

A MicroRNA Panel in Pancreatic Cyst Fluid for the Risk Stratification of Pancreatic Cysts in a Prospective Cohort

WK Utomo¹, LH Looijenga², MJ Bruno¹, BE Hansen¹, AJM Gillis², K Biermann², MP Peppelenbosch¹, GM Fuhler¹ and H Braat¹

A subset of pancreatic cystic neoplasms are regarded as precursor lesions of pancreatic cancer, but only a minority of all pancreatic cystic neoplasms will undergo malignant transformation. MicroRNAs are increasingly recognized as molecular targets in carcinogenesis. Previously, a 9-microRNA (miR) signature was suggested to discriminate between high risk and low risk pancreatic cystic neoplasm. In this study, we aimed to validate this 9-miR panel in a prospective cohort. Total miR was isolated from pancreatic cyst fluid and expression of miR18a, miR24, miR30a-3p, miR92a, miR99b, miR106b, miR142-3p, miR342-3p, and miR532-3p was analyzed by singleplex Taqman MicroRNA Assay. A total of 62 patient samples were analyzed. During follow-up, 24 (38.7%) patients underwent resection, of which 6 (9.7%) patients showed at least high grade dysplasia. A logistic regression model presented a “predicted risk” score which significantly differed between low and high risk cysts, either including all patients or only those with histological confirmation of diagnosis. Using a set cut-off of 50%, the sensitivity of the model for the total cohort was 10.0%, specificity 100.0%, positive predicted value 100.0%, negative predicted value 85.2%, and diagnostic accuracy of 85.5%. Thus, while observing a significant difference between low and high risk cysts, clinical implementation of this biomarker panel is as yet unlikely to be beneficial in the management of pancreatic cysts.

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Subject Category: siRNAs, shRNAs and MiRNAs

Introduction

Pancreatic Cystic Neoplasms (PCNs) are potential precursors of pancreatic cancer.¹ With the widespread use of radiologic cross-sectional imaging, PCNs are discovered with increasing frequency. The prevalence of pancreatic cysts in patients undergoing cross-sectional imaging of the abdomen is estimated to be ~2.4%.² Patients with a PCN have been shown to have a 35 times higher age- and gender-adjusted incidence of pancreatic malignancy compared with a noncyst population.¹ Therefore, PCNs are generally regarded as pre-malignant lesions and preemptive resections are often considered necessary.

Surgical treatment of cysts varies from pancreaticoduodenectomy to distal pancreatectomy with or without splenectomy. These invasive procedures are associated with a significant risk of morbidity and mortality, which may be unnecessary when the cyst has little to no malignant potential. In the past, some studies showed that half of the pancreatic cysts resected turned out histopathologically to be a pseudocyst, while the remainder consisted of true PCNs of various origins and dysplastic grade.³ In recent years, diagnostic performance has improved considerably, but nevertheless there is need for better identification of cysts with malignant potential. Studies validating the initial criteria for resection of PCN (*i.e.*, Sendai guidelines⁴) have shown a high sensitivity, close to 100%, but very low specificity of 25–30%.⁵ The revised guidelines released in 2012 (Fukuoka guidelines⁶) appear to perform better,⁷ but nevertheless, have shown that 67.7% of resected patients are still exposed to unnecessary surgery

for cysts which in hindsight were low risk PCN.⁸ Thus, there is an urgent need for additional predictors to aid in the clinical decision making whether to operate or not.

Endoscopic ultrasound (EUS) is considered a valuable asset to aid in the differentiation between various pancreatic cysts including intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasms, however, it unfortunately does not allow reliable identification of the presence and/or risk of malignant transformation other than the identification of nonspecific morphological features like mural nodules or asymmetric cyst wall thickening. Unfortunately, there is a poor correlation between morphological features and dysplastic grade. Biomarkers such as carcinoembryonic antigen, while able to help distinguish between mucinous and nonmucinous cysts, have shown limited sensitivity and specificity for diagnosis and prognosis of pancreatic cancer.^{9–12} Several other markers have been investigated, including carbohydrate antigen 19-9, although, again, poor sensitivity and specificity preclude the use of this marker for pancreatic cancer diagnosis.¹³

Currently, there is an increasing interest in the potential use of pancreatic cyst fluid in the treatment algorithm of patients with pancreatic cysts. Cyst fluid obtained by EUS-fine needle aspiration (EUS-FNA) may contain precancerous cells which potentially can be identified by mutational status analysis. KRAS mutations are common in pancreatic cancer and the largest study to date investigating the use of KRAS mutation detection in pancreatic cyst fluids was the pancreatic cyst DNA analysis (PANDA) study.¹⁴ They showed that while KRAS mutations were more prevalent

¹Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, The Netherlands; ²Department of Pathology, Erasmus MC University Medical Center Rotterdam, The Netherlands. Correspondence: Gwenny M Fuhler, Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, Gravendijkwal 230, 3015CE, Rotterdam, The Netherlands. E-mail: g.fuhler@erasmusmc.nl

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in mucinous cysts, the presence of KRAS mutations did not distinguish premalignant from malignant cysts, and while additional allelic loss was highly specific for malignant cysts (96%), sensitivity was low (37%). However, next generation sequencing may improve sensitivity, and combining molecular markers (*i.e.*, BRAF, CDKN2A, CTNNB1, GNAS, KRAS, NRAS, PIK3CA, RNF43, SMAD4, TP53, and VHL and loss of heterozygosity thereof) together with clinical markers may improve diagnostic accuracy of PCN.^{15–17}

Biomolecules released by dysplastic cells may also be present in cyst fluid, and may aid in the identification of premalignant cysts. One such potential biomarker is the presence of miRs in pancreatic cyst fluid, which could be differentially expressed in benign, dysplastic, and malignant cysts.

MicroRNAs (MiRs) are small, noncoding, functional RNAs of ~21–23 nucleotides in length that are involved in epigenetic regulation by binding to specific mRNAs, leading to their degradation and translational repression.¹⁸ It has been shown that expression levels of miRs differ between various pathological conditions, including cancer.¹⁹ Furthermore, miRs can be quantified in several types of bodily fluid²⁰ and are extremely stable, showing resistance against endogenous RNase activity.²¹ In 2012, Matthaehi *et al.* published a 9-miR model, which showed that a panel of nine miRs measured in pancreatic cyst fluid were able to differentiate between low risk cysts and high risk cysts, which allowed stratification of patients into those eligible for either surgery or watchful waiting with a sensitivity of 89%, specificity of 100%, and area under the curve of 1.²² However,

clinical implementation of these promising results requires validation. Hence, in this study, we aimed to prospectively validate this 9-miR model in a cohort of pancreatic cyst patients.

Results
Descriptives

In total, 92 pancreatic cyst fluid samples were collected during the study period. Of those, 12 samples were excluded due to technical difficulties during isolation or inconclusive diagnosis. Samples were spiked with a known concentration of the nonhuman miRNA cel-miR39 in order to confirm efficient isolation of miRs from the sample. In three cases, levels of cel-miR39 after isolation were too low (Ct >30) to assure efficient miR isolation, and samples were discarded (see **Supplementary Figure S1**). Finally, unreliable measurement of one of the miRs in the panel occurred in 15 samples. Final analysis was performed in 62 patients — see **Figure 1** for the flowchart of the study and **Table 1** for the clinical patient characteristics. In total, 52 patients with a low risk cyst were included, versus 10 patients with a high risk cyst. Histological confirmation of diagnosis was available through EUS-FNA for 11 patients (17.8%) and by histology after surgery for 24 (38.7%) of which, 25% (6 out of 24) had high grade dysplasia or invasive carcinoma. The remainder of the patients (27; 43.5%) was followed up for a mean duration of 26.59 (0–74.25) months, and for all patients diagnosis known at the end of the follow-up was used.

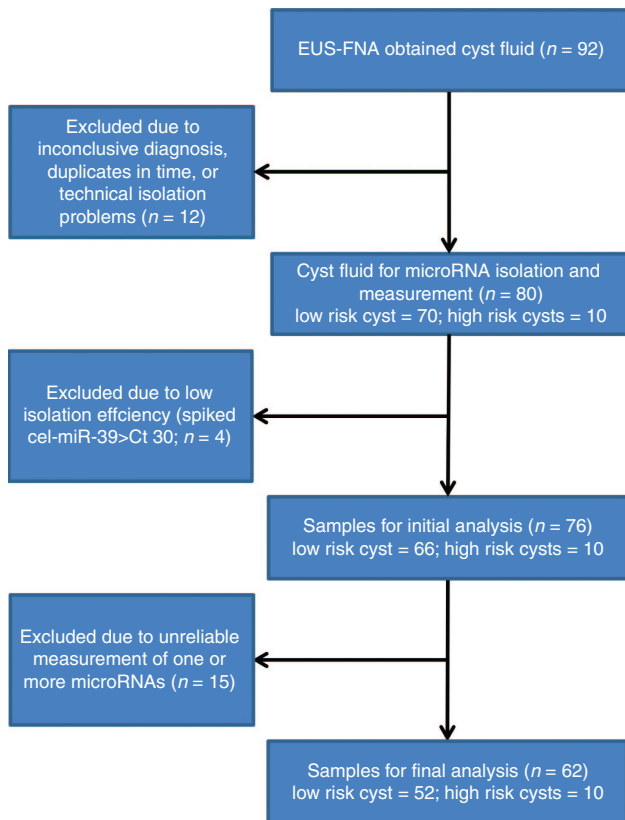


Figure 1 Flowchart of the study population.

Table 1 Patient characteristics of the cohort

	Total (n = 62)	Low risk cyst (n = 52)	High risk PCN (n = 10)
Age, years			
Range	19.9–79.5	19.9–79.5	45.5–78.4
Mean (SD)	59.6 (13.4)	58.5 (13.7)	65.5 (10.6)
Gender, n (%)			
Male	20 (32.3)	14 (26.9)	6 (60.0)
Female	42 (67.7)	38 (73.1)	4 (40.0)
Diagnosis, n (%)			
Main Branch IPMN	3 (4.8)	1 (1.9)	2 (20.0)
Side Branch IPMN	18 (29.0)	16 (30.8)	2 (20.0)
Mixed Type IPMN	4 (6.5)	0 (0)	4 (40.0)
Mucinous Cystic Neoplasm	13 (21.0)	11 (21.2)	2 (20.0)
Serosus Cystadenoma	15 (24.2)	15 (28.8)	0 (0)
Pseudocyst	9 (14.5)	9 (17.3)	0 (0)
Histological confirmation, n (%)			
None	27 (43.5)	23 (44.2)	4 (40.0)
EUS-FNA	11 (17.8)	11 (21.2)	0 (0)
Surgery	24 (38.7)	18 (34.6)	6 (60.0)
Dysplasia, of resected (n = 24)			
No dysplasia	6 (25.0)		
Low grade dysplasia	9 (37.5)		
Moderate grade dysplasia	3 (12.5)		
High grade dysplasia	5 (20.8)		
Invasive carcinoma	1 (4.2)		

PCN, pancreatic cystic neoplasms; EUS-FNA, endoscopic ultrasound-fine needle aspiration; IPMN, intraductal papillary mucinous neoplasm.

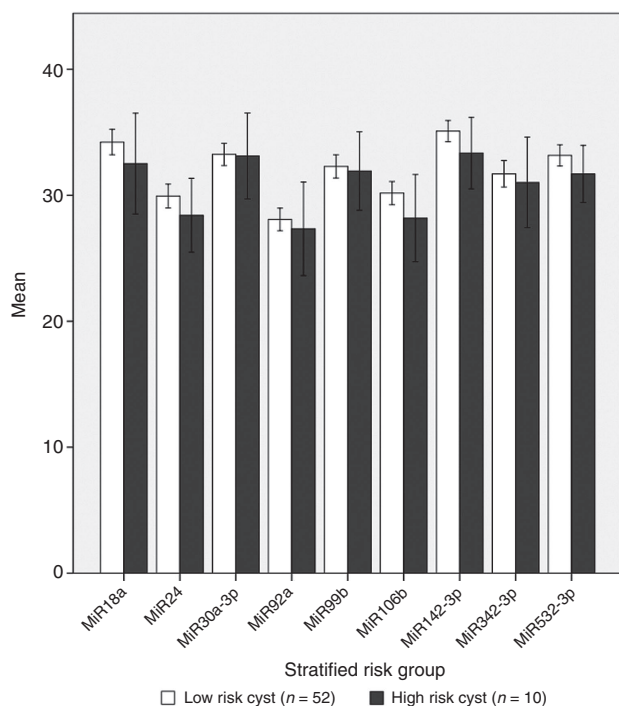


Figure 2 The mean expression of individual microRNAs of the 9-microRNA panel. There were no significant differences in the expression of the microRNAs between low risk cysts and high risk cysts. Error bars denote the 95% confidence interval.

Individual miRs and calculated diffpairs show no significant difference between low risk cysts and high risk cysts

In total, nine miRNA expression levels were measured. Comparing the total expression of these individual miRs did not show significant differences in mean Ct values between low risk and high risk cysts (Figure 2). MiR expression levels were subsequently normalized by making seven distinct “diffpairs” (i.e., seven combinations of two miRNAs), where values were generated by comparing the expression levels of the two miRNAs in that diffpair. The choice of miRNA pairing was based on earlier results by Matthaai *et al.*, who defined these diffpairs based on the most differentially expressed diffpairs used in a L1-penalized logistic regression model (miR18 was combined with miR92a, miR24 with miR30a-3p, miR30a-3p with miR532-3p, miR24 with miR99b, miR106b with miR92a, miR142-3p with miR92a, and miR24 with miR342-3p). One of these diffpair combination (diffpair (miR106b;miR92a)) was found to be significantly different between low risk cysts and high risk cysts (Figure 3). This difference did not hold up in the logistic regression and was therefore considered not clinically relevant. Of note, generating other diffpairs also did not result in significant differences in diffpairs observed between high risk and low risk cysts (see Supplementary Table S1).

The logistic regression model using diffpairs is significantly different between low risk cysts and high risk cysts

The diffpairs were entered in a logistic regression model using previously published regression coefficients.²² The regression

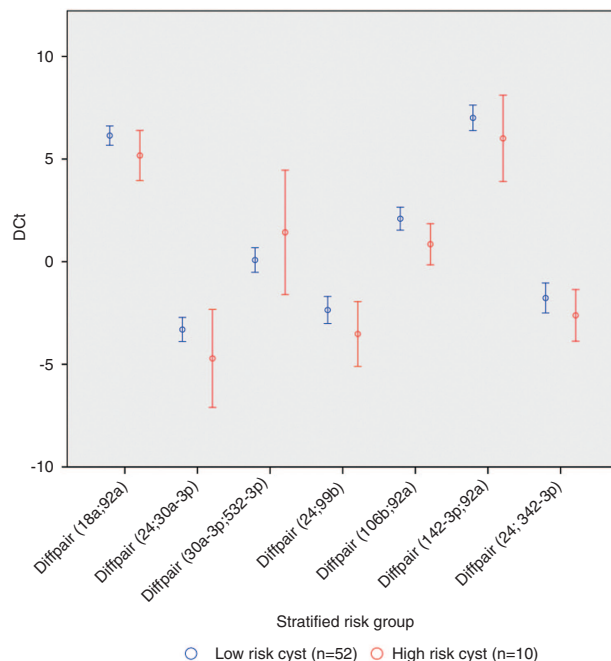


Figure 3 Graph depicting the diffpair expression between low risk cysts and high risk cysts. Diffpair (106b;92a) was significantly different ($P = 0.028$) between low risk and high risk cysts, however, this did not hold up in the regression model. Error bars denote the 95% confidence interval.

coefficient represents the weight given to each diffpair in the model, i.e., in this weighted model each diffpair does not contribute equally to the equation. Diffpair (miR24;miR30a-3p) and diffpair (miR18a;miR92a) contributed the most in this model (see Supplementary Table S2). Exploring the possibility for recalibration did not lead to improved discrimination between low risk and high risk cysts (data not shown). The diffpairs in the logistic regression model together generate a risk score (named the “predicted risk” (13)) which ranges from 0% and 100%. The mean predicted risk was 13.97% for low risk cysts versus 27.3% in the high risk cysts (Figure 4; $P = 0.013$). Comparing high risk IPMN to either low risk IPMN alone or low risk mucinous cystic neoplasms alone also showed significant higher predicted risk in the high risk IPMN ($P = 0.0286$ and 0.0093 , respectively; Supplementary Figure S2). The diagnostic performance of the logistic regression model classified as fair in separating low risk cysts from high risk cysts, with an area under the receiver operator curve of 0.75 (Figure 5a). Excluding all cases without a histological confirmation, the diagnostic performance increased to good discrimination with an area under the receiver operator characteristic curve of 0.83 (Figure 5b). These data demonstrate that the 9-miR panel shows potential for distinguishing high risk and low risk cysts.

The established cut-off value of 50% is not clinically implementable

Reliable implementation of a diagnostic tool requires reliable cut-off levels with sufficient sensitivity and specificity. From the coordinates of the receiver operator characteristic curve, we explored various cut-off values and evaluated diagnostic

characteristics of each of these cut-off points (Table 2). First we employed a cut-off value of 50%, as this was the value suggested in the study of Matthaiei *et al.*²² In our study, a cut-off of 50% yielded a sensitivity of 10.0%, specificity of 100.0%, positive predicted value of 100.0%, negative predicted value of 85.5%, and diagnostic accuracy of 85.5%, which was significantly poorer than the previously reported results. In our study, the optimal cut-off value was 25.66%, as determined by the sensitivity and specificity curve using Graphpad Prism 5 (version 5.01) (Figure 6a), and confirmed using an online cut-off calculator tool. The diagnostic characteristics of the optimal cut-off were 60.0% sensitivity, 90.4%

specificity, 54.5% positive predicted value, 92.2% negative predicted value, and 85.5% diagnostic accuracy (Table 2). Only taking samples with the golden standard into account yielded an optimal cut-off value of 25.0% (Figure 6b) which results in a sensitivity of 66.7%, specificity of 89.7%, positive predicted value 57.1%, negative predicted value 92.9%, and diagnostic accuracy of 85.7% (Table 3).

Discussion

With the growing detection of pancreatic cysts of various sizes, it is becoming increasingly important to discriminate between harmless cystic lesions and cysts that have high malignant potential. We prospectively validated a promising 9-miR panel as biomarker for the detection of high risk cysts which should be resected based on the dysplastic transformation within the epithelial lining.²² While in our study we observed a significant difference between low risk cysts and high risk cysts in the predicted risk of the 9-miR model, the threshold of 50% as proposed by Matthaiei *et al.* does not unequivocally segregate low risk from high risk cysts and is accompanied by a very low sensitivity of 10%.

Recently published guidelines of the American Gastroenterological Association regarding asymptomatic cysts have led to a major debate regarding the implementation of these guidelines.²³⁻²⁶ Nine out of ten recommendations for surgery were deemed to be based on very low quality evidence,²³ underlining the necessity for a more objective measurement in patients with this increasingly frequent lesion. The importance of finding and validating novel biomarkers for identification of high risk cysts is further emphasized by the high rate of resections performed for low risk PCN (specifically in mucinous cystic neoplasms and side branch-IPMN).⁸

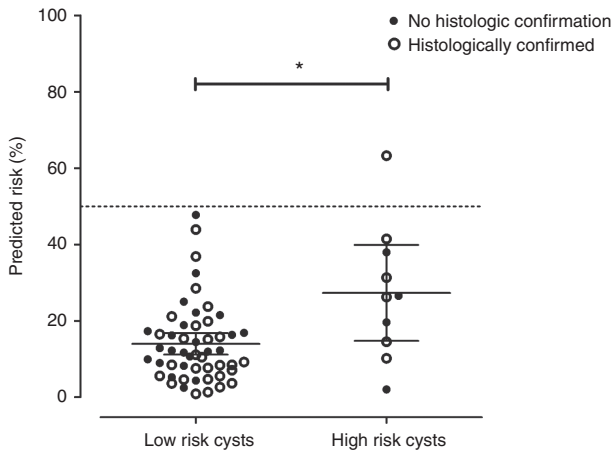


Figure 4 Graph depicting the predicted probability for resection. The predicted probability is significantly higher in the high risk cyst group ($P = 0.013$). In the paper published by Matthaiei *et al.*, a threshold of 50% (dotted line) was used to determine the need for resection. Error bars denote the 95% confidence interval.

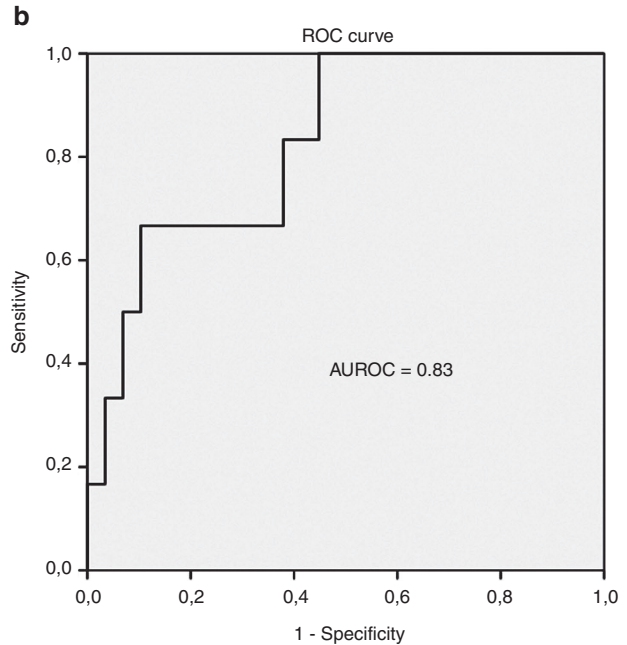
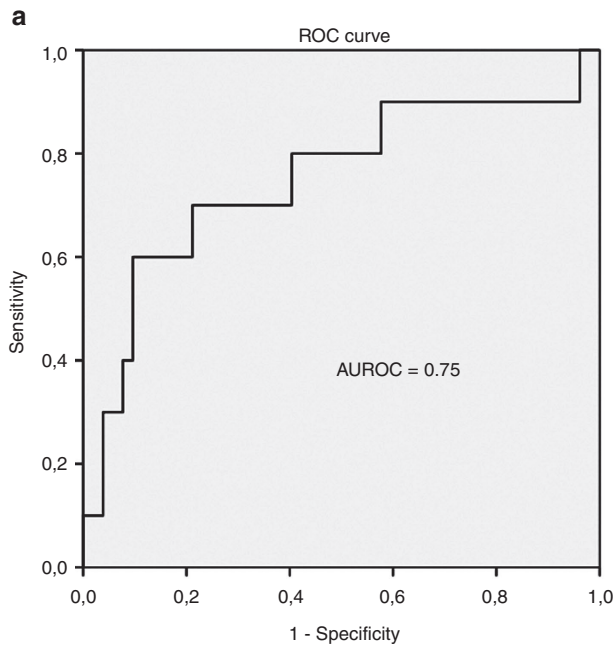


Figure 5 Receiver operator characteristic (ROC) curve of the logistic regression model in predicting cysts that require resection. (a) ROC of the whole cohort. (b) ROC excluding samples without histologic confirmation.

Table 2 Diagnostic characteristics of the logistic regression model

Cut-off, %	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %	Diagnostic accuracy, %
8	90.0	28.8	19.6	93.8	38.7
10	90.0	42.3	23.1	95.7	50.0
25	50.0	88.5	50.0	92.0	83.9
25.66	60.0	90.4	54.5	92.2	85.5
50	10.0	100.0	100.0	85.2	85.5

Cut-off value in bold marks the calculated optimal threshold.

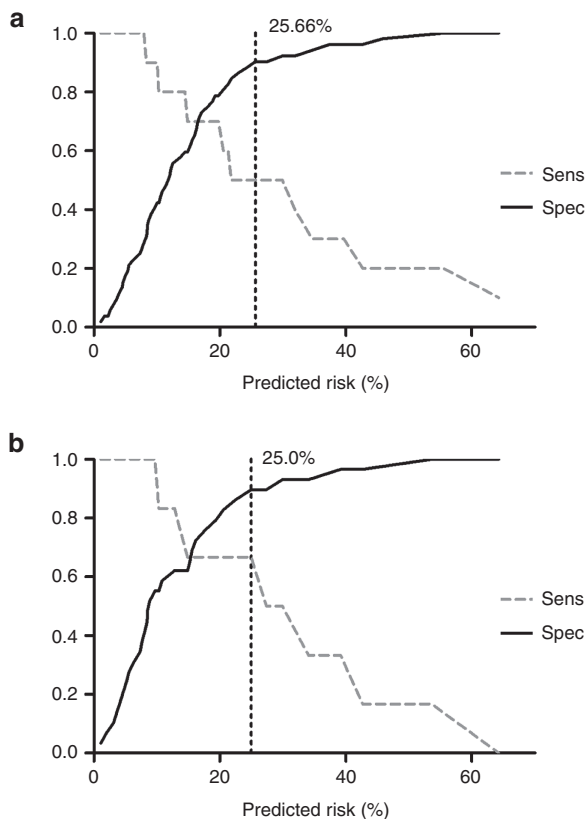


Figure 6 Calculation of the optimal threshold value. Calculation of the optimal threshold value as derived from (a) the whole cohort and (b) the cohort with histologic confirmation only.

MiRs are being widely investigated as a biomarker for the early detection, diagnosis, prognosis, and treatment response of numerous cancers. The field is rapidly evolving and the first phase 1 trial using a miRNA mimic in liver cancer is currently ongoing.²⁷ As a biomarker, they hold several advantages — *e.g.*, they are very stable and thus measurable across different tissues and different moments in time. Furthermore, miR signatures have been shown to be specifically associated with various types of cancer.²⁸ Most importantly, it is assumed that distinct miRs are released by tumor cells into their environment, and make their way to the periphery, thereby making it possible to measure them in for example blood samples and reducing the need for invasive procedures. The downside to this approach is that circulating miR levels can be sensitive to subtle changes in the human body, including diet and viral infections, leading to conflicting results.^{29,30} Moreover, the optimal compartment of blood (whole blood, serum, or plasma) in which miRNAs should be measured is still matter of discussion.³¹

A multitude of circulating miRs has been associated with the detection of pancreatic ductal adenocarcinoma.^{32–34} These studies, however, generally show considerable dissimilarities in reported miRNAs.³⁵ The use of serum miRs for pancreatic lesion distinction has been described, albeit in a low number of cases, and could be hampered by the fact that the cyst is a closed compartment.³⁶ This may impede the dispersion of molecular markers in the circulation. Using pancreatic cyst fluid as the source for miR measurements may overcome this problem. Several studies have indicated that the use of miRs as a biomarker for malignant degeneration of pancreatic cysts may show promise.^{22,37,38} However, similar to the problems in circulating miRs, there is no consensus in the best endogenous control for the normalization in pancreatic cyst fluid. Two of these studies have chosen the small nucleolar RNA, RNU6B, as their endogenous control. However, the use of RNU6B for expression normalization has been reported to result in bias and erroneous findings, as they are not stably expressed in all tissues and fluids.^{39–42} The study by Matthaei *et al.* was unique by a different approach of normalization using diffpairs which was described earlier in ref. 43.

The 9-miR model as described by Matthaei *et al.* was derived from high throughput analysis of 11 cyst fluid samples and subsequently validated using singleplex quantitative reverse transcription-polymerase chain reaction (PCR) in 37 cyst fluid samples. In the validation cohort a threshold of 50% yielded a sensitivity of 89% and specificity of 100%.²² In our study, we performed an external validation of the use of this model and observe a significant difference in predicted risk of low risk and high risk cysts. Despite this difference, the proposed cut-off value of 50% yielded a sensitivity of only 10% and specificity of 100.0%. While our study showed a diagnostic accuracy of 85.5%, we feel this may be biased by the low numbers of high risk cysts in our cohort. In addition, with the sensitivity differing so much between these studies, further validation in other cohorts is warranted before any clinical implementation may be considered. Employing various other cut-off values improved diagnostic accuracy to levels that are theoretically suitable for clinical implementation, however, this is most likely due to overfitting of our study population and lacks generalizability. Even though miRs have been studied for a couple of decades in particular for application in the field of cancer, only few have made it into clinical trials, and even less are being used in the clinic despite initial promising results.⁴⁴ For a biomarker to be implemented in routine clinical care, reproducibility across different hospitals or laboratories must be shown, making this study important for the validation of the 9-miR model in pancreatic cyst fluid.⁴⁵

This study has several limitations. First, as pancreatic cyst fluid is only collected from a lesion requiring a EUS-FNA,

Table 3 Diagnostic characteristics of the logistic regression model in patients with histological confirmation only

Cut-off, %	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %	Diagnostic accuracy, %
8	100.0	41.4	26.1	100.0	51.4
10	100.0	55.2	31.6	100.0	62.9
25	66.7	89.7	57.1	92.9	85.7
50	16.7	100.0	100.0	85.3	85.7

Cut-off value in bold marks the calculated optimal threshold.

the study group suffers from a selection bias. However, we believe that this is exactly the population that is most likely to benefit from a molecular marker considering the poor diagnostic yield of cytology in itself.⁹ Second, validation of disease by the golden standard of histology was not performed in all patients in our cohort. This is potentially problematic, as the current guidelines are not 100% accurate in predicting malignancy. However, we have previously shown that classification of PCN was >95% accurate in our tertiary referral center. Furthermore, excluding patients without histological confirmation, while improving the sensitivity slightly, still did not result in the clear discrimination as reported by Matthaei *et al.* Third, patients with a small cyst which cannot be reached with an EUS-FNA will not benefit from a biomarker based on cyst fluid, however this holds true for most biomarker studies in pancreatic cyst fluid unless the use of secretin is considered.⁴⁶ Prospective studies to follow the natural development of these small asymptomatic lesions are needed.

MiRs can be rapidly and efficiently isolated from pancreatic cyst fluid, making them ideal biomarkers for monitoring dysplastic changes within the cyst. However, in our study, we were unable to replicate the diagnostic characteristics of a promising 9-miR model in pancreatic cyst fluid. More studies are needed to find the holy biomarker grail in pancreatic cyst fluid, whether it be DNA-based, (micro)RNA-based, or protein-based.

Conclusions

With cystic lesions ever more frequently found in the pancreas, there is a pressing need for an objective marker to aid the management of pancreatic cysts. Here, we validated a 9-miR panel that has shown great promise in the discrimination between low and high risk cysts. In our study, while observing a significant difference between the two groups, the diagnostic characteristics corresponding to various cut-off values were poor. Thus, the clinical implementation of this biomarker panel is as yet unlikely to be beneficial in the management of pancreatic cysts.

Materials and methods

Pancreatic cyst fluid acquirement. From January 2009 to October 2013 all patients analyzed for a pancreatic cyst, who subsequently underwent EUS-FNA were included. This study received approval of the Institutional Review Board (MEC-2008–233 and MEC-2012–107). A declaration of Helsinki protocols was followed and all patients provided written informed consent. The pancreatic cyst fluid was stored sterile at –80°C until analysis.

The primary objective of the study was the validation of the 9-miR panel for the prediction of a low risk or high risk cyst.

Low risk cysts consist of cystic lesions without dysplasia, low grade dysplasia and moderate grade dysplasia of which follow-up is indicated. Low risk cysts were defined as no dysplasia, low grade dysplasia or moderate dysplasia. High risk cysts were defined as cystic lesions with high grade dysplasia or invasive carcinoma, which should be resected. The risk classification of PCN occurred based on histopathology, or updated Sendai guidelines when histopathological data was unavailable.

Total MiR isolation. Total miR was isolated from pancreatic cyst fluid using the Taqman miRNA ABC Purification Kit–Human Panel A (Applied Biosystems, Glasgow, UK), according to the manufacturer's protocol. In short, lysates were prepared from pancreatic cyst fluid and subsequently transferred to tubes containing anti-miRNA Dynabeads. These are superparamagnetic beads bound to a set of 377 anti-miRNA oligonucleotides. As a positive control and measure for isolation efficiency, the samples were spiked with 1 nmol/l of nonhuman cel-miR-39 prior to isolation. After hybridization, the beads were washed and the miRs were eluted in 100 µl elution buffer from aforementioned purification kit.

cDNA synthesis. Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) was used to convert the selected miRs into cDNA according to the manufacturer's protocol. A total volume of 15 µl (11.25 µl miRNA RT mix + 3.75 µl miRNA sample) was used for the RT reaction. Samples were incubated at 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and kept at 4°C using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA).

MiR expression analysis by quantitative reverse transcriptase-PCR. The reverse transcription (RT) product from the cDNA synthesis step was used in a singleplex Taqman MicroRNA Assay reaction according to the manufacturer's protocol. In short, 18 µl of the Taqman PCR mix was mixed with 2 µl of the RT reaction solution obtained from the RT step. The PCR was run at 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 60 seconds for 40 cycles using the StepOnePlus Real-Time PCR System (Life Technologies). MiR expression levels were measured in duplicate.

Selected miRs. We measured the expression levels of miR18a, miR24, miR30a-3p, miR92a, miR99b, miR106b, miR142-3p, miR342-3p, and miR532-3p as previously reported.²²

Statistical analysis

Exclusion. Samples were excluded when the diagnosis was inconclusive, or when there were technical problems with the miR isolation procedure such as clumping of beads.

Furthermore, samples that showed cel-miR39 expression of Ct values above 30 were excluded, as these present samples in which the RNA isolation was inefficient. Of the remaining samples, individual quantitative PCR measurements of miRNAs were excluded when duplicates showed large deviations. Large deviations of duplicates were defined as duplicates that differed >1.96 times the SD of the miR measured in the whole cohort. When both Ct values were >35, deviation correction was not applied due to the imprecision in detection of such low concentrations of the miR.

Diffpairs, risk score, and logistic regression. The generation of diffpairs was performed for normalization of the pancreatic cyst fluid as described elsewhere.⁴³ The nine individual miRNAs measured were combined into seven pairs, the so called “diffpairs”, as defined by Matthei *et al.* Diffpair values were generated by subtracting normalized Ct values of the two miRs in that pair (*e.g.*, diffpair (miR18;miR92a) was generated by subtracting Ct(miR18) – Ct(miR92a)). The values obtained from these diffpairs were used to generate a risk score based on published regression coefficients, and subsequently entered in a logistic regression model to generate a score between 0 and 100%.²² Differences between the scores of low risk cysts and high risk cysts were tested by the Mann-Whitney U-test. A *P*-value of < 0.05 was considered statistically significant. The diagnostic performance of high risk cysts of this model was analyzed with a receiver operator characteristic curve. Sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were calculated using various cut-off values of the model. The optimal cut-off value was determined using a sensitivity and specificity curve, and confirmed using the freely available online tool “Cutoff Finder”.⁴⁷

Supplementary material

Figure S1. Ct values of spiked *C. elegans* miR-39. Samples that showed cel-miR39 expression of Ct values above 30 were excluded, as these present samples in which the RNA isolation was inefficient.

Figure S2. Graph depicting the predicted probability for resection.

Table S1. Table showing the p-value of a Mann-Whitney U test comparing all possible diffpair combinations between low and high risk cysts.

Table S2. Regression coefficients used in the logistic regression model, adapted from Matthei *et al.*

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