

## Original Article

# Noninvasive urinary miRNA biomarkers for early detection of pancreatic adenocarcinoma

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**Abstract:** Currently, the majority of patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) present with locally invasive and/or metastatic disease, resulting in five-year survival of less than 5%. The development of an early diagnostic test is, therefore, expected to significantly impact the patient's prognosis. In this study, we successfully evaluated the feasibility of identifying diagnostic cell free microRNAs (miRNAs) for early stage PDAC, through the analysis of urine samples. Using Affymetrix microarrays, we established a global miRNA profile of 13 PDAC, six chronic pancreatitis (CP), and seven healthy (H) urine specimens. Selected differentially expressed miRNAs were subsequently investigated using an independent technique (RT-PCR) on 101 urine samples including 46 PDAC, 29 CP and 26 H. Receiver operating characteristic (ROC) and logistic regression analyses were applied to determine the discriminatory potential of the candidate miRNA biomarkers. Three miRNAs (miR-143, miR-223, and miR-30e) were significantly over-expressed in patients with Stage I cancer when compared with age-matched healthy individuals ( $P=0.022$ ,  $0.035$  and  $0.04$ , respectively); miR-143, miR-223 and miR-204 were also shown to be expressed at higher levels in Stage I compared to Stages II-IV PDAC ( $P=0.025$ ,  $0.013$  and  $0.008$ , respectively). Furthermore, miR-223 and miR-204 were able to distinguish patients with early stage cancer from patients with CP ( $P=0.037$  and  $0.036$ ). Among the three biomarkers, miR-143 was best able to differentiate Stage I ( $n=6$ ) from healthy ( $n=26$ ) with area under the curve (AUC) of  $0.862$  (95% CI  $0.695-1.000$ ), with sensitivity (SN) of  $83.3\%$  (95% CI  $50.0-100.0$ ), and specificity (SP) of  $88.5\%$  (95% CI  $73.1-100.0$ ). The combination of miR-143 with miR-30e was significantly better at discriminating between these two groups, achieving an AUC of  $0.923$  (95% CI  $0.793-1.000$ ), with SN of  $83.3\%$  (95% CI  $50.0-100.0$ ) and SP of  $96.2\%$  (95% CI  $88.5-100.0$ ). In this feasibility study, we demonstrate for the first time the utility of miRNA biomarkers for non-invasive, early detection of PDAC in urine specimens.

**Keywords:** Pancreatic ductal adenocarcinoma, diagnostic biomarkers, urine, miR-143, miR-223, miR-30e, miR-204

## Introduction

Due to the late diagnosis and the aggressive nature of pancreatic adenocarcinoma (PDAC), median survival of patients with the disease is usually 5-6 months and five-year survival <5%. Highly accurate biomarkers for early detection are thus expected to significantly impact patient's prognosis; a five-year survival approaching 70% has been reported after inci-

dental diagnosis of Stage I tumors, when they were still confined to the pancreas and smaller than 2 cm [1]. MicroRNAs (miRNAs), small non-coding evolutionarily conserved RNAs, are critically implicated in the regulation of a whole host of cellular processes [2], and their aberrant expression is associated with cancer in a variety of tissues [3], including pancreas [4, 5]. Recently, it has been shown that cell-free circulating miRNAs are highly stable (i.e. resilient to

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**Table 1.** Demographics of analyzed healthy and patient groups

A. Microarray analysis (Total n=26*)				
Stage	H (n=7)	CP (n=6)	PDAC (n=13)	
			I (n=4)	II-IV (n=9)
Average Age (y)	61.6	69.5	64.6	
<60	3	0	1	3
60-70	3	3	1	3
>70	1	3	2	3
Gender				
Female	4	3	3	5
Male	3	3	1	4
Diabetes	0	0	0	0
B. RT-PCR validation (Total n=75)				
Stage	H (n=19)	CP (n=23)	PDAC (n=33)	
			I (n=2)	II-IV (n=31)
Average Age (y)	60.4	57.7	63.9	
<60	9	14	0	9
60-70	6	5	1	15
>70	4	4	1	7
Gender				
Female	10	7	0	10
Male	9	16	2	21
Diabetes	0	5	1	4

\*These samples were also used for the RT-PCR validation experiment.

unfavorable physiological conditions such as extreme pH variation and multiple freeze-thaw cycles [6-8]) and protected from degradation through protein binding or inclusion into sub-cellular particles [9]. Their quantification in body fluids, such as plasma, serum and saliva, has a huge diagnostic potential as it has already been demonstrated for several solid tumors, including PDAC [7, 10-12]. However, the high specificity and sensitivity of circulating miRNAs for the early detection of PDAC has still not been achieved, even when combined with serum CA19.9 [13, 14], the only PDAC biomarker commonly used in clinics [15]. At least in blood, this is partly due to contamination with cellular miRNAs of hematopoietic origin [16].

This prompted us to explore the landscape of miRNAs in urine, as this body fluid represents an attractive alternative to plasma/serum for biomarker discovery. Urine is less complex than blood; while blood is the site of continuous metabolic and homeostatic regulation, urine is inherently stable [17]. In addition, it allows com-

pletely noninvasive sampling, high volume collection and ease of repeat measurements. Not surprisingly, blood and urine thus demonstrate different profiles of various biomarkers, including miRNAs [6].

The association of urine miRNA expression and various tumors has recently been reported. Importantly, this has been demonstrated not only for urothelial cancers [18, 19], but also for cancers originating outside of urogenital tract, such as hepatocellular [20] and breast carcinomas [21].

We have previously shown that urine is a valid body fluid for detecting PDAC and chronic pancreatitis (CP) [22] and have recently identified a three-biomarker protein panel that can detect early stage cancer [23]. In order to further improve the accuracy of early detection, in the present study we have analyzed the expression of circulating miRNAs in urine specimens of healthy individuals and the same patient's groups.

### Materials and methods

#### Samples

The study was performed using urine specimens collected at the Barts and the London HPB Centre, The Royal London Hospital, and University College Hospital, London, after obtaining patients' consent and with full Ethical approval (REC reference number 05/Q0408/65). The samples were kept on ice upon collection, aliquoted before freezing within two hours of collection and maintained at -80°C for long-term storage. Both collection and storage were performed according to standard operating procedures, compliant with Tissue bank requirements under Human Tissue Act.

A total of 101 urine samples were analyzed in this study, which included 46 PDAC at different stages of disease, 29 chronic pancreatitis (CP), and 26 healthy individuals (H) (**Table 1**). In addition to verification of clinical notes for the absence of any renal diseases, all urine samples were also first routinely tested with Urine dipstick (Combur10Test, Roche) in order to eliminate samples with pathological values in any of the parameters measured (protein, glucose, bilirubin, leukocytes, specific gravity).

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### *Low Molecular Weight (LMW) RNA isolation*

LMW RNAs (<200 bp) that include miRNAs and small nucleolar (sno) RNAs were extracted from 3-5 ml of cryopreserved urine, using the Urine microRNA Purification Kit from NORGEN, followed by concentration with RNastable (Biomatrica). Quantification was performed with Quant-iT OliGreen Kit (Invitrogen), and quality assessed on Bioanalyser using the Agilent Small RNA kit.

### *Global miRNA expression profiling*

The microarray profiling was performed using the Affymetrix GeneChip microRNA v. 3.0 Arrays, which comprise 5,607 human small RNAs, including 1,733 mature miRNAs, 1,658 precursor miRNAs and 2,216 snoRNAs. 100 ng of LMW RNAs was labeled using FlashTag Biotin HSR RNA Labeling Kit (Affymetrix) according to the manufacturer's protocol, and ELOSA QC Assay performed in order to confirm the successful labeling. Hybridization to the GeneChip microRNA array, staining, washing and scanning was performed according to standard protocols on Affymetrix workstation.

Raw data files were first analyzed with the Expression Console Software v 1.2 (Affymetrix); a quality control (QC) report with information concerning the performance of the experiment was obtained for each array. Samples were normalized using the Robust Multi-Array (RMA) algorithm. Pearson correlation coefficient ( $r$ ) was used to determine the reproducibility of labeling and hybridization.

### *Validation of MicroRNA expression*

Differential miRNA expression was validated by real-time PCR using the TaqMan MicroRNA Assays (Life Technologies) and carried out on a Fluidigm BioMark HD System, a microfluidic platform for high-throughput real-time PCR quantification [24, 25]. Two 24.192 Dynamic Array™ Integrated Fluidic Circuits (IFCs) were employed and reactions performed in triplicate. PCR assays were performed as previously described [26]. The specific miRNA primers for reverse transcription and pre-amplification reactions were pooled following Life Technologies's instructions (User Bulletin PN 4465407). Multiplexed reverse transcriptions (RT) were carried out using the TaqMan®MicroRNA

Reverse Transcription Kit from 20 ng of LMW RNA in 15  $\mu$ l of final volume. To test the limit of sensitivity and the dynamic range of the method, the reverse transcription reaction was performed on a control sample from a healthy individual using an increasing amount of input LMW RNA: 10, 15, 20, 30, 40, 50, 100, and 200 ng.

The pre-amplification reactions were performed from 2.5  $\mu$ l of RT products in 10  $\mu$ l volume. The mix was first incubated at 95°C for 10 min, 55°C for 1 min, followed by 12 cycles of amplification at 95°C for 15 sec and 60°C for 4 min. Pre-amplification products were diluted 1:5 in TE 1X and 1.35  $\mu$ l used to prepare the quantitative PCR reaction mix according to Fluidigm's protocol (192.24\_GE\_TaqMan\_Std PN 100-6170 B1). All the reactions were performed in triplicates.

The sample and the assay mixes were loaded onto a 24.192 Dynamic Array™ Integrated Fluidic Circuits (IFCs), and then placed in the BioMark Instrument for PCR amplification. The chip was first incubated at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. Data were analyzed using the Real-Time PCR Analysis Software, which is integrated in the Fluidigm system.  $C_t$  values that did not pass the quality threshold of 0.6 (default setting) were discarded.

The average  $C_t$  value was calculated for each miRNA assay in each sample. The two plates were scaled and normalized to a value centered around 0 as follows:

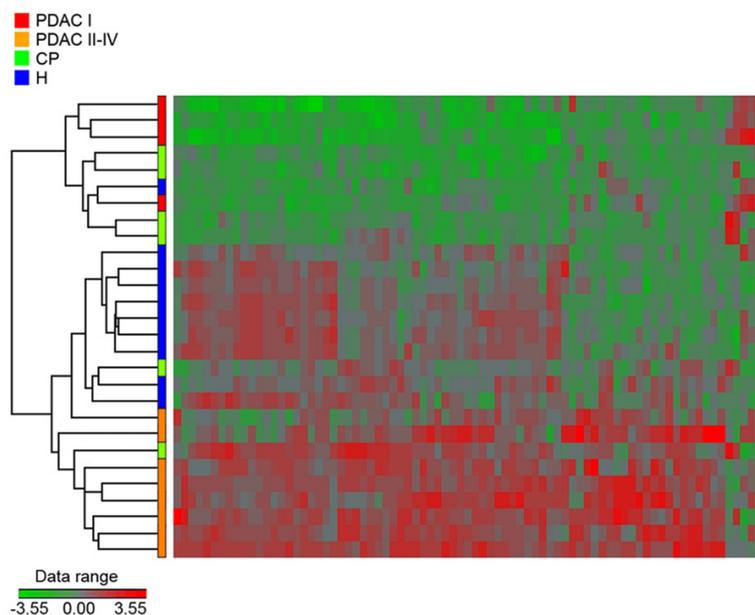
$$[\text{Sample value} - \text{mean (plate)}] / \text{Standard deviation (plate)}$$

### *Statistical analysis*

Data files generated by Affymetrix microarrays were imported into Partek® Genomics Suite™ 6.6 for statistical analysis and hierarchical clustering. Statistically significant differences in miRNAs expression among the examined groups were identified using ANOVA and a 5% false discovery rate (FDR) threshold (Benjamini & Hochberg method [27]). Hierarchical clustering of the most differentially expressed miRNAs was conducted using Partek default settings.

Normalized  $C_t$  values were imported in GraphPad PRISM Software for statistical analysis. The nonparametric Mann-Whitney-U test

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**Figure 1.** Hierarchical cluster analysis of 79 differentially expressed miRNAs (FDR <0.05). The disease status of the samples is shown at the top. Each column represents a miRNA and each row a sample. (The color display indicates the logarithm of the expression changes, where varying shades of red and green indicate up and down-regulation, respectively).

was applied to calculate the *p*-values when comparing the miRNA expression levels between two groups.

Selected miRNA biomarkers were investigated for their ability to discriminate between samples from PDAC Stage I patients and healthy control samples using an exploratory Receiver Operating Characteristics (ROC) curve analysis approach based on all available samples. Logistic regression was applied to each miRNA log base 2-transformed average  $C_t$  data values. The model was adjusted for plate (experimental run) and the individual's age. ROC curves were generated for each of the miRNAs; the area under the curve (AUC), the sensitivity (SN) and the specificity (SP) at the 'optimal' cut-point for discrimination between the two groups were obtained. The optimal cut-point corresponded to the point closest to the top-left part of the plot in the ROC plane (coordinates 0, 1) with optimal SN and SP according to the following criterion:

$$\min ((1-\text{sensitivities})^2 + (1-\text{specificities})^2)$$

as calculated by the 'ci.threshold' procedure of the R 'pROC' package [28]. This approach has been shown to have good performance in the estimation of the optimal cut-point of a biomarker [29].

MiRNAs were then combined to assess the discriminative power of the combination. MiRNAs that correlated with each other (significant Spearman's correlation coefficient) were not combined to avoid collinearity issues in the model.

Confidence intervals (CI, 95%) for AUCs were derived based on DeLong's asymptotically exact method to evaluate the uncertainty of an AUC [30]; SN and SP, 95% CI were derived using non-parametric stratified resampling with the percentile method (2,000 bootstrap replicates) as described by Carpenter et al. [31]. AUCs were compared using DeLong's 1-sided test for correlated/paired [30].

ROC curve analyses were performed in R version 2.13.0 (The R Foundation for Statistical Computing, <http://www.r-project.org/foundation>) using procedures from the Epi [32], pROC [28] and ROCR [33] packages.

## Results

### *Urine miRNA expression profiling*

Global LMW RNA expression profile was determined for 26 urine samples, which included 13 PDAC samples (four Stage I, three Stage II, six Stage III-IV), six CP and seven healthy individuals (**Table 1A**). For one of the healthy samples, two biological replicates (independent RNA extractions) and two technical replicates were also performed to assess the reproducibility of the isolation method and the robustness of the profiling platform, respectively. Therefore, in total, 30 arrays were interrogated. (Microarray data are deposited in GEO, accession number GSE71962). Both the isolation method and the Affymetrix platform proved highly reproducible, resulting in correlation coefficients >0.95 between duplicates (data not shown). The Expression Console Software v 1.2 (Affymetrix) applied to the whole set of experiments, confirmed the expression of an average of 815 (ranging from 748 to 1003) miRNAs per sam-

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**Table 2.** List of 15 miRNAs selected for validation. The fold change [FC] and the significance level of adjusted *p-values* (*P*) are reported for every pairwise comparison

	St. I v H		St. II-IV v H		St. I v II-IV	
	[FC]	(Adj P)	[FC]	(Adj P)	[FC]	(Adj P)
miR-30e	Up [2.2]	0.00002	Up [1.2]	0.21	Up [1.9]	0.0003
miR-143	Up [2.0]	0.0001	Up [1.1]	0.62	Up [1.9]	0.0004
miR-223	Up [4.3]	0.0002	Up [1.0]	0.89	Up [4.2]	0.0005
miR-204	Up [1.4]	0.0004	Up [1.2]	0.013	Up [1.2]	0.06
miR-30b	Up [1.6]	0.13	Up [1.1]	0.63	Up [1.4]	0.27
miR-149*	Down [5.9]	1.80E-07	Down [1.3]	0.15	Down [4.4]	3.20E-06
miR-1915	Down [2.9]	0.00001	Up [1.5]	0.017	Down [4.2]	1.80E-07
miR-3141	Down [7.1]	1.90E-07	Down [1.1]	0.64	Down [6.5]	5.60E-07
miR-4739	Down [3.9]	0.00002	Up [3.8]	0.009	Down [6.9]	1.50E-07
miR-4750	Down [6.4]	0.00002	Down [2.1]	0.012	Down [3.0]	0.004
miR-3663-3p	Up [1.3]	0.5	Up [17.7]	3.00E-08	Down [13.3]	4.80E-06
miR-665	Up [1.1]	0.66	Up [5.6]	3.00E-07	Down [5.0]	0.00002
miR-483-5p	Down [1.1]	0.7	Up [2.0]	0.00001	Down [2.1]	0.00008
miR-1275	Down [2.0]	0.016	Up [3.1]	0.00004	Down [6.2]	1.20E-06
miR-1207-5p	Down [2.0]	0.12	Up [2.0]	0.06	Down [3.9]	0.005
	CP v H		CP v St. I		CP v St. II-IV	
	[FC]	(Adj P)	[FC]	(Adj P)	[FC]	(Adj P)
miR-30e	Up [1.2]	0.26	Down [1.9]	0.002	Up [1.0]	0.9
miR-143	Up [1.2]	0.14	Down [1.6]	0.006	Up [1.2]	0.31
miR-223	Up [1.2]	0.63	Down [3.7]	0.002	Up [1.1]	0.73
miR-204	Down [1.1]	0.35	Down [1.5]	0.0001	Down [1.3]	0.003
miR-30b	Up [3.3]	0.00008	Up [2.1]	0.03	Up [3.0]	0.0004
miR-149*	Down [2.5]	0.0008	Up [2.4]	0.005	Down [1.9]	0.02
miR-1915	Down [1.4]	0.07	Up [2.0]	0.005	Down [2.1]	0.0007
miR-3141	Down [5.8]	9.70E-07	Up [1.2]	0.51	Down [5.3]	2.90E-06
miR-4739	Down [4.2]	8.80E-06	Down [1.1]	0.8	Down [7.5]	8.00E-08
miR-4750	Down [6.9]	0.00001	Down [1.1]	0.86	Down [3.2]	0.003
miR-3663-3p	Down [1.1]	0.82	Down [1.5]	0.45	Down [19.5]	6.80E-07
miR-665	Down [1.3]	0.4	Down [1.5]	0.28	Down [7.2]	1.30E-06
miR-483-5p	Down [1.1]	0.7	Down [1.0]	0.99	Down [2.1]	0.00008
miR-1275	Down [3.1]	0.0004	Down [1.5]	0.19	Down [9.5]	5.00E-08
miR-1207-5p	Down [4.1]	0.003	Down [2.1]	0.16	Down [8.1]	0.0001

St=Stage.

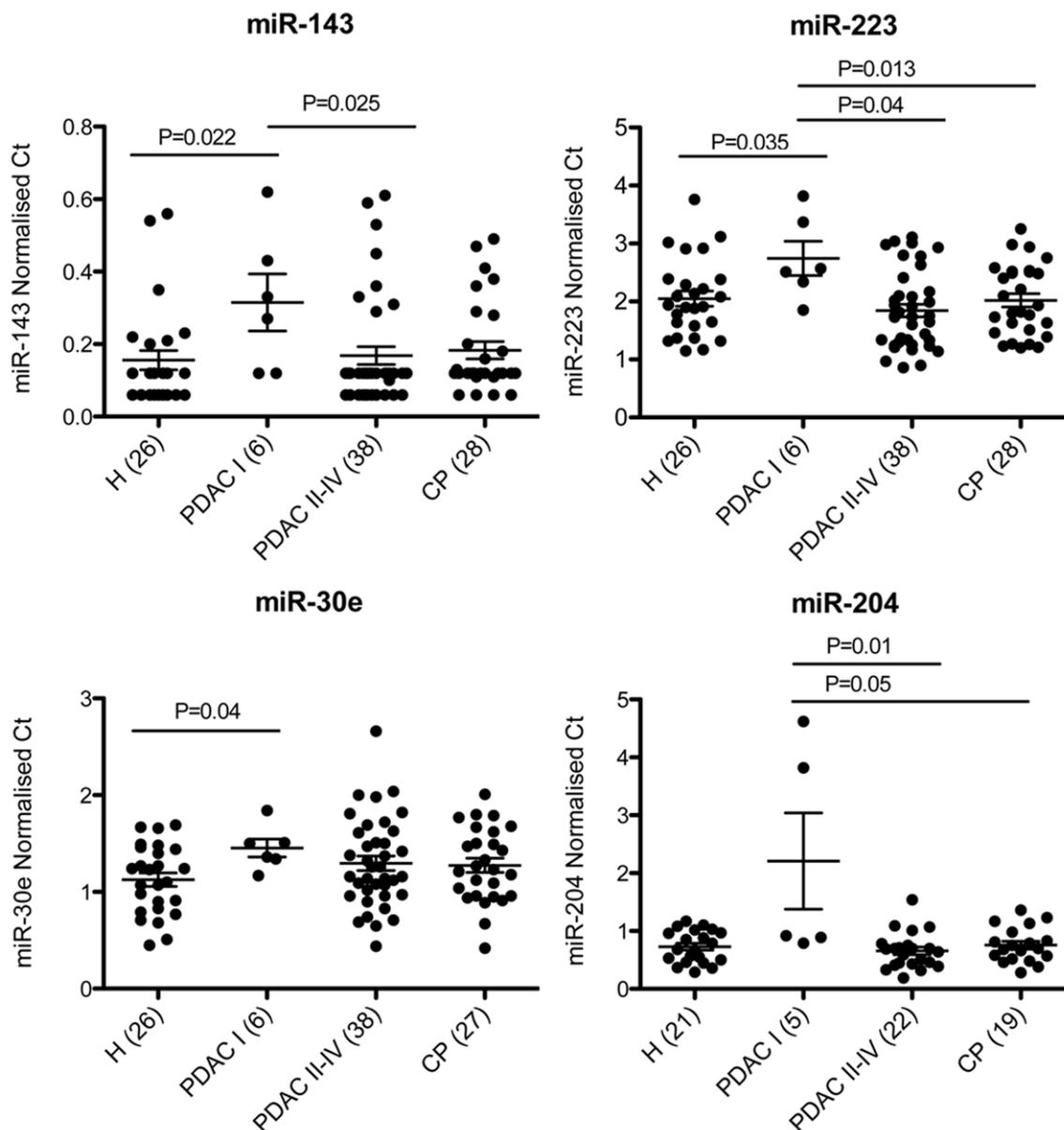
ple. One sample (PDAC Stage III) failed QC and was thus not further analyzed.

ANOVA applied to identify differentially expressed miRNAs between the sample groups (H, CP and PDAC Stage I and Stage II-IV), led to the identification of 79 statistically significant differentially expressed miRNAs (FDR <0.05). A supervised hierarchical cluster analysis of these 79 miRNA profiles showed clear grouping of the healthy, PDAC stage I and PDAC stage II-IV samples according to their disease status (**Figure 1**); in contrast, CP samples showed high heterogeneity and did not cluster.

### *Biomarker selection and validation by RT-PCR*

Out of the 79 differentially expressed miRNAs, urine expression levels of 15 miRNAs showing the lowest *p-values* (**Table 2**) were selected for validation.

The 26 samples previously profiled by microarrays, and a further 75 new urine specimens, including 33 PDAC (two Stage I, 31 Stage II-IV), 23 CP and 19 healthy controls (**Table 1B**), were interrogated using the TaqMan/Fluidigm BioMark platform. However, three samples (one PDAC Stage II, one PDAC Stage III, and one



**Figure 2.** Validation of the four potential miRNAs biomarkers using RT-PCR. The number of samples analyzed in each group is indicated in brackets. Significant adjusted *p*-values (*P*) are shown.

CP) were excluded from the analysis as the  $C_t$  values for the miRNA assays measured failed the quality control.

Among the selected 15 miRNAs, four miRNAs (miR-30e, miR-143, miR-204 and miR-223) were found in significantly higher amounts in the urine of PDAC Stage I patients compared to the healthy population. These miRNAs (except for miR-204) also showed a decreased expression in Stage II-IV compared to Stage I (Table 2). Another three miRNAs, (miR-3141, miR-4739 and miR-4750) were significantly down-

regulated in CP compared to healthy, whereas miR-30b was the only miRNA with increased expression in CP compared with both the healthy and PDAC (all stages) groups. The expression of miR-3663-3p, miR-665 and miR-483-5p was significantly higher in later PDAC stages than in healthy samples. The remaining four candidate miRNAs were differentially expressed between PDAC Stages II-IV and I and/or CP (Table 2).

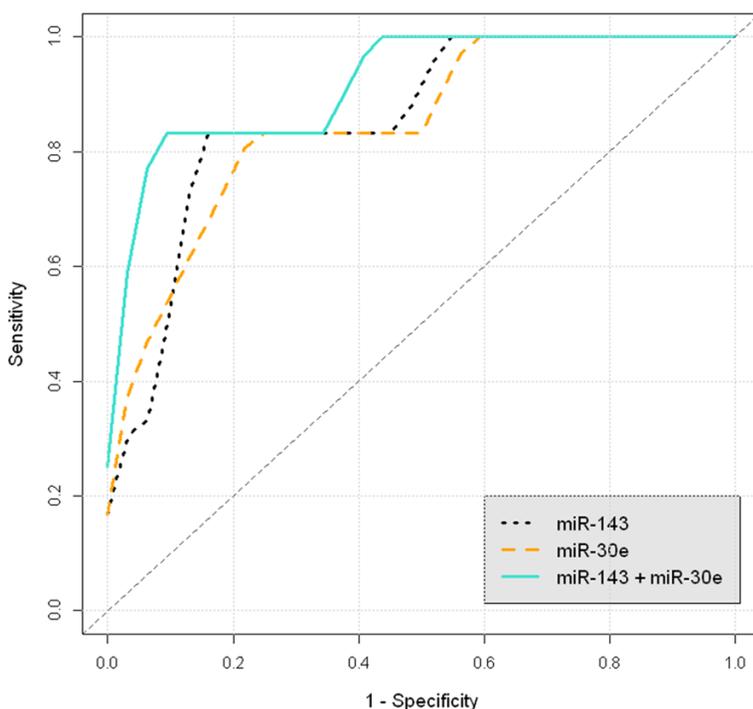
Significant over-expression in PDAC Stage I when compared with healthy controls was con-

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**Table 3.** Results of the ROC analyses for the discrimination between healthy and PDAC stage I individuals

miRNAs	AUC (95% CI)	% SN (95% CI)*	% SP (95% CI)*
<b>Individual markers</b>			
miR-143	0.862 (0.695-1.000)	83.3 (50.0-100.0)	88.5 (73.1-100.0)
miR-30e	0.853 (0.673-1.000)	83.3 (50.0-100.0)	80.8 (65.4-96.2)
miR-223	0.795 (0.586-1.000)	83.3 (50.0-100.0)	76.9 (61.5-92.3)
<b>Combinations<sup>§</sup></b>			
miR-143+miR-30e <sup>§§</sup>	0.923 (0.793-1.000)	83.3 (50.0-100.0)	96.2 (88.5-100.0)
miR-30e+miR-223 <sup>§§§</sup>	0.891 (0.714-1.000)	83.3 (50.0-100.0)	92.3 (70.8-100.0)

\*At optimal cut-point. <sup>§</sup>miR-30e did not significantly correlate with miR-223, while miR-143 correlated significantly with miR-223 (P=0.59, P<0.001) and resulted in collinearity. <sup>§§</sup>DeLong's 1-sided test for correlated/paired AUCs to assess whether the addition of miR-30e significantly increases the AUC obtained with miR-143 alone (0.923 versus 0.862), P=0.04. <sup>§§§</sup>DeLong's 1-sided test for correlated/paired AUCs to assess whether the addition of miR-223 significantly increases the AUC obtained with miR-30e alone (0.891 versus 0.853), P=0.1.



**Figure 3.** ROC curves for individual miRNAs, miRNA-143 and miRNA-30e, and their combination.

firmed for miR-30e, miR-143 and miR-223. MiR-143, miR-223 and miR-204 also showed statistically higher levels in Stage I when compared to Stages II-IV. Furthermore, miR-223 and miR-204 were also able to distinguish patients with Stage I from patients with CP (Figure 2). A significant differential expression between PDAC Stage II-IV and CP was also confirmed for miR-1915 (data not shown). The expression of six miRNAs (miR-30b, miR-1207-5p, miR-1275, miR-483-5p, miR-3141, and

miR-4739), while correlating with the results obtained by Affymetrix array, resulted in *p-values* that were just below the threshold for statistical significance. The differential expression of one miRNA, miR-3663-5p, was not confirmed and the remaining three (miR-4750, miR-149\*, miR-665) failed the  $C_t$  quality filter set by the analysis program.

### *Diagnostic potential of the miRNAs to discriminate between healthy and PDAC Stage I individuals*

Logistic regression analysis was applied to the Fluidigm data obtained for miR-143, miR-30e and miR-223 (miR-204 was not included in this analysis as RT-PCR did not perform well for several samples). Among the three bio-

markers, miR-143 was best able to differentiate Stage I (n=6) from healthy (n=26) (AUC=0.862 (95% CI 0.695-1.000), with SN of 83.3% (95% CI 50.0-100.0) and SP of 88.5% (95% CI 73.1-100.0) at optimal cut-point; Table 3 and Figure 3). The combination of miR-143 with miR-30e was significantly better at discriminating between the two groups, achieving an AUC of 0.923 (95% CI 0.793-1.000), with SN of 83.3% (95% CI 50.0-100.0) and SP of 96.2% (95% CI 88.5-100.0) at optimal cut-point (Table

**3 and Figure 3).** Combining miR-30e with miR-223 only achieved an AUC of 0.891 (95% CI 0.714-1.000), which was not significantly better at the 5% level compared to miR-30e alone (AUC=0.853 (95% CI 0.673-1.000), P=0.1; **Table 3 and Figure 3**) although a larger sample size may reveal this increase in AUC to be significant.

### Discussion

In this study, we demonstrated, for the first time, feasibility of a genome-wide expression analysis of miRNAs in the urine of patients with PDAC and CP and compared them to healthy controls. Moreover, we established the significant over-expression for a subset of miRNAs in PDAC Stage I versus healthy individuals (miR-143, miR-223, miR-30e) and Stage I versus Stages II-IV PDAC (miR-204, miR-143, miR-223).

All four miRNAs have previously been detected in pancreatic tissues. MiR-223 has been shown to be over-expressed in resectable PDAC tissues, and associated with good patients' outcome and miR-143 has been shown to be up-regulated in resectable PDAC tissues and down-regulated in liver metastases [34]. Enrichment of miR-204 was reported in the cyst fluid from high-grade pancreatic cystic lesions when compared with low grade-benign cystic lesions in a study with the goal of classifying IPMN cases by risk of progression to pancreatic cancer [35]. These data thus independently corroborate the potential of the selected miRNAs to serve as early diagnostic biomarkers.

Both miR-143 and miR-204 have previously been described as tumor suppressors, with their down-regulation associated with proliferation and invasion in a number of solid tumors [36-39], including PDAC [40, 41]. MiR-143 is of particular interest because it has not only been shown to promote apoptosis and suppresses tumorigenesis by targeting Bcl-2 [42, 43], but it has also been shown to be involved in a regulatory pathway in KRAS mutant pancreatic cancers [40]. Activated KRAS was demonstrated to lead to the loss of expression of the miR-143/145 cluster through the activation of the Ras-responsive element-binding protein (RRB1), which directly represses the two miRNAs in order to maintain the tumorigenic potential of PDAC cells. However, both KRAS and RRB1 transcripts are targets of these miRNAs,

so their restoration abrogates tumorigenesis [40]. Our results showing the up-regulation of miR-143 in Stage I tumors and its decreased level in later PDAC stages are in agreement with the correlation between miR-143 down-regulation and the development of a more aggressive tumor. This hypothesis is also supported by a study reporting the negative correlation of miR-143 expression with tumor spread in lymph nodes [44]. Interestingly, up-regulation of miR-143 was also reported in a meta-analysis of miRNAs differentially expressed between type 2 diabetic patients and non-diabetic controls; miR-143 expression was found to be pancreas and liver specific [45], therefore pointing to its potential role as an early tissue biomarker of type 2 diabetes. As for the tumor suppressor role of miR-204, Chen et al. have shown that it represses the expression of the Myeloid cell leukaemia-1 gene (Mcl-1) in PDAC cell lines, with subsequent decrease in cell viability. In PDAC patients, the over-expression of Mcl-1 is linked to tumor progression [41].

In contrast, miR-223 has been previously described as an oncomiR and has been shown to promote the invasion and metastasis of gastric cancer and glioblastoma cells by targeting tumor suppressor genes [46, 47]. In PDAC, inhibition of miR-223 expression by Genistein treatment causes inhibition of cell growth and induces apoptosis, and it has thus been suggested that down-regulation of miR-223 could be a novel therapeutic strategy for pancreatic cancer [48]. While the increase of miR-223 in urine seems counterintuitive, in some cases an inverse relationship between miRNA expression in tissues and in body fluids has been seen, although the underlying mechanism is still unclear [49].

Interestingly, the de-regulation of miR-223 was, similarly to miR-143, also seen in a meta-analysis of miRNA expression in type 2 diabetes [45], and both mi-RNAs have been identified as biomarkers for metabolic changes in obesity [50]. As these conditions are recognized risk factors for PDAC [51, 52], aberrant expression of miR-143 and miR-223 seen in our study is intriguing, particularly as none of the PDAC samples used in the initial microarray screening were from patients with manifested diabetes.

MiR-30e is down-regulated in resectable PDAC according to Jamieson et al. [34], which is consistent with the down-regulation of this miRNA

in several epithelial cancers [53]. MiR-30e suppresses cell growth by directly targeting ubiquitin-conjugating enzyme E2I (Ubc9) in breast, head and neck, and lung cancer [54]. However, opposing functions have also been attributed to this miRNA, such as promoting cell invasion in gliomas [55].

A number of studies have been conducted to identify PDAC miRNA biomarkers in body fluids, e.g. serum [56, 57], plasma [10, 13], pancreatic juice [58], whole blood [14, 59] and saliva [12]. Of interest, miRNA-223 was previously included in two panels consisting of four and 10 miRNAs in order to discriminate PDAC from healthy controls combined with CP using whole blood; however this miRNA was not tested in early stages of the disease [14]. Thus, not only the expression of miRNAs in urine specimens from PDAC patients has not previously been interrogated, more importantly, miRNAs capable of detecting PDAC patients at Stage I have not yet been described.

The four miRNAs reported here, as well as the combination of miR-143 and miR-30e, require further mechanistic analysis and testing in larger, independent cohorts of urine samples. These should be procured through multicenter collaborative studies; this is particularly important for obtaining additional samples from Stage I disease, which is typically obtained only incidentally. Further validation of the miRNAs in samples collected from high-risk groups, cystic lesions and other benign and malignant diseases of pancreas also needs to be performed. Finally, the diagnostic accuracy of urinary miRNAs in comparison to, and in combination with serum CA19.9 needs to be established.

In summary, in this feasibility study we determined the expression profiles of miRNAs in urine samples of patients with PDAC, CP and healthy individuals. We demonstrate that miRNA levels in urine can not only distinguish between healthy and diseased individuals, but importantly, can differentiate early from late stage tumors. Using the same samples and an independent urine sample cohort, we successfully validated four differentially expressed miRNAs, showing their potential diagnostic value at an early stage of disease. Subsequent to large-scale validation, the seamless translation of these miRNAs into the clinical setting as a RT-PCR-based urine test for early detection,

could ultimately make a huge impact on the prognosis and survival of pancreatic cancer patients.

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### Disclosure of conflict of interest

None.

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