adjuvant chemotherapy or radiation therapy, prior to surgery. Sixty-three percent were post-menopausal and 37% were premenopausal. The control serum samples were collected concurrently, using the same collection and sampling procedures, from healthy women with no history of malignant diseases and no inflammatory conditions. Furthermore, none of the healthy controls were diagnosed with cancer or any inflammatory condition within 5 years after collection of the blood sample. The age-matched healthy women were randomly selected from the Danish Civil Registration System for a given case with a birthday of  $\pm$ one day. The precise menopausal status for the healthy controls were not available, however 65% were above the age of 50 and thus were likely post-menopausal. All cases and controls were of Western European descent.

### 2.2. Study design

The study consisted of three stages (Figure 1): Marker discovery, Marker selection and Marker validation. *Marker discovery:* Global miRNA analysis was performed on serum from 48 post-



**Figure 1 – Consort diagram illustrating the work flow.**

menopausal patients with estrogen receptor (ER)-positive early-stage breast cancer (24 with and 24 without lymph node metastasis) and 24 disease-free healthy controls using Exiqons LNA-based quantitative PCR (qRT-PCR) Plasma/serum Focus panel (174 miRNAs). Each control was age-matched to one patient in the lymph node-positive breast cancer group and one patient in the lymph node-negative breast cancer group, 17/24 of whom were the same age within 1 year and the remaining within 3 years. *Marker selection*: based on automated selection of microRNAs by a statistical approach in combination with resampling, 9 microRNAs were selected for further validation. *Marker validation*: Selected miRNAs were investigated in an independent cohort of 111 serum samples from 60 post-menopausal patients with ER-positive early-stage breast cancer and 51 healthy controls. Specificity for breast cancer was evaluated by examining the selected miRNAs in an independent data set from a study of circulating miRNAs in patients with mucosal lichen planus (Nylander et al., 2012), as well as a data set from a study of circulating miRNAs in patients with oral cancer (Maclellan et al., 2012). These data sets were chosen because the investigators used the same LNA-based platform for their serum analysis and also to investigate whether the findings represented only the degree of inflammation rather than the presence of cancer, since lichen planus by WHO is considered to be an inflammatory precancerous condition (van der Meij et al., 2007).

Furthermore, to test for reproducibility of the miRNA signature to discriminate breast cancer patients from healthy controls, the signature was evaluated in two data sets from two other studies that compared circulating miRNAs in breast cancer patients and healthy controls (GSE41526 and GSE42128), one using the same platform as our study (Chan et al., 2013) and another using a different genome-wide platform (Illumina Human v2 Microarray) (Leidner et al., 2013).

The study was approved by the regional ethics committee (Project-ID: S-20100132) and the REMARK guidelines (McShane et al., 2005) were followed where possible.

### 2.3. RNA isolation from serum

Total RNA was extracted from 250  $\mu$ l of serum using the miRNeasy<sup>®</sup> Mini Kit (Qiagen, Hilden, Germany) according to

manufacturer instructions. The standard protocol was modified according to Exiqon's application note "RNA Purification from Blood Plasma & Serum" (<http://www.exiqon.com/ls/Documents/Scientific/serum-plasma-RNA-isolation.pdf>) by adding 1.25  $\mu$ l MS2 carrier RNA (Roche) to the QIAzol Reagent prior to RNA purification to maximize the yield and minimize purification efficiency variation (Andreassen et al., 2010). Total RNA was eluted by adding 50  $\mu$ l of RNase-free water to the membrane of the spin column and incubating for 1 min before centrifugation at 15,000  $\times$  g for 1 min at room temperature. The RNA was immediately stored in a  $-80^{\circ}$  C freezer.

### 2.4. miRNA real-time PCR

RNA (4  $\mu$ l) was reverse-transcribed in 20  $\mu$ l reactions using the miRCURY LNA<sup>™</sup> Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50 $\times$  and assayed in 10  $\mu$ l PCR reactions according to the manufacturer's protocol for miRCURY LNA<sup>™</sup> Universal RT microRNA PCR; each microRNA was assayed once on microRNA Ready-to-Use PCR Serum/Plasma Panel (Exiqon, Vedbaek, Denmark). Negative controls, excluding the template from the reverse transcription reaction, were profiled similarly to the test samples. As technical controls, an RNA spike-in (Sp6) was added in the reverse transcription reaction to evaluate the RT reaction. In addition, a DNA spike-in (Sp3) was included in triplicates on all panels as an indicator of possible inhibitions at the qPCR level. The amplification was performed by a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche, Basel, Switzerland) in 384-wells. The amplification curves were analyzed using the Roche LC software both for determination of Cp (by the 2nd derivative method) and for melting curve analysis. All assays were inspected for distinct melting curves and the Tm was confirmed to be within known assay specifications. Any data points that showed multiple peaks were excluded from the data set.

Assay efficiencies were determined by analysis of the amplification curves using algorithms similar to the LinReg software package. The efficiencies range between 1.8 and 2.1. Individual reactions with efficiencies <1.6 were excluded from the dataset.

### 2.5. Sample quality

Initial determinations of data quality for each sample were achieved by comparing the level of microRNAs (number of microRNAs detected as well as the average Cp for each sample) in all samples. In this study, the samples showed little variation in microRNA content, suggesting that the samples were of similar quality.

### 2.6. Normalization and data analysis

Only samples that exhibited a 5 Cps lower value than the negative control and Cp < 37 were included in the data analysis.

Normalization was performed based on the mean of the assays detected in all samples, a reliable method for normalization of gene expression studies involving numerous assays (Mestdagh et al., 2009). The present study included 65 assays.

The equation used to calculate the normalized Cp values was: Normalized Cp = mean Cp ( $n = 65$ ) – assay Cp.

For experiments involving only few assays, such as our validation study, the most stably-expressed miRNAs (with least variability) in the discovery set were considered potential normalization candidates. For this purpose, miR-10b and miR-30a were selected since high expression and low variability of these two miRNAs was seen in almost all individuals, while in the remaining few, the expression was measurable and sufficient for normalization.

### 2.7. Evaluation for potential hemolysis of the blood samples

To determine whether hemolysis of the blood samples had occurred during sample preparation and thus released excess blood component-related miRNAs, the ratio between the expression level of two miRNAs was determined; miRNA-451, expressed in red blood cells and miRNA-23a, relatively stable in serum and plasma and not affected by hemolysis (Blondal et al., 2013). Blondal et al. found that a delta Cp (miR-23a–miR-451) of more than five was an indicator of possible erythrocyte miRNA contamination and a delta Cp of 7–8 or more indicated a high risk of hemolysis. These parameters were evaluated for both the discovery and validation set.

### 2.8. Statistical analysis

To compare the 3 sample groups, we performed a one factor ANOVA. MiRNAs with different expression levels between BC patients and controls were identified by unpaired two-tailed parametric t-test. P-values obtained for each miRNA were adjusted for multiple testing by Bonferroni adjustment. Component-wise likelihood-based boosting (Tutz and Binder, 2007) was used as a statistical approach for automated selection of a small set of important microRNAs for a logistic regression model. The number of boosting steps, i.e. the tuning parameter that determines the number of selected microRNAs, was chosen by 10-fold cross-validation to optimize prediction performance. Resampling was performed to identify stably-selected miRNAs informative concerning breast cancer status (Sauerbrei et al., 2011). Specifically, the data was randomly split into training and test data 100 times, with training sets containing 63.2% of the observations (Binder and Schumacher, 2008). A signature was developed in each training data set and the resampling inclusion frequency, meaning the relative frequency of signatures where a certain miRNA is contained, was calculated. MicroRNAs with a resampling inclusion frequency  $\geq 0.3$  were selected for further validation. To evaluate whether BMI, smoking status, tumor grade, tumor size, or lymph node status affected the signature, we also provided these factors as candidates for the boosting approach, which would assure their inclusion in the model if they were important confounders. Receiver Operating Characteristics (ROC) curve was calculated for the validation samples to determine the discrimination performance between new samples from women with or without breast cancer. As the boosting approach estimates a logistic regression model, odds ratios are obtained for interpretation, where it should be noted that the odds ratios are biased

toward zero because of using this estimation approach for a high-dimensional setting. Odds ratios larger than 1 indicate increased levels of miRNA in cases, while values below 1 indicate decreased levels of miRNA in cases.

All statistical analyses were performed using normalized data. Any missing values were replaced by the median of a given miRNA in the case or control group. Overall, there were few missing values (Supplementary Table S1). This did not change any p-values obtained when comparing the two groups, but it does add to the statistical power. All p-values were two-tailed and p-value  $< 0.05$  was considered statistically significant. Power simulations were conducted to find the minimal sample size necessary to find a true 1.5-fold change with at least 80% statistical power. All statistical calculations were performed using the statistical environments R and STATA.

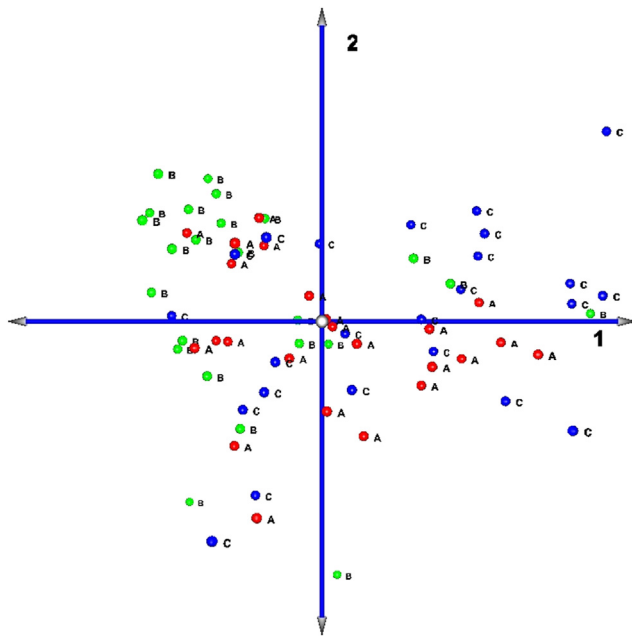
## 3. Results

### 3.1. Marker discovery and marker selection

Global miRNA analysis was performed on serum from 48 postmenopausal patients with ER-positive early-stage breast cancer (24 with and 24 without lymph node metastasis) and 24 age-matched and disease-free healthy controls using LNA-based qRT-PCR (Table 1). Among the 175 miRNAs analyzed, 67 were differentially expressed in serum of breast cancer patients vs. healthy controls. No miRNA signature was identified that could significantly distinguish breast cancer patients with vs. without lymph node metastasis. As a result, the two groups were pooled in the further analysis.

**Table 1 – Characteristics of the individuals included in the discovery set.**

Parameters	Lymph node-positive breast cancer (%)	Lymph node-negative breast cancer (%)	Controls (%)
Total	24	24	24
Median age, yr	58	58	57
Range	47–71	50–69	45–69
BMI	26	25	24
Range	19–35	20–37	19–39
Smoking status			
Yes	6 (25)	4 (16)	6 (25)
No	13 (54)	10 (42)	18 (75)
Unknown	5 (21)	10 (42)	0
Post-menopausal	23 (96)	22 (92)	
Pre-menopausal	1 (4)	2 (8)	
ER+	24 (100)	24 (100)	
Median tumor size, mm	16.5	16.5	
Range	5–36	2–35	
Node-positive	24 (100)	0	
1–4	19 (79)		
5–9	2 (8)		
10+	3 (13)		
Ductal carcinoma	24 (100)	21 (88)	



**Figure 2 – Principal Component Analysis (PCA) plot showing unsupervised clustering analysis of the top 50 miRNAs with the highest standard deviation (expressed in all samples) from the discovery test set. The healthy controls (green dots) appear different from the breast cancer patients with (red dots) and without lymph node metastasis (blue dots).**

Principal Component Analysis (PCA), a method used to reduce the dimension of large data sets, was performed on the top 50 microRNAs that had the largest variation across all samples. An overview of how the samples clustered based on this variance was obtained (Figure 2).

Based on a multivariable logistic regression model estimated by component-wise likelihood-based boosting and

**Table 3 – Characteristics of the individuals included in the validation set.**

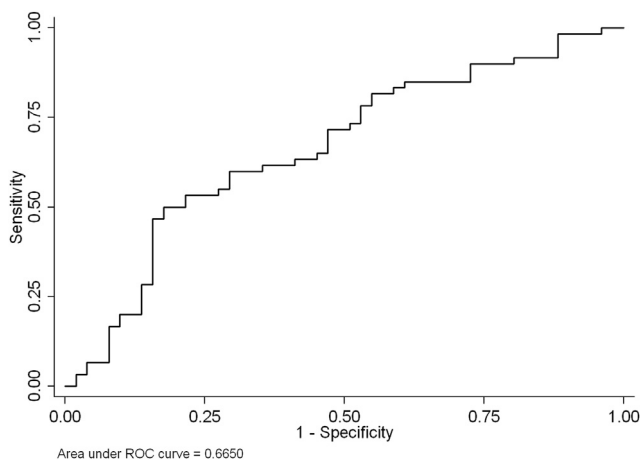
Parameters	Patients (%)	Controls (%)
Total	60	51
Median age, yr	57	58
Range	32–69	33–70
Post-menopausal	38 (63)	(65% above age 50)
Pre-menopausal	22 (37)	
ER+	60(100)	
Median tumor size, mm	15	
Range	2–65	
Nodal status		
0	42 (70)	
1–4	15 (25)	
5–9	1 (2)	
10+	2 (3)	

resampling inclusion frequencies, a 9 miRNA signature, including miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-425, was identified. Four of these miRNAs were more highly expressed in the breast cancer samples vs. healthy controls (miR-15a, miR-18a, miR-107, and miR-425), while 5 were expressed at lower levels (miR-133a, miR-139-5p, miR-143, miR-145, and miR-365). Neither BMI, smoking status, tumor grade, tumor size, nor lymph node status was selected by the boosting approach for inclusion and thus do not appear to be important confounders. Results of the univariate analyses as well as resampling inclusion frequencies for each miRNA in the profile are summarized in Table 2. The parameters of the logistic regression model, as estimated by the boosting approach, provide an algorithm for obtaining predictions for future individuals based on the 9 miRNA signature. Basically, the algorithm provides a risk score for each sample analyzed. Subsequently, this score can be converted into a predicted probability of having breast cancer by the following equation  $1/(1 + \exp^{-\text{score}})$ .

**Table 2 – Circulating microRNAs differentially expressed in the serum of patients with early-stage breast cancer compared to healthy controls. Odds ratios selected by the multivariate model. Discovery data.**

MicroRNA	Odds ratio	Resampling inclusion frequencies	Mean $\Delta$ Cp cases (N = 48)	Mean $\Delta$ Cp controls (N = 24)	Univariate p-values	Adjusted p-value (Bonferroni)
<b>hsa-miR-145</b>	0.52	0.87	-1.95	-1.32	2.08E-06	0.0004
<b>hsa-miR-107</b>	1.23	0.42	0.44	-0.17	3.63E-05	0.0062
<b>hsa-miR-365</b>	0.89	0.32	-4.17	-3.29	3.59E-05	0.0061
<b>hsa-miR-18a</b>	1.12	0.31	-3.29	-3.93	4.06E-05	0.0069
<b>hsa-miR-425</b>	1.1	0.30	-0.57	-0.82	0.0007	0.1191
<b>hsa-miR-139-5p</b>	0.72	0.40	-3.19	-2.66	0.0037	0.6231
<b>hsa-miR-143</b>	0.88	0.40	-2.61	-1.88	0.0004	0.0727
<b>hsa-miR-15a</b>	1.33	0.33	3.13	2.87	0.0111	>1
<b>hsa-miR-133a</b>	0.75	0.37	-4.42	-3.67	0.0028	0.4789
<b>hsa-miR-142-3p</b>	1.51	0.24	2.46	2.15	0.0086	>1
<b>hsa-miR-378</b>	0.95	0.21	-2.12	-1.56	0.0015	0.2579
<b>hsa-let-7b</b>	0.79	0.21	0.36	0.58	0.0436	>1
<b>hsa-miR-423-5p</b>	1.05	0.20	-1.04	-1.28	0.0111	>1

MicroRNAs selected for validation are in bold.



**Figure 3** – ROC curve analysis using the 9 miRNA profile (miR-15a, miR-18a, miR-107, miR-133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-425) for discriminating breast cancer cases from healthy controls in the validation set, which consisted of serum from 60 early-stage, ER-positive, breast cancer patients and 51 healthy age-matched controls.

As an example, the probability of having breast cancer with a score of 1.1 is  $1/(1 + \exp^{-1.1}) = 0.75$ .

Based on the ratio between miR-451 and miR-23a, which have been shown to be associated with hemolysis under the blood drawing process, we found a mean delta Cp (miR-23a–miR-451) of 5.6 among the controls and 6.0 among cases, indicating possible erythrocyte miRNA contamination, but no overall high risk of hemolysis in the two groups.

### 3.2. Profile validation

To validate the ability of the 9 miRNA signature to stratify breast cancer patients vs. healthy controls, we analyzed the expression levels of these 9 miRNAs in serum from an independent group of 60 early-stage, ER-positive, breast cancer patients and 51 healthy age-matched controls (Table 3). When evaluating the samples for possible risk of hemolysis, we found a mean delta Cp (miR-23a–miR-451) of 5.4 among the controls and 5.8 among cases. Again, this indicates possible erythrocyte miRNA contamination, but no overall high risk of hemolysis in the two groups. Eight samples (7%) had a mean delta Cp

between 7 and 8, but no samples had a mean delta Cp above 8. Most important, no significant difference in potential risk of hemolysis was found between the two groups.

The 9 miRNA signature significantly discriminated between patients with breast cancer and healthy controls ( $p = 0.012$ , 95% CI 1.12–2.39). A ROC curve was calculated with an Area Under the Curve (AUC) of 0.665 (95% CI 0.562–0.768; Figure 3) to discriminate early-stage breast cancer patients from healthy controls. Using a probability cutoff of 0.48, the sensitivity was 83.3%, specificity 41.2%, positive predictive value (PPV) 62.5% and a negative predictive value (NPV) of 67.4%. To assess whether the 9 miRNA signature was specific to post-menopausal breast cancer patients, we compared the expression of the selected miRNAs in post- (63%) and pre-menopausal (37%) breast cancer patients and found no significant difference, suggesting that the signature is not related to menopausal status.

Examining our 9 miRNA signature in two separate data sets using the same platform, one comparing serum miRNA expression in patients with lichen planus to healthy controls (Nylander et al., 2012), and the other comparing serum miRNA expression in patients with oral cancer to healthy controls (Maclellan et al., 2012), we found no difference in signature expression of the 9 miRNAs between either patient set and healthy controls, indicating that the signature is not just a marker of inflammation or cancer in general (data not shown).

### 3.3. Test of reproducibility

We also evaluated our signature in data sets from two studies of circulating miRNAs in early-stage breast cancer (Chan et al., 2013; Leidner et al., 2013), applying our developed formula after normalizing to miRNA-10b. Thus, scores for cases and controls were obtained and compared by t-tests. A significant difference between cases and controls was observed in the data set of Chan et al. ( $p = 0.024$ ), while no significant difference was observed for the data of Leidner et al. ( $p = 0.626$ ) (Table 4).

## 4. Discussion

In this study, we identified and validated a signature, consisting of 9 circulating miRNAs (miR-15a, miR-18a, miR-107, miR-

**Table 4** – Signature miRNAs from current study compared in Leidner, Chan and Maclellan data sets.

miRNA	Kodahl et al.		Chan et al.		Leidner et al.		Maclellan et al.	
	Log2 FC	P value	Log2 FC	P value	Log2 FC	P value	Log2 FC	P value
miR-143	0.72	0.0004	−1.09	6.30E−04	0.90	0.054	N/A	N/A
miR-145	0.64	2.08E−06	1.24	0.293	N/A	N/A	N/A	N/A
miR-139-5p	0.53	0.004	−0.30	0.223	N/A	N/A	N/A	N/A
miR-365	0.91	3.59E−05	−1.01	0.014	1.44	0.016	0.72	0.017
miR-133a	0.75	0.003	−3.68	7.65E−09	N/A	N/A	0.57	0.104
miR-15a	−0.26	0.011	−0.67	0.015	−0.25	0.041	N/A	N/A
miR-18a	−0.63	4.06E−05	−0.45	0.0028	N/A	N/A	N/A	N/A
miR-107	−0.61	3.63E−05	−0.42	7.13E−04	N/A	N/A	N/A	N/A
miR-425	−0.25	0.0007	−0.57	0.0021	N/A	N/A	0.25	0.171

133a, miR-139-5p, miR-143, miR-145, miR-365, and miR-425), that discriminates between patients with newly diagnosed, ER-positive, early-stage breast cancer and healthy controls. A particular strength of this study is the long follow-up of the DBCG-cohort and healthy controls that assured the latter were not diagnosed with any cancer or inflammatory diseases within 5 years from blood collection. A second strength is the identified miRNA signature consisting of several miRNAs, as such signatures are likely less vulnerable than single miRNAs to biological differences, which makes them more applicable to daily clinical use.

In our miRNA selection for the signature, we accounted for potential co-regulation of different miRNAs by using a multi-variable approach that simultaneously considered all microRNAs as candidates and automatically adjusted for other microRNAs in the signature. Since miRNAs have numerous target mRNAs, and a single mRNA is targeted by numerous miRNAs, the effect of one dysregulated miRNA is most likely dependent on the dysregulation of several others that might perform similar biological roles. Our 9 miRNAs signature, when put into a specific algorithm, generates a score that reflects the risk of having breast cancer. With only a moderate performance (AUC of 0.665), the signature alone is not an effective diagnostic test, but it may show promise as a valuable risk assessment tool in breast cancer screening in combination with screening mammography. In comparison, AUC for screening mammography was found to be between 0.554 and 0.877, depending on age and breast tissue density (Pisano et al., 2008). However, the performance of the test in combination with screening mammography needs further evaluation in larger, pre-diagnostic, studies.

One of the major concerns when analyzing circulating miRNAs is the normalization procedure. In the discovery analysis, we normalized to the mean Cp-value of all samples, which is recommended when dealing with multiple assays (Mestdagh et al., 2009). However, this method was not applicable in the validation study since it included only a few assays. Previous single assay studies lacked reliable endogenous control microRNAs, and most used miR-16 for endogenous normalization. However, recent studies have found miR-16 to be unsuitable as reference (Cookson et al., 2012; Kirschner et al., 2011). As an alternative, the most stably and consistently expressed miRNAs may be used for normalization, in our case miR-10b and miR-30a. MiRNA-10b, however, has previously been described in relation to metastatic breast cancer (Biagioni et al., 2012; Chen et al., 2013; Eichelser et al., 2013; Iorio et al., 2005; Ma et al., 2007; Zhao et al., 2012) with conflicting findings. Ma et al. found miR-10b to be up-regulated in tumor tissues from patients with metastatic breast cancer, but not in tissue from patients with no metastatic disease (Ma et al., 2007). In contrast, Iorio et al. found miR-10b to be down-regulated in primary breast cancer tissue compared to normal breast tissue (Iorio et al., 2005), while Eichelser et al. recently reported no association between serum miR-10b expression and the presence of overt metastasis (Eichelser et al., 2013) despite having previously found serum MiR-10b to be related to breast cancer metastasis (Roth et al., 2010). Finally, Heneghan et al. did not find that circulating miR-10b could discriminate between patients with breast cancer (stages I-IV) and healthy controls

(Heneghan et al., 2010). Thus, no clear evidence exists linking miR-10b to early-stage breast cancer.

With regard to the other control miRNA, miR-30a, it has been suggested that it might act as a tumor suppressor by targeting Vim, a gene coding for vimentin (Cheng et al., 2012). Examined on micro-dissected tumor cells, patients with decreased levels of miRNA-30a at the site of primary tumors had increased hazard ratios (HR) of recurrence. To our knowledge, no studies have shown a linkage between the expression of circulating miR-30a and early-stage breast cancer.

One of the major concerns when investigating circulating miRNAs is the lack of reproducibility and the prevalence of inconsistent findings between studies. This issue was previously addressed by Leidner et al., who examined the degree of consensus between several genome-wide circulating miRNA studies as well the reproducibility of these results in a study involving serum samples from 20 women with newly diagnosed breast cancer before surgery, 20 women after tumor resection, 20 healthy controls and 10 women with cancers other than breast (Leidner et al., 2013). Comparing their results to other published studies revealed a similar lack of consistency for which there may be several reasons, such as differences in study populations, sample material and preparation, platforms, miRNA extraction procedures, normalization techniques as well as statistical approaches.

We investigated whether our miRNA signature discriminated between early-stage breast cancer patients and healthy controls in a study using the same platform as we (Chan et al., 2013), as well as in the data from the above-mentioned study (Leidner et al., 2013), using a different platform. Comparison of results across platforms has clear limitations, and thus we were not surprised to find that the signature did not replicate in the data set by Leidner et al. However, using the same normalization approach on the data set by Chan et al., we successfully validated our 9 miRNA signature in this data set, supporting the possible clinical utility of the signature although this needs further investigation in a larger prospective study.

Several others have examined the expression of circulating miRNAs (Supplementary Table S2) in relation to breast cancer (Asaga et al., 2011; Chan et al., 2013; Cuk et al., 2013; Heneghan et al., 2010; Leidner et al., 2013; Ng et al., 2013; Roth et al., 2010; Schrauder et al., 2012; Wang et al., 2010; Wu et al., 2011, 2012; Zhao et al., 2010), some using very limited numbers of probe-sets (Asaga et al., 2011; Heneghan et al., 2010; Roth et al., 2010) prone to becoming biased due to the way the selected probes define the final observations. Others have used genome-wide platforms to profile all circulating miRNAs (Cuk et al., 2013; Leidner et al., 2013; Ng et al., 2013; Schrauder et al., 2012; Wu et al., 2011, 2012; Zhao et al., 2010), a method that provides unbiased discovery of putative circulating miRNA biomarkers. It has been suggested that miRNA signatures consisting of a combination of multiple miRNAs are superior to single miRNA models in discriminating blood samples from breast cancer patients and healthy controls (Cuk et al., 2013; Ng et al., 2013). Ng et al. found that a combination signature consisting of miR-451 (up-regulated) and miR-145 (down-regulated) discriminated between breast cancer cases and healthy controls as well as other cancer types with an AUC of 0.96. However, this signature could not distinguish between benign tumors and invasive breast cancer. In our study, we also found

miR-145 to be down-regulated in breast cancer vs. healthy controls, while miR-451 expression did not differ between the two groups. Cuk et al. showed that a signature consisting of miR-148b, miR-409-3p and miR-801 was able to distinguish between 127 female sporadic breast cancer patients and 80 healthy controls (AUC = 0.69). MiR-801 was not included in our study, and no difference in expression between miR-148b and miR-409-3p was observed. These differing results may be due to differences in the sample populations, since Cuk et al. allowed inclusion of patients who were not primarily operable.

All samples in the discovery set were analyzed for 174 miRNAs (Exiqon Cancer Focus Panel). However, important miRNAs of more than 1800 human miRNAs currently listed in the miRBase (<http://www.mirbase.org/>, accessed September 13, 2013) may have been missed.

Several of the miRNAs in our 9 miRNA signature have been investigated by others. Interestingly, using prospectively collected samples from the large Sister Cohort Study, serum miR-18a was found to be expressed at higher levels in women who subsequently developed breast cancer (within 18 months from blood collection) compared to those who remained breast cancer-free, although this could not be validated in the relatively small validation set (Godfrey et al., 2013). Similar to our findings, Chan et al. found down-regulation of miR-145 in serum of women with breast cancer compared to normal controls, whereas in the same study, miR-133a and miR-143 were up-regulated, in contrast with our findings (Chan et al., 2013). In agreement with our findings, Chen et al. also found higher expression of miR-107 in breast cancer vs. normal tissue (Chen et al., 2011). Finally, as in our study, low levels of miR-139-5p and high levels of miR-425 were observed in breast cancer vs. adjacent normal tissue, whereas, in contrast to our findings, miRNA-365 was found to be more highly expressed in breast cancer tissue (Romero-Cordoba et al., 2012; Yan et al., 2008).

Recently, spike-in controls to evaluate the quality of the RNA extraction have been introduced, but this technique was not commonly used at the time of serum sample analysis for this study and no control for the RNA extraction procedure was performed. While this may be a potential bias, all samples in each study (discovery and validation set) were carefully processed simultaneously and by the same bioanalyst to standardize the extraction procedure.

Technical replicates were not included in the present study, however the qPCR platform used has previously demonstrated a high reproducibility with median Pearson correlation coefficients of 0.985, minimizing the risk of technical variance (Jensen et al., 2011). Furthermore, technical validation is usually performed when the biological material is sparse and the information gained by running more biological samples instead of technical replicates is preferred.

To investigate whether the difference in miRNA expression between the breast cancer and the control group was simply due to an inflammatory response, we investigated whether our signature could distinguish mucosal lichen planus patients from healthy controls using a published circulating miRNA data set (Nylander et al., 2012). This study was specifically chosen because it was not a cancer being studied, but a precancerous inflammatory disease, and because this study

employed the same platform as we. A limitation in this comparison is that the previous study included both men and women, although 80% were women, and they were slightly older (mean age women: 64 years, men: 58 years) than those in our study (mean age: 55 years).

Sample handling, preparation and the use of different collection tubes in serum vs. plasma could possibly affect the miRNA profile. In our study, serum was used for the analysis of circulating miRNAs. Measurements of miRNAs in serum and plasma have previously been found to be highly correlated (Kroh et al., 2010; Mitchell et al., 2008). However, serum samples have more detectable miRNAs than plasma samples, and most miRNAs in serum show higher concentrations than corresponding plasma samples (Wang et al., 2012). Compared to whole blood, we would expect a difference since miRNAs extracted from whole blood would include those within blood cells. All patients in this study had diagnostic biopsies taken prior to blood sampling, and whether this procedure affected detection of circulating miRNAs remains to be determined and thus should be considered a potential bias.

One method to increase the statistical power when analyzing a limited amount of material is using a more homogenous group, which is why we focused on only women with ER-positive breast cancer. The ATAC trial comprised of 9366 post-menopausal women with early-stage breast cancer showed that 84% of the population was ER-positive (Baum et al., 2002). Since then, the cut-off of 10% staining cells for ER, required for classification as ER-positive, has been changed to 1% (Viale et al., 2007), which means the number of ER-positive breast cancers might be even higher than 84%. Whether the signature presented in this study can distinguish between ER-negative breast cancer and healthy controls has not been investigated, but based on the above, the patient characteristics in this study are close to the general Danish mammography screening population. To assess whether our signature is applicable to both pre- and post-menopausal women requires larger studies. The present study did not provide enough power for such subgroup analysis.

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## 5. Conclusion

We identified a novel circulating 9-miRNA signature that discriminates between serum from early-stage, ER-positive, breast cancer patients and healthy control subjects. The signature was successfully validated in an independent cohort of 111 serum samples as well as an independent data set from the GEO database. This signature may be of use in the development of a non-invasive, multi-marker blood test to improve early detection of breast cancer. Specifically, the signature provides a risk score reflecting the possibility of having early-stage breast cancer. To further validate the potential of this signature, we are planning a prospective study in the pre-diagnostic setting at a mammography screening center.

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## Authors' contributions

ARK, AK and HD participated in study design. ARK coordinated the project, performed data interpretation and wrote



the first draft of the manuscript. HB performed the majority of the statistical analysis. HD, ML, HB and KG provided critical advice on study design and data interpretation. HB, ML, KG, and HD assisted with writing the manuscript. SC provided all samples and patient data. All authors read and approved the final manuscript.

### Conflict of interest

The authors declare no conflict of interest.

### Funding

The public and private foundations that supported the study had no role in the design and conduct of the study; in the collection, management, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript. The authors had full access to all the data in the study and had the final responsibility for the decision to submit the manuscript for publication.

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### Appendix A.

#### Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molonc.2014.03.002>.

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