**Direct Comparison of Metastasis-Related miRNAs Expression Levels in Circulating Tumor Cells, Corresponding Plasma, and Primary Tumors of Breast Cancer Patients**

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**BACKGROUND:** Circulating tumor cells (CTCs) and microRNAs (miRNAs) are important in liquid biopsies in which peripheral blood is used to characterize the evolution of solid tumors. We evaluated the expression levels of miR-21, miR-146a, miR-200c, and miR-210 in CTCs of breast cancer patients with verified metastasis and compared their expression levels in corresponding plasma and primary tumors.

**METHODS:** Expression levels of the miRNAs were quantified by quantitative reverse transcription PCR (RT-qPCR) in (a) 89 primary breast tumors and 30 noncancerous breast tissues and (b) CTCs and corresponding plasma of 55 patients with metastatic breast cancer and 20 healthy donors. For 30 of these patients, CTCs, corresponding plasma, and primary tumor tissues were available.

**RESULTS:** In formalin-fixed, paraffin-embedded tissues, these miRNAs were differentially expressed between primary breast tumors and noncancerous breast tissues. miR-21 (P < 0.001) and miR-146a (P = 0.001) were overexpressed, whereas miR-200c (P = 0.004) and miR-210 (P = 0.002) were underexpressed. In multivariate analysis, miR-146a overexpression was significantly [hazard ratio 2.969 (1.231–7.157), P = 0.015] associated with progression-free survival. In peripheral blood, all miRNAs studied were overexpressed in both CTC and corresponding plasma. There was a significant association between miR-21 expression levels in CTCs and plasma for 36 of 55 samples (P = 0.008). In plasma, ROC curve analysis revealed that miR-21, miR-146a, and miR-210 could discriminate patients from healthy individuals.

**CONCLUSIONS:** Metastasis-related miRNAs are overexpressed in CTCs and corresponding plasma; miR-21 expression levels highly correlate in CTCs and plasma; and miR-21, miR-146a, and miR-210 are valuable plasma biomarkers for discriminating patients from healthy individuals.

The liquid biopsy is a minimally invasive blood-based analysis approach that can give information on a solid tumor’s evolution in real time. Information is derived mainly from circulating tumor cells (CTCs), circulating tumor DNA, circulating microRNAs (miRNAs), and exosomes, and changes in their concentrations have been associated with tumor burden and malignant progression (1). Numerous studies have demonstrated the feasibility of cancer liquid-biopsy testing, and the clinical utility of CTCs and cell tumor DNA (ctDNA) in many types of cancer (2). Many international consortia are working on the clinical validation of a variety of liquid-biopsy tests (3, 4). Liquid biopsy–based tests that are specifically designed to predict resistance to specific drugs and select patients for specific molecular-targeted therapies have advantages over classical surgical biopsies. These blood-based targeted molecular assays have a strong potential to be applied as companion diagnostics, in disease monitoring, and even for early cancer detection (5, 6).

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It is well known that the presence of tumor cells in the peripheral blood of patients with operable breast cancer is an early indicator of metastasis and may signal tumor spread sooner than clinical symptoms appear and imaging results confirm a poor prognosis (7–10). CTC enumeration with CellSearch has been acquired for use as a prognostic factor when measured in patients with metastatic breast, colorectal, and prostate cancer (11, 12). CellSearch is the only US Food and Drug Administration CTC assay approved for prognostication and disease monitoring in patients with metastatic breast, colorectal, and prostate cancer (13). The clinical utility of CTCs in breast cancer for treatment decisions is currently being evaluated in ongoing clinical studies (14). However, besides detection and enumeration, molecular characterization of CTCs is extremely important because it offers the potential to provide unique information that can be further used to guide individualized targeted treatments (7, 15, 16).

In addition to CTCs, miRNAs seem to be involved in metastasis and might offer the unique potential to better understand the biology of metastasis and resistance mechanisms to established therapies (17). miRNAs are key regulators of gene expression because each miRNA targets, on average, 200 mRNA transcripts (18). The expression of specific miRNAs in primary breast tumors has been associated with the clinical outcome in several studies (19, 20). Because of their small size and remarkable stability (21), miRNAs can also be detected in serum and plasma as cell-free circulating miRNAs (21–23).

To the best of our knowledge, the expression of miRNAs has not been studied so far in CTCs, nor have they been compared to their expression in plasma and corresponding primary tumors. For our study, we selected 4 miRNAs that critically regulate various stages of migration and invasion and play critical roles in the multistep metastatic process, namely miR-21, miR-146a, miR-200c, and miR-210. In this study, we evaluated the expression levels of 4 metastasis-related miRNAs in the epithelial cell adhesion molecule (EpCAM)-positive CTC fraction of patients with metastatic breast cancer and compared our findings with the expression levels in corresponding plasma and paired formalin-fixed, paraffin-embedded primary tumors (FFPEs). In this study, we aimed to answer the following questions: (a) Could we detect metastasis-related miRNAs in CTCs? (b) Was there an association between CTC and plasma in terms of miRNAs expression? (c) Did the expression levels of miRNAs in CTC and plasma reflect those of the primary tumor? and (d) Did the expression levels of miRNAs in CTC and plasma provide any prognostic significance?

Materials and Methods

PATIENTS
All study participants signed an informed consent form to participate in the study, which was approved by the ethics and scientific committees of our institutions. Using quantitative reverse transcription PCR (RT-qPCR), we quantified the expression of miR-21, miR-146a, miR-200c, miR-210, and miR-191 (used as a normalizer) in (a) 55 EpCAM-positive CTC fraction samples isolated from peripheral blood of patients with metastatic breast cancer, (b) 55 corresponding plasma samples (same vein-puncture), and (c) 89 FFPEs. For 30 of these patients, there were available samples of the EpCAM-positive CTC fraction, corresponding plasma, and FFPEs. As a control population, peripheral blood was collected from 20 healthy individuals, and 30 noncancerous breast tissues (mamoplasties) were used as noncancerous tissue controls. In the EpCAM-positive CTC fraction, 1 sample was defined as miRNA overexpressed based on the fold change of miRNA expression in respect to the group of 20 healthy individuals used as a control group. More specifically, a cutoff value was estimated according to the expression of miRNA in the EpCAM-positive fraction of healthy individuals analyzed in exactly the same way as the patient’s peripheral blood samples.

EXTRACTION OF MIRNAS FROM FFPEs
For miRNAs analysis in FFPE blocks, the samples were cut into 10-mm-thick slices, and each tissue slice was placed into a 1.5 mL nuclease-free microcentrifuge tube. Extraction of miRNAs was performed by using the mirNeasy FFPE kit (Qiagen) according to the manufacturer’s instructions.

EXTRACTION OF MIRNAS FROM THE EPCAM-POSITIVE CTC FRACTION
We isolated CTCs from 20 mL peripheral blood by following a positive immunomagnetic selection approach, using anti-EpCAM-coated immunomagnetic beads, as previously described (24). All peripheral blood samples for controls and patients were processed in exactly the same way. Peripheral blood in EDTA-containing tubes was obtained from breast cancer patients with verified metastasis and from female healthy volunteers. To reduce blood contamination by epithelial cells from the skin, the first 5 mL of blood were discarded, and the final collection tube was disconnected before withdrawing the needle. The peripheral blood mononuclear cells were isolated within 2 h of venipuncture by gradient centrifugation with Ficoll, and cell pellets were kept at −70 °C until RNA extraction.

Isolation of total RNA was performed with TRIZOL (Invitrogen). The isolated RNA from each fraction was dissolved in 10 μL RNA storage buffer. RNA con-


centration was determined by using the Nanodrop-1000 spectrophotometer. As a negative control, we followed exactly the same procedure and evaluated miRNAs expression levels in the EpCAM-positive CTC fraction isolated from peripheral blood of 20 healthy blood donors.

**EXTRACTION OF CIRCULATING MIRNAS FROM PLASMA**

The extraction of miRNAs from plasma (200 μL) was performed as previously described by using the mirVana™ PARIS™ Kit (25). More specifically, whole-blood samples were subjected to centrifugation at 2000g for 10 min at room temperature. The upper plasma layer was immediately collected after centrifugation and saved in new RNase-free tubes. Before starting the miRNA extraction procedure, plasma samples were subjected to a second centrifugation at 12 000g for 15 min at 4 °C to remove all cellular debris and the vast majority of platelets. Subsequently, 25 fmol of cel-miR-39 used as an exogenous miRNA-spiked-in control was added to all plasma aliquots to allow for normalization of sample-to-sample variations in the RNA-isolation procedure. We evaluated the efficacy and analytical performance of the whole procedure by quantifying the concentrations of the spiked-in exogenous control miRNA cel-miR-39. Recovery of cel-miR-39 in each case was estimated with respect to the concentrations of an equivalent amount of cel-miR-39 copies that we added to the eluted RNA after the extraction step in each case (representing 100% recovery). In parallel, endogenous tested miRNAs and miR-191 concentrations were also quantified by RT-qPCR in all samples.

**REVERSE TRANSCRIPTION OF MIRNAS**

In all cases, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers in a 15 μL total-volume reaction. Each reaction consisted of (a) 3 μL of miRNAs eluted from plasma, (b) 30 ng total RNA from the EpCAM-positive CTC fraction, and (c) 30 ng total RNA from FFPEs tissues and 10 μL of Master Mix. The RT reaction mixture was incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min and then held at 4 °C. A no-RT negative control was included in each experiment to ensure that PCR products were not because of contamination by genomic DNA.

**QUANTIFICATION OF MIRNAS EXPRESSION BY RT-QPCR**

The expression levels of each miRNA were quantified by RT-qPCR, using the TaqMan microRNA assays (Applied Biosystems), according to the manufacturer’s protocols. RT-qPCR was performed in a final volume of 10 μL, containing 2 μL of cDNA template, 2 μL nuclease free water, 1 μL of 20X primer/probe mix from the TaqMan MicroRNA assay, and 5 μL of 2X TaqMan Universal PCR master mix. All reactions were run in triplicate in the Light Cycler 2.0 Real-Time PCR Instrument (Roche). The reaction mixture was incubated at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression values were normalized in respect to miR-191, which has been shown to be a suitable reference miRNA for breast cancer (26). We evaluated 4 different miRNAs (miR191, RNU44, RNU48, and cel-7a) as calibrators by measuring their expression levels in 40 primary breast tumors. RNU44 was excluded from the analysis because it was not expressed in the majority of the samples. For the remaining miRNAs, we performed NormFinder algorithm analysis, and according to our results, miR-191 was ranked as the most-stable RNA (see Supplemental Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue7). Relative quantification was based on the ΔΔCq method, as described by Livak and Schmittgen (27). All RT-qPCR experiments were performed according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (28).

**STATISTICAL ANALYSIS**

Statistical analysis was performed using the SPSS statistical package. RT-qPCR data were analyzed by Wilcoxon signed rank tests to statistically evaluate differences in miRNA expression between breast cancer and healthy breast tissues. For the survival analysis, we divided breast cancer patients into 2 different groups, high expression and low expression, using the median expression cutoff. Survival time was calculated from the date of endpoint event or last follow-up. The association between survival and miRNA expression was estimated using the median ΔΔCq of noncancerous samples for each miRNA studied at the corresponding cutoffs. Survival time was calculated from the date of endpoint event or last follow-up. The association between survival and miRNA expression was statistically evaluated using the Kaplan–Meier method and 2-sided log-rank test. To evaluate the prognostic potential of individual miRNAs and sets of miRNAs, we generated ROC curves and areas under the curves (AUC).

**Results**

**EXPRESSION OF METASTASIS-RELATED MIRNAS IN PRIMARY BREAST TUMORS**

The experimental flowchart of our study is outlined in Fig. 1. We initially evaluated the expression levels of miR-21, miR-146a, miR-200c, and miR-210 in primary breast tumors. Overexpression and underexpression of these 4 miRNAs were estimated by evaluating the differences in their expression levels between 89 breast cancer tumors (FFPEs) and 30 noncancerous breast tissues (mammaplasties). The expression levels of all 4 miRNAs were significantly different between primary tumors and noncancerous breast tissues. More specifically, miR-21 (P < 0.001) and miR-146a (P = 0.001) were overexpressed,
whereas miR-200c ($P = 0.004$) and miR-210 ($P = 0.002$) were found to be underexpressed (Fig. 2A).

The correlation of the expression levels of the 4 miRNAs with patients' clinical outcome revealed that patients with miR-21 overexpression had significantly shorter disease-free intervals (DFIs) than those with miR-21 underexpression (43 months vs 84 months, respectively, $P = 0.040$, Fig. 3A). Moreover, patients who had miR-146a overexpression had shorter DFIs than those with miR-146a underexpression (19 months vs 59 months, respectively; $P = 0.018$, Fig. 3B). The expression levels of miR-200c and miR-210 in FFPEs were not correlated with DFI and overall survival (OS).

Univariate analysis demonstrated that miR-21 overexpression [hazard ratio (HR): 1.846; 95% CI 1.016–3.355, $P = 0.044$] and miR-146a overexpression (HR: 2.696; 95% CI 1.143–6.357, $P = 0.025$) were significantly associated with DFI; conversely, there was no association with the ER/PR (estrogen receptor/progesterone receptor) and HER2 (human epidermal growth factor receptor 2) status with DFI in these patients (see online Supplemental Table 1). Multivariate analysis demonstrated that only miR-146a overexpression was an independent factor associated with DFI (HR: 2.969; 95% CI 1.231–7.157, $P = 0.015$) (see online Supplemental Table 1).

EXPRESSION OF METASTASIS-RELATED MIRNAS IN THE CORRESPONDING EPCAM-POSITIVE CTC FRACTION

We further evaluated the expression of these 4 metastasis-related miRNAs in the corresponding EpCAM-positive CTC fraction isolated from peripheral blood. All miRNAs were overexpressed in the EpCAM-positive CTC fraction of the patients in respect to the corresponding healthy controls; miR-21 ($P < 0.001$, 17.6-fold rise), miR-146a ($P < 0.001$, 6.5-fold rise), miR-200c ($P < 0.001$, 81.0-fold rise), and miR-210 ($P < 0.001$, 81.0-fold rise) (Fig. 2B). Univariate analysis revealed that miR-146a overexpression ($P = 0.068$) was not statistically associated with OS in this group of patients. Kaplan–Meier survival analysis and log-rank tests showed that none of the tested miRNAs was correlated with OS (results not shown).

EXPRESSION OF METASTASIS-RELATED CIRCULATING MIRNAS IN CORRESPONDING PLASMA

The expression levels of these 4 metastasis-related miRNAs were further evaluated in corresponding plasma samples isolated from the same peripheral blood samples on the same day and through the same veinpuncture as CTC. All of these 4 metastasis-related miRNAs were significantly overexpressed in patients’ plasma compared with healthy donors (miR-21, $P < 0.001$; miR-146a,
$P = 0.004$; $\text{miR-200c}, P = 0.002$; and $\text{miR-210}, P < 0.001$ (Fig. 2C).

The diagnostic utility of these circulating miRNAs in plasma was evaluated by ROC curve analysis. ROC curves showed that $\text{miR-21}$, $\text{miR-146a}$, and $\text{miR-210}$ were valuable biomarkers for discriminating patients from healthy individuals, with AUCs of 0.820 (95% CI = 0.691–0.948, $P < 0.0001$), 0.912 (95% CI = 0.847–0.977, $P < 0.0001$), and 0.959 (95% CI = 0.917–1.000, $P < 0.0001$), respectively (Fig. 4). When the plasma concentrations of a combination of $\text{miR-21}$, $\text{miR-146a}$, and $\text{miR-210}$ were subjected to combined

**Fig. 2.** Expression levels of miRNAs in (A) FFPEs, (B) EpCAM-positive CTC fraction, and (C) plasma.

**Fig. 3.** (A), Kaplan–Meier estimates of DFI or breast cancer patients with verified metastasis in respect to $\text{miR-21}$ overexpression. (B), Kaplan–Meier estimates of DFI for breast cancer patients with verified metastasis in respect to $\text{miR-146a}$ overexpression.
analysis by multiple logistic regression, the generated ROC curve reflected a higher ability to differentiate patients from healthy controls (AUC value: 0.997; 95% CI: 0.989 – 1.000), demonstrating the diagnostic accuracy of these miRNAs as effective biomarkers in combination.

DIRECT COMPARISON BETWEEN MiRNAS EXPRESSION LEVELS IN THE EPCAM-POSITIVE CTC FRACTION, IN PLASMA, AND IN CORRESPONDING PRIMARY TUMORS

We further investigated whether there was a direct association between the expression of these miRNAs in the EpCAM-positive CTC fraction, in corresponding plasma, and paired FFPEs. A heat map summarizing our results is shown in Fig. 5.

For a direct comparison of miRNAs expression between the EpCAM-positive CTC fraction and corresponding plasma, material for matched EpCAM-positive CTC fraction and plasma was available for 55 patients. The results for the direct comparison with respect to CK-19 expression and death are shown in Fig. 5A. The higher percentage agreement between circulating miRNAs in plasma and miRNAs in the EpCAM-positive CTC fraction was observed for miR-21 (67.2%, χ² = 0.187; Table 1). Contingency tables for these statistics are presented in Table 1.

For the direct comparison of miRNAs expression between the EpCAM-positive CTC fraction, plasma, and corresponding FFPEs, material for matched EpCAM-positive CTC fraction, corresponding FFPEs, and plasma was available for 30 patients. miR-21 expression levels were concordant between the EpCAM-positive CTC fraction and FFPEs in 18 of 30 (60%) patients (k = 0.143, Fig. 5B). miR-21 expression levels between plasma and corresponding FFPEs showed a concordance for 16 of 30 (53.3%) cases (k = 0.16, Fig. 5B). Among the remaining 12 discordant cases between CTC and plasma, the expression of miR-21 in CTC matched the corresponding FFPEs in 6 cases, and the expression of miR-21 in plasma matched the FFPEs in the other 6 cases. However, the expression levels of all other miRNAs studied were not correlated between the EpCAM-positive CTC fraction and paired FFPEs (Fig. 5B). The concordance of miRNAs expression levels between plasma and corresponding FFPEs varied between 46.6% and 66.6%.

Fig. 4. ROC curve analysis for (A) miR-21, AUCs : 0.820 (95% CI 0.691– 0.948, P < 0.0001); (B) miR-146a, AUC: 0.912 (95% CI 0.847– 0.977, P < 0.0001); and (C) miR-210, AUC: 0.959 (95% CI 0.917–1.000, P < 0.0001).
Discussion

The liquid biopsy is a very promising approach for cancer diagnosis, prognosis, and evaluation of therapy response. However, there is a constantly increasing number of biomarkers for evaluation in CTC and plasma, and well-designed comparison studies between different liquid-biopsy information sources, such as CTC, ctDNA, and

### Table 1. Direct comparison of miRNAs expression levels in the EpCAM-positive CTC fraction and corresponding paired plasma samples in metastatic breast cancer patients (n = 55).

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<tr>
<th>miRNA</th>
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<th>EpCAM-positive CTC fraction</th>
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<td>Total</td>
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<td>Total</td>
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* Underexpression.
* Overexpression.
* Nonsignificant.

Fig. 5. Heat maps of 4 miRNAs in FFPEs, EpCAM-positive fraction, and plasma samples as quantified by RT-qPCR. Red and green indicate overexpression and underexpression, respectively.
circulating miRNAs and conventional-biopsy samples are still missing.

There have been only a few studies that correlated circulating miRNAs with CTC enumeration. Sieuwerts et al., using CellSearch, reported that 55 miRNAs and 10 miRNAs were more abundantly expressed in blood of patients with at least 5 CTC/7.5 mL of blood compared with samples from patients without detectable CTC and healthy blood donors. Recently, Gasch et al., using in-situ-hybridization protocols combined with antibody-based immunofluorescence, in combination with CellSearch, demonstrated a high heterogeneity in miR-10b expression in CTCs isolated from blood of metastatic-cancer patients.

In the present study, all peripheral blood samples were collected in a way that CTC and plasma were isolated from the same blood draw, and all samples were analyzed in parallel with the same methodologies. The expression levels of 4 metastasis-related miRNAs in the EpCAM-positive CTC fraction of patients with metastatic breast cancer was investigated; moreover, the expression levels of the same miRNAs in corresponding plasma and paired primary tumors was evaluated. The selection of these miRNAs was based on their critical role in the multistep metastatic process. Indeed, miR-21 is involved in invasion, miR-146a in invasion and migration, miR-210 in tumor proliferation and hypoxia, and miR-200c in mesenchymal-to-epithelial transition.

Our results demonstrate that all of these miRNAs were significantly differentially expressed in primary breast tissues when compared with noncancerous breast tissues. miR-21 overexpression in FFPEs was associated with reduced progression-free survival, but not with OS. This finding is in concordance with our previous results concerning the prognostic significance of miR-21 in breast cancer FFPEs as well as with other studies. Furthermore, miR-146a overexpression was found to be an independent factor associated with progression-free survival.

We report for the first time that, in the EpCAM-positive CTC fraction, the expression levels of all 4 of these miRNAs were significantly different between cancerous and noncancerous breast samples. A striking finding of our study is the upregulation of miR-21 both in the EpCAM-positive CTC fraction and in corresponding plasma. miR-21 expression levels were concordant between CTC and paired plasma in the majority of the patients. This is the first time that a high correlation for miR-21 expression is reported between plasma and CTC. It is important to note that miR-21 is a well-studied miRNA and the only miRNA known to be upregulated in all types of human malignancies. Our group has reported that miR-21 is an independent negative prognostic factor for OS in non-small-cell lung cancer patients. Very recently, Ortega et al. have developed a protocol for the detection of miRNAs in CTC using in-situ hybridization combined with immunomagnetic selection based on cytokeratin expression and immunocytochemistry. According to their data, all CTC-positive samples were expressing both cytokeratin and miR-21. It is also important to note that miR-21 plays an important role in the regulation of anticancer drug sensitivity and resistance because aberrant miR-21 expression can reduce the sensitivity of cancer cells to anticancer agents such as tamoxifen, gemcitabine, docetaxel, and 5-fluorouracil.

According to our findings, although miR-200c and miR-210 were down-regulated in primary tissue samples, we found them up-regulated in corresponding plasma of patients at the time they developed metastasis. We believe that these findings can be explained by the fact that we are analyzing liquid-biopsy material from metastatic breast cancer patients. It is now known that tumors are continuously evolving during time and that the metastatic sites are not identical to the primary tumors.

It is known that miR-200c possesses regulatory functions in the EMT pathway and tumor-suppressive features, and in vivo studies suggest that overexpression of the miR-200 family increases the metastatic potential in breast cancer by inducing mesenchymal-to-epithelial transition (MET). MET is required for successful colonization and establishment of metastasis. Alternatively, low expression of miR-200c in the primary tissue might reflect changes taking place in the primary tumor associated with EMT that facilitate exit from the primary tumor, a process that is later reversed in the metastatic deposit.

Based on our findings, we suggest that plasma miR-200c and miR-210 from patients with metastasis might originate from the metastatic site, from which the cancer cells are secreted abundantly into the systemic circulation in patients. Our results are in full agreement with the findings of Madhavan et al. that have also shown that CTC-positive metastatic breast cancer patients had significantly higher levels of miR-200c and miR-210 than CTC-negative metastatic breast cancer patients.

The choice of a reference gene remains problematic and can have a serious impact on the actual available transcript levels and, consequently, on the biological interpretation of data. Although several groups have been focused on studying the expression levels of circulating miRNAs in plasma, it is remarkable that limited overlap has been observed between the findings of even very similar studies of the same disease. Regarding breast cancer, as Thompson et al. mentioned, there is a dampening enthusiasm for circulating miRNAs because after the analysis of 15 reports on circulating miRNAs in breast cancer patients revealed a very low reproducibility between the datasets published previously. The lack
of reproducibility may have several reasons: (a) sample type (plasma, serum, whole blood), (b) differences in blood processing protocols, (c) differences in study populations, (d) differences in time points of sample collection (46). General pitfalls concerning circulating miRNAs as biomarkers, not the least of which is the need for more validation and the contribution to miRNA secretion by blood cells, together with possible solutions, have been extensively reviewed elsewhere (46).

In conclusion, this first direct-comparison study of miRNAs expression in CTCs, corresponding plasma, and paired primary tissues demonstrates that: (a) metastasis-related miRNAs are overexpressed in CTCs and corresponding plasma, (b) miR-21 expression levels are highly correlated in CTCs and plasma, and (c) miR-21, miR-146a, and miR-210 are valuable plasma biomarkers for discriminating patients from healthy individuals.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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