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Early detection of tumour recurrence in pancreatic cancer patients by the detection of specific microRNA and protein signatures in peripheral blood

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aus

dem Jemen

2023

Dekan: Herr Prof. Dr. Hans-Georg Kräusslich...……

Doktorvater: Herr Prof. Dr. med. Thilo Hackert ……

DEDICATION

To the soul of my father,

my mother,

my brothers and sisters.

To my wife Amira

and my lovely children Naji, Khawlah, and Abdulmajeed

I dedicate this work

Table of Contents

TABLE OF ABBREVIATIONS

ABCG2	ATP Binding Cassette Subfamily G Member 2
ACTR10	Actin Related Protein 10
ALDH1	Alkaline dehydrogenase 1
ALG2	Alpha-1,3/1,6-Mannosyltransferase
Arid1a	AT-Rich Interaction Domain 1A
ATG14	Autophagy Related 14
AUC	Area under the curve
AURKA	Aurora Kinase A
AXIN2	Axin 2
BAX	BCL2 Associated X, Apoptosis Regulator
Bcl-2	BCL2 Apoptosis Regulator
BMPR2	Bone Morphogenetic Protein Receptor Type 2
BRD3	Bromodomain Containing 3
Bsp	Integrin Binding Sialoprotein
CA19-9	Carbohydrate antigen 19-9
CALML5	Calmodulin Like 5
CBX2	Mitogen-Activated Protein Kinase 1
CCL21/CCR7	C-C Motif Chemokine Ligand 21
CCN1	Cellular Communication Network Factor 1
CD133	Cluster of differentiation 133
CD24	Cluster of differentiation 24
CD44	Cluster of differentiation 44
CD7	Cluster of differentiation 7
CDC34	Cell Division Cycle 34, Ubiqiutin Conjugating Enzyme
c-Myc	MYC Proto-Oncogene, BHLH Transcription Factor
COL4A1	Collagen Type IV Alpha 1 Chain

CRAF-ERK	Mitogen-Activated Protein Kinase 3
CSCs	Cancer stem cells
ctDNA	Circulating tumour DNA
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member 5
Dab2	DAB Adaptor Protein 2
DCLK1	Doublecortin Like Kinase 1
DDI2	DNA Damage Inducible 1 Homolog 2
DMSO	Dimethyl sulfoxide
DNAJC3	DNA J C3
DNMT1	DNA Methyltransferase 1
DYNC2H1-4	Dynein Cytoplasmic 2 Heavy Chain 1
ELISA	Enzyme Linked immunoassay
EMT	Epithelial mesenchymal transition
EMT-ATF	Vimentin
EpCAM	Epithelial Cell Adhesion Molecule
EPZ	European pancreas centre
Erk/NF-κB	Mitogen-Activated Protein Kinase 1
ESPAC	European Study Group for Pancreatic Cancer
FHL2	Four And A Half LIM Domains 2
FoxM1	Fork head Box M1
FOXP3	Fork head Box P3
FUT3	Fructosyltransferase 3 (Lewis Blood Group)
FZR1	Fizzy And Cell Division Cycle 20 Related 1
GATA6	GATA binding protein 6
GCA	Grancalcin
GFRP	GTP Cyclohydrolase I Feedback Regulator
GLI1	GLI Family Zinc Finger

GPX1	Glutathione Peroxidase 1
GPX4	Glutathione Peroxidase 4
GTPase A	Rac Family Small GTPase 1
HDAC1	Histone Deacetylase 1
HIF-α	Hypoxia Inducible Factor 1 Subunit Alpha
HM13	Histocompatibility Minor 13
HMGB1	High Mobility Group Box 1
HOTTIP	HOXA Distal Transcript Antisense RNA
Hoxa2	Homeobox A2
HOXB5	Homeobox B5
IFNG	Interferon Gamma
IFNGR1	Interferon Gamma Receptor 1
IGF-1R	Insulin Like Growth Factor 1 Receptor
IGFBP3	Insulin Like Growth Factor Binding Protein 3
IL-6	Interleukin 6
INPP5D	Inositol Polyphosphate-5-Phosphatase D
IPA	Inguinity Pathway Analysis
IPMNs	Intraductal papillary mucinous neoplasms
ITGA1	Integrin Subunit Alpha 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
Klf4	KLF Transcription Factor 4
KLF8	KLF Transcription Factor 8
KRAS	Kirsten rat sarcoma virus
L1CAM	L1 Cell Adhesion Molecule
LASSO	Least Absolute Shrinkage and Selection Operator
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
LIMMA	Linear Models for Microarray
LIN28	Lin-28 Homolog A

Linc-ROR	Long Intergenic Non-Protein Coding RNA, Regulator of Reprogramming
LIV-1	Solute Carrier Family 39 Member 6
LOXL2	Lysyl Oxidase Like 2
MALAT-1	Metastasis Associated Lung Adenocarcinoma Transcript 1
MAPK/ERK	Mitogen-Activated Protein Kinase 1
MART	Melan-A
MAZ	MYC Associated Zinc Finger Protein
MCNs	Mucinous cystic neoplasms
MEG3	Maternally Expressed 3
MMP2	Matrix Metallopeptidase 2
MMP3	Matrix Metallopeptidase 3
MSX2	Msh Homeobox 2
MTDH	Metadherin
MYC	MYC Proto-Oncogene
NANOG	Nanog Homeobox
NCCN	National Comprehensive Cancer Network
NFATc1	Nuclear Factor of Activated T Cells 1
NF-κB	Nuclear Factor Kappa B Subunit 1
Notch-1	Notch Receptor 1
NOTSCH	Notch Receptor 3
NR5A2	Nuclear Receptor Subfamily 5 Group A Member 2
NUSAP1	Nucleolar And Spindle Associated Protein 1
Oct-4	Octamer-binding transcription factor 4
OSM	Oncostatin M
OSMR	Oncostatin M Receptor
OTUB1	OTU Deubiquitinase, Ubiquitin Aldehyde Binding 1
p53	Tumour Protein P53
PanINs	Pancreatic intraepithelial neoplasms

PBMC	Peripheral blood monocyte cells
PDAC	Pancreatic ductal adenocarcinoma
PD-L1	Programmed Cell Death 1
PEAK1	Pseudopodium Enriched Atypical Kinase 1
PIGK	Phosphatidylinositol Glycan Anchor Biosynthesis Class K
Prx III	Protein Kinase C Delta
PTPRS	Protein Tyrosine Phosphatase Receptor Type S
PVT1	Pvt1 Oncogene
qPCR	Quantative polymerase chain reaction
RER1	Retention In Endoplasmic Reticulum Sorting Receptor 1
REST	RE1 Silencing Transcription Factor
RFE	Recursive feature elimination
RIFK	Riboflavin Kinase
ROC	Receiver operating characteristics
ROS	ROS Proto-Oncogene 1, Receptor Tyrosine Kinase
RSPO2	R-Spondin 2
SALL4	Spalt Like Transcription Factor 4
SATB2	SATB Homeobox 2
Sck	SHC Adaptor Protein 2
SDCBP	Syndecan Binding Protein
SERPINE1	Serpin Family E Member 1
SHC1	SHC Adaptor Protein 1
SHh	Sonic Hedgehog Signaling Molecule
SLC1A6	Solute Carrier Family 1 Member 6
Slug	Snail Family Transcriptional Repressor 2
SMAD	Sma- And Mad-Related Protein
SMARCAD1	SWI/SNF-Related, Matrix-Associated Actin-Dependent Regulator of Chr Subfamily A, Containing DEAD/H Box 1

Snai1	Snail Family Transcriptional Repressor 1
SOD1	Superoxide Dismutase 1
SOD2	Superoxide Dismutase 2
SOX2	SRY-Box Transcription Factor 2
SOX9	SRY-Box Transcription Factor 9
ST6Gal1	ST6 Beta-Galactoside Alpha-2,6-Sialyltransferase 1
STAT1	Signal Transducer and Activator of Transcription 1
STAT3	Signal Transducer and Activator of Transcription 3
SVM	Support vector machine
TBS	Tris-buffered
TGF-b/Smad	Transforming Growth Factor Beta 1
TGF-β1	Transforming Growth Factor Beta 1
TIM-3	Hepatitis A Virus Cellular Receptor 2
TNC	Tenascin C
TNFAIP1	TNF Alpha Induced Protein 1
TUFT1	Tuftelin 1
Twist	Twist Family BHLH Transcription Factor 1
URG11	Von Willebrand Factor C And EGF Domains
UV	Ultraviolet
VASH2	Vasohibin 2
VCAM1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor A
VPS4B	Vacuolar Protein Sorting 4 Homolog B
WNK1	WNK Lysine Deficient Protein Kinase 1
WNT	Wnt Family Member 1
WWC2	WW And C2 Domain Containing 2
XBP1	X-Box Binding Protein 1
XIAP	X-Linked Inhibitor of Apoptosis

XMRVP12	Xenotropic murine leukaemia virus-related virus P12
YIPF6	Yip1 Domain Family Member 6
Zeb1	Zinc Finger E-Box Binding Homeobox 1
ZNF32	Zinc Finger Protein 32
ZO-1	Tight Junction Protein 1
α-SMA	Alpha smooth muscle actin

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The dissertation aimed at the establishment of blood-based microRNA and protein signatures for prediction and early detection of tumour recurrence in patients who had undergone resection of pancreatic ductal adenocarcinoma (PDAC). Utilising a microarray of 2,977 antibodies, variations were detected in the protein content of serum samples collected from 101 patients, who had experienced tumour recurrence or not, including consecutively collected samples from the same patients. Secretome analyses of non-tumours and cancer cells indicated tumour-related variations. Selected biomarkers were utilised to train support vector machine classifiers. They were validated on new, prospectively collected samples from 36 patients in order to document applicability. By combination of biomarkers selected by both a focussed tumour-centred approach and a broader systemic analysis, a classifier of 10 proteins was defined that discriminated patients with recurrence from those without at 91% accuracy. Validation on prospectively collected samples achieved an accuracy of 85%. Recurrence detection was on average 3.5 months earlier than that with current processes. Besides diagnosis, protein signatures were established that allow predicting the period, after which tumour recurrence is likely to occur.

I studied the microRNA (miRNA) content of 149 serum samples by means of small RNA sequencing. For the discovery phase, 75 serum samples were analysed. Libraries were prepared and sequenced at the sequencing core facility of DKFZ. Data was obtained in Fastq files and processed using the Heidelberg Unix System Analysis Resource (HUSAR). In total, I analysed 135 miRNA variations between recurrence and nonrecurrence samples using logistic regression and selected informative miRNA biomarkers after removal of unnecessary covariates by Least Absolute Shrinkage Selection Operator (LASSO) regression. To find the best possible miRNA combination, I used Recursive Feature Elimination (RFE) with 5-fold cross validation. A miRNA classifier made of hsa-mir-100, hsa-mir-215, hsa-mir-3916, hsa-mir-484, hsa-mir-6752, hsa-mir-6773, hsa-mir-6883-5P was constructed and trained. The algorithm parameters were optimized to avoid over- or underfitting. The signature was validated in an independent cohort with all parameters being fixed. The miRNA classifier could discriminate between recurrence from nonrecurrence at an accuracy of 97% and 91% in the discovery and validation cohort, respectively. Furthermore, I established four miRNAs classifiers that accurately predicted the time when recurrence is likely to happen.

Combining both miRNAs and proteins was done using the samples that were tested simultaneously by antibody microarray and small RNA sequencing. The data was randomly divided into a training and validation cohorts and RFE with 5-fold cross validation was applied to the 17 miRNAs and protein markers. Using a marker signature of two miRNAs and two proteins, I was able to detect pancreatic cancer recurrence at 91% accuracy, which was slightly reduced to 83% upon validation.

Analysis of the protein and miRNA contents of blood permits prediction and detection of tumour recurrence in PDAC patients after curative surgery with an accuracy that substantially surpasses the performance of currently used processes, in particular CA19-9 testing. The analysis also indicated the existence of changes that are either directly due to the tumour's presence or based on the body's systemic reaction to it. Combining both miRNA and proteins reduced the number of molecules required to achieve an accurate and robust diagnosis. The results could have a direct and immediate benefit for patients with pancreatic cancer and could be translated to clinical practice quickly. In addition, the process could proof the applicability of the signatures for early diagnosis of the primary tumour. Thus, the results could be applied to screening individuals who are at high risk of pancreatic cancer, potentially having a clinical impact beyond the detection of tumour relapse. In addition, the established assays could serve as a means for monitoring disease progression during chemotherapeutic treatment.

1. INTRODUCTION

Pancreatic tumours are two types: Endocrine and exocrine. Endocrine tumours start in hormone-producing cells and are quite rare whereas exocrine lesions originate from enzyme-producing glands or pancreatic ducts. A relatively scarce exocrine tumour is acinar carcinoma. A likely uncommon are malignant pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). The three are non-malignant lesion that become PDAC if they undergo a transformation process. Pancreatic ductal adenocarcinoma (PDAC) is the most common exocrine neoplasm accounting for 90–95% of all pancreatic cancer cases (Hidalgo et al., 2015), from which 10% of them have a familial background. Due to its aggressiveness and frequency, PDAC is a primary reason for pancreatic cancer dismal prognosis.

1.1. Pancreatic cancer epidemiology

The seventh leading cause of mortality in developed countries is pancreatic cancer and the third most common one in the Western world (Bray et al., 2018). Globally, pancreatic cancer is the 11th common cancer entity accounting for 458,918 new cases; 432,242 of them die from the disease (Bray et al., 2018). Irrespective of the advancement in the awareness of the disease risk factors and new detection and treatment strategies, pancreatic cancer incidence is expected to increase by 355,317 new cases in 2040 (Rawla et al., 2019). In Germany, roughly 19,685 people were diagnosed with pancreatic cancer and almost the same number of people died from this disease in 2019(“Krebs - Cancer in Germany,” n.d.). The disease is multifactorial and has a complex molecular and cellular biology. However, chronic pancreatitis, smoking and familial history are dominant risk factors (Cid-Arregui, 2015).

The survival rate of pancreatic ductal adenocarcinoma (PDAC) is 8%, which is the lowest among all malignancies worldwide, making PDAC a devastating disease with a projection that in 2030 pancreatic cancer will be the second most common cause of cancer related mortality (Rahib et al., 2014). This dismal prognosis is due to ineffective screening procedures, late diagnosis, high metastatic rate and inefficient systemic therapy (Neoptolemos et al., 2017; Wolfgang et al., 2013). For up to 20% of the patients, in whom the tumour is identified early, there is a better chance of cure through radical surgery

(Aier et al., 2019). Unfortunately, however, 80% of the patients have recurrent tumour within the next two years after surgery (Groot et al., 2019a). For the lack of sensitive diagnostic methods, the recurrent tumour is also frequently diagnosed late. However, if the recurrent tumour is found early, surgical resection can be repeated with high chances of a good outcome (Strobel et al., 2013).

1.2. Classification of recurrence

Histopathological and molecular analyses can determine the metachronous lesion type in the pancreatic residues. An amiable agreement in both histological and molecular patterns between the primary and the relapse lesion is defined as a "true" recurrence of PDAC primary tumour. In contrast, the lesion is defined as "second primary" when it presented distinct morphological and molecular features. The third type of recurrent tumour is classified as "undetermined" since it has not met any of the above criteria (Luchini et al., 2019).

1.3. PDAC recurrence patterns and timing

Retrospective studies estimated that PDAC recurrence patterns after resection include both local and distant metastases (Groot et al., 2018; Hishinuma et al., 2006). These findings have been confirmed with data collected prospectively in the first trail conducted by European Study Group for Pancreatic Cancer (ESPAC) (Neoptolemos et al., 2004). In a large retrospective analysis that included 1103 patients at John Hopkins Medical school, 531 patients had recurrence (76.7%). Out of them local recurrence was in 126 (23.7%) patients and metastases were detected in 307 (57.8%) patients. About 98 (19%), however, experienced local recurrence and distant metastases (Groot et al., 2018). This study and other retrospective series are limited by a significant amount of missing data and other biases (Groot et al., 2018; Hishinuma et al., 2006; Johnstone and Sindelar, 1993; Suenaga et al., 2014). These limitations were minimized in a large prospective multicentre study conducted by ESPAC (Neoptolemos et al., 2004). The study involved 730 patients, from whom 479 patients experienced recurrence. Local recurrence occurred earlier than distant recurrence, but the survival remains the same. Key message from this clinical trial is that there is no correlation between the overall survival time and recurrence

pattern since pancreatic cancer is a systemic condition that requires efficient systemic therapy (Jones et al., 2019).

To improve pancreatic cancer management after surgery, robust molecular diagnostics to detect tumour recurrence early and personalized therapeutics to overcome chemotherapy resistance and unfavourable side effects are required. A key step beforehand is understanding the disease biological characteristics. Our understanding about pancreatic cancer recurrence molecular drivers indicates that pancreatic cancer recurrence is initiated by genomic alterations and cancer stem cells. The recurrence progression is also driven by epithelial mesenchymal transition (EMT), treatment failure, immunological suppressive tumour microenvironment, metastasis, and tumour dormancy **(Fig. 1)**.

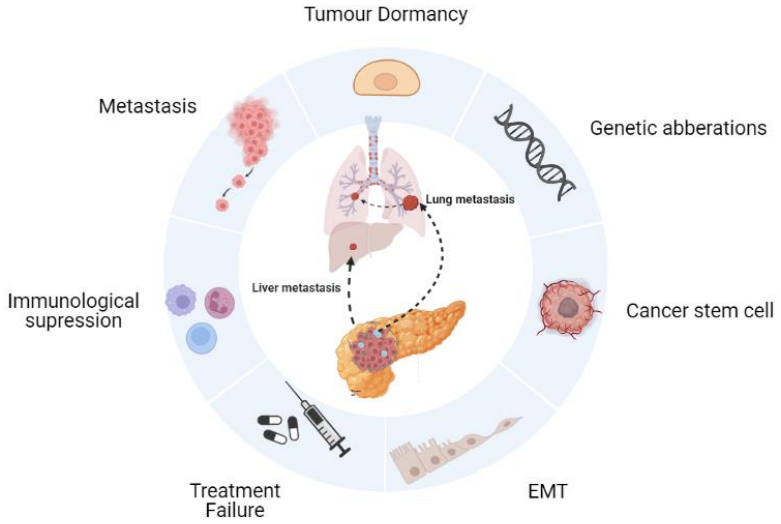

Figure 1. **Mechanisms involved in pancreatic cancer recurrence.** Therapy induced genetic rearrangements and cancer stem cell (CSCs) are important hits for tumour relapse. In addition, CSCs develop EMT and treatment resistance. The immunological suppressive nature of pancreatic cancer, metastasis and dormancy are also important characteristics for recurrent tumours at distant sites.

1.4. Genomic and clonal landscape

In comparison to treatment-naïve, recurrent tumour displayed different genomic features with huge heterogeneity, especially at metastatic sites, variable clonality, and higher mutational load (Sakamoto et al., 2020). By combining targeted and whole-exome sequencing with phylogenetic analysis of resected primary tumours and relevant recurrences, Sakamoto and colleagues suggested two methods by which tumours relapse: A monophyletic and polyphyletic recurrences. In the monophyletic recurrence, a single sub-clone is the source of recurrence; whereas multiple sub-clones produce multiple

recurrences and this is called polyphyletic recurrence **(Fig. 2).** Additional complex genomic alterations (e.g. *KRAS* allele gain and allelic imbalance) lead to metastases compared with the resected primary tumours. In addition, changes to the distribution of chromatin regulators and transcription (e.g. GATA6 and MYC) advance PDAC evolution (Bednar and Pasca di Magliano, 2020; Hayashi et al., 2020; Mueller et al., 2018; Cancer Genome Atlas Research Network. Electronic address: andrew_aguirre@dfci.harvard.edu and Cancer Genome Atlas Research Network, 2017).

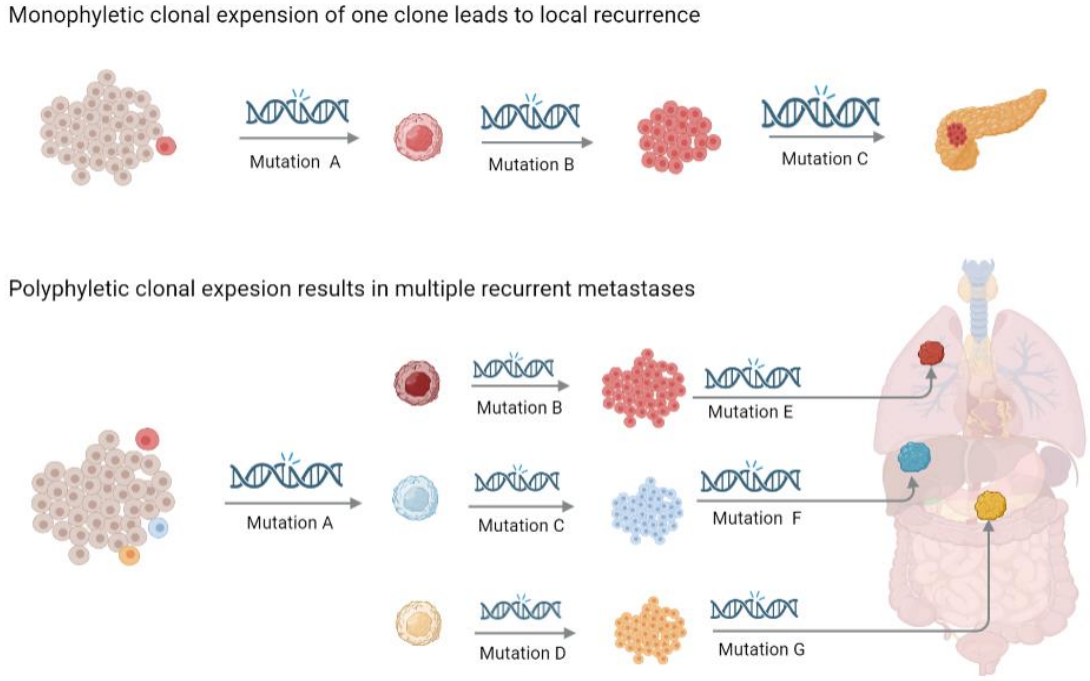

Figure 2. Modes of pancreatic cancer recurrence. Mutations induced by chemotherapy in various subclones initiates new oncogenic drivers. The recurrent tumour genetic heterogeneity enables metastatic potentials.

Progressive loss of the acinar cell compartment and cystic lesions progression is correlated with mutation in *Arid1a* (Wang et al., 2019). In addition, the majority of specimens from patients with distant recurrence are SMAD-deficient (Yamada et al., 2015). Polyclonal development within the tumour potentiates the ability to metastasize. Therefore, curative resection timing determines the mode of recurrence (**Fig. 2**) (Bednar and Pasca di Magliano, 2020). Phylogenetic analysis suggested that squamous differentiation arises from classic glandular tumours, but metastases stems from clones of both subtype, since the metastatic phenotype acquisition is associated with mutant p53-driven secretome stimulation (Butera et al., 2020; Hayashi et al., 2020).

1.5. Cancer stem cells

The main drivers of pancreatic cancer recurrence are cancer stem cells (CSCs) (Li et al., 2007). CSC essential genes are *ALDH1*, *CD44*, *CD133*, *NANOG*, and *SOX2* (Fu et al., 2017b). Transcription factors such as Zeb1, Snail (Zhou et al., 2014) and SOX2 (Herreros-Villanueva et al., 2013) are crucial to maintain and impart stem cell phenotype in pancreatic cancer. An important event for robust transcriptional activation of genes implicated in CSCs stemness is the loss of p53, which induces Zeb1, Twist, and Snai1 in p53-depleted cancer cells (Singh et al., 2015).

In hypoxia, CSCs display resistance to hypoxic exposure by inducing the expression levels of SOD1, SOD2, GPX1 and GPX4, regulators of oxidative homeostasis and effectors of cell viability (Peng et al., 2019). Oxidative phosphorylation promotes the stemness and immunoreactive properties of pancreatic cancer stem cells via increasing mitochondrial function and upregulating stemness related genes (e.g., *SOX9*) or pluripotency (e.g., *NANOG*) (Valle et al., 2020). Stemness in human pancreatic cancer is also stimulated by a STAT3-dependent mechanism (Panni et al., 2014) and LIV-1, which upregulates cancer stem cell molecules e.g. LIN28 and ATP-binding cassette sub-family G member 2 (Unno et al., 2014), EMT-ATF (Kaşıkcı et al., 2020), Linc-ROR (Fu et al., 2017b), HOXB5 (Li et al., 2020), NR5A2 (Luo et al., 2017), HOTTIP (Fu et al., 2017a), and NFATc1 (Singh et al., 2015) **(Fig. 3).** In contrast, miR-200a overexpression in CSCs resulted in the up-regulation of mRNA level of *E-cadherin* but down-regulates *ZEB1*, *N-cadherin* and *vimentin*. Loss of miR-200a is crucial for EMT phenotypes (Lu et al., 2014).

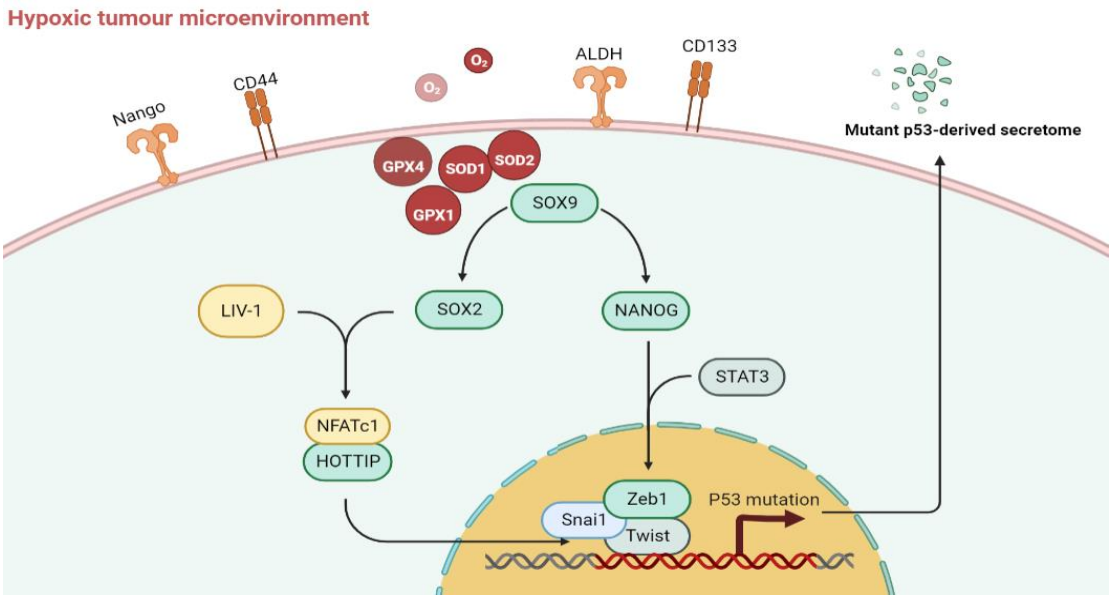

Figure 3. Pancreatic cancer stem cell stimulation mechanism. The desmoplastic tumour microenvironment of pancreatic cancer induces transcriptional activation leading stem cells activation. Activated CSC promotes chemotherapy resistance and epithelial mesenchymal transition.

1.6. Epithelial mesenchymal transition

In the process of epithelial-mesenchymal transition (EMT), the basal polarity as well as their tight cell-to-cell junctions in pancreatic epithelial cells are lost. Mesenchymal cells acquire fibroblast like phenotype instead. The mesenchymal markers (e.g. E-cadherin) are predominant instead of epithelial markers (e.g. vimentin) (Thiery, 2002). Activated stem cells (e.g., CD133+) induce EMT predominantly in pancreatic cancer. Critical transcription factor for CSC stimulation is Zinc Finger E-Box Binding Homeobox (ZEB1) **(Fig.4)**. ZEB1 promotes EMT via activation of stemness transcription factors Snail, Sox2, Bmi1 and p63 and inhibition of stemness inhibiting miRNAs (Wellner et al., 2010). ZEB1 is also activated by NF-κB (Nomura et al., 2015), STAT3 (Panni et al., 2014), SHh (Tang et al., 2012), WNT (Ilmer et al., 2015), NOTSCH, miRNA200 (Brabletz et al., 2011), DCAMKL-1 (Sureban et al., 2011), CD44 (Preca et al., 2015), OSM (Smigiel et al., 2017) and VASH2 (Zhang et al., 2018) **(Fig.4)**.

The EMT phenotype of pancreatic stem cells is also maintained in hypoxia, which induces the upregulation of glutathione peroxidase (GPX4) and N-cadherin in pancreatic stem cell (Peng et al., 2019). In addition, HIF-α in $CD133^{+}$ promotes the upregulation of Slug (Maeda et al., 2016), VEGF, IL-6 (Bao et al., 2012), and RER1. Overexpression of RER1 upregulates N-cadherin, vimentin, and snail

in one hand, and downregulates E-cadherin and claudin-1 on the other hand. Markers relevant to stemness and EMT phenotypes e.g. Sox2, Bmi1, Lin28 and Nanog were suppressed in RER1 knockdown cells (Chen et al., 2019).

Intermittent hypoxia increases HIF-1α-induced autophagy, which endorses human pancreatic cancer cells migration and invasion (Zhu et al., 2014). HIF-1α upregulates β-catenin via Wnt3a, which increases vimentin expression and reduces expression of E-cadherin (Zhang et al., 2017). Another paracrine osteopontin/integrin αvβ3 signalling mechanism promotes EMT by modulating fork-head box protein M (Cao et al., 2019). Cyr61/CCN1 signalling is crucial for epithelial-mesenchymal transition as it cooperates with mesenchymal/stem cell molecules (i.e., Vimentin, Notch-1, Oct-4, ABCG2 and CD44) (Haque et al., 2011). Downstream from CCN1 is MAZ transcription factor, a stimulator of pancreatic cancer cell invasion via CRAF-ERK signalling (Maity et al., 2018).

Canonical Wnt signalling is heterogeneously activated in PDAC. However, stemness-identical genes (e.g., *SNAI1*, *NANOG*, and *ZEB1*) and Wnt targets/enhancers (*AXIN2*, *LGR5*, *RSPO2*) are upregulated in pancreatic cancer cells. Significantly, higher expression of Wnt enhancer gene *RSPO2* in PDAC tumours is correlated with *ZEB1* overexpression (Ilmer et al., 2015). *MTDH* is overexpressed in metastatic PDAC cells. *MTDH* aides EMT by abolishing *Twist1* in metastatic PDAC cells since the expression of *Twist1* is significantly increased at EMT initiation only (Suzuki et al., 2017).

CCL21/CCR7 enhances CD133+ pancreatic cancer stem cell metastasis and migration potential to lymph node or distant sites via EMT and Erk/NF-κB Pathway (Zhang et al., 2016). Nestin promotes tumour cell migration and induces EMT in PDAC. Nestin expression is induced by TGF-β1–Smad4 pathway. TGF-b/Smad pathway induces Nestin expression, downregulating E-cadherin, and increasing protein expression of vimentin, α-SMA, and N-cadherin (Su et al., 2013). Dab2 loss of expression likely to happeen early in pancreatic cancer progression, as Dab2 mRNA levels are higher in normal pancreatic tissue samples in comparison to pancreatic cancer stages III or metastatic tumour samples. Dab2 low expression affects EMT and CSC markers and stimulates TGF-β, Bmi-1 and Sox2 (Hocevar, 2019). Tumour-derived exosomes promote invasion of PDAC cells via enhancement of EMT and stem cell properties. Overexpression of Sox2ot induces mesenchymal-like morphological features in cancer cells and stemness properties via Sox2 activation (Li et al., 2018). Pancreatic cancer invasion ability and

expression of EMT-related genes is correlated with linc00675 as low levels of linc00675 decreased expression of mesenchymal markers N-cadherin and vimentin, and upregulated epithelial marker E-cadherin (Li et al., 2015).

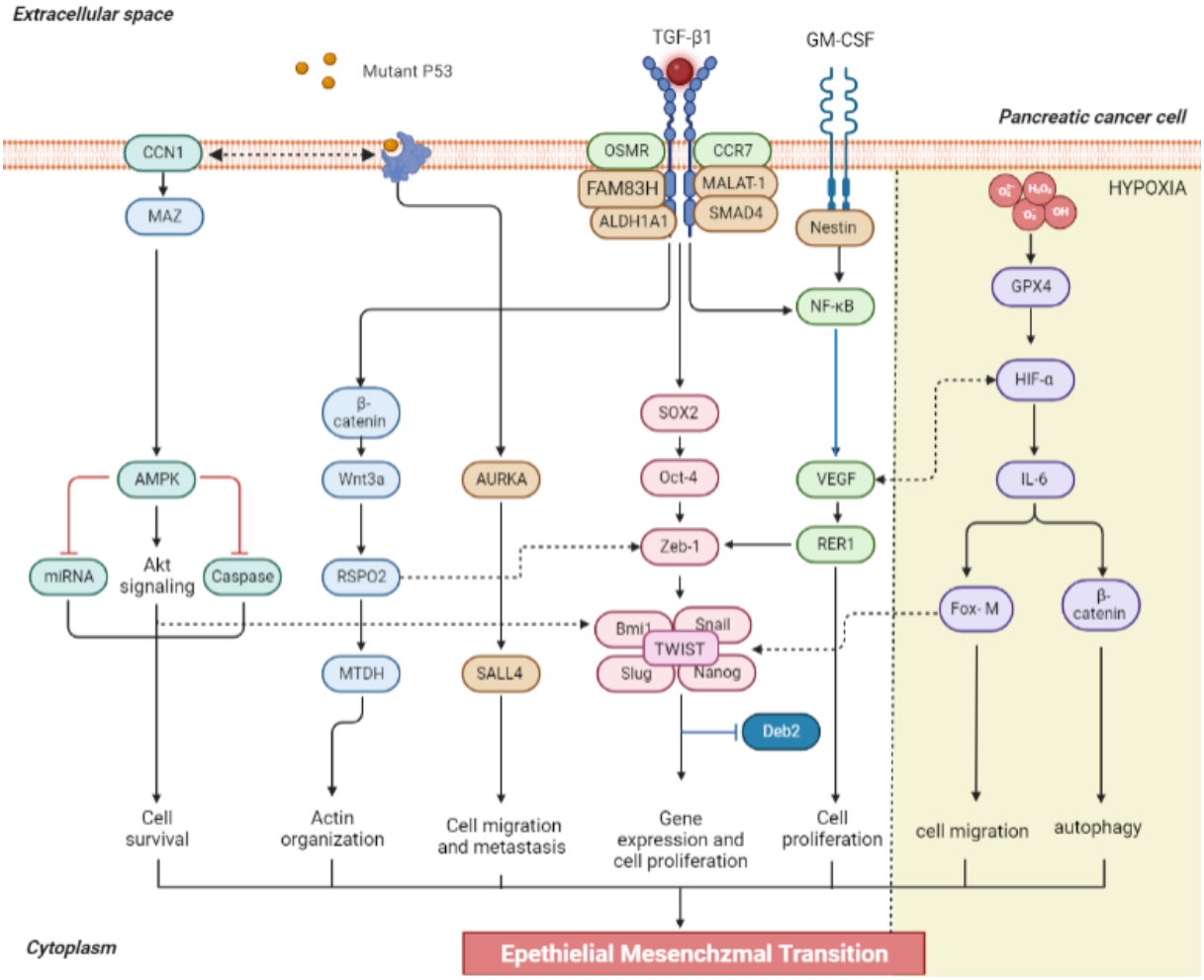

Figure 4. Epithelial-mesenchymal transition molecular drivers. ZEB1 drives EMT via activation of TWIST, Slug, Snail and Bmi1 and inhibition of miRNAs inhibitory molecules.

GM-CSF is necessary for transendothelial migration and invasion (Waghray et al., 2016). An RNA-Binding Protein, Hu-antigen R, enhances pancreatic cancer cell EMT, migration, and CSCs by upregulation of Snail (Dong et al., 2020). Aberrant co-overexpression of KLF8 and FHL2 was associated with tumour metastasis in pancreatic cancer. KLF8 upregulates FHL2 levels to induce EMT and promote cell invasion in pancreatic cancer (Yi et al., 2017). TUFT1 is involved in lymph node metastasis of advanced pancreatic cancer patients and overexpressed in tumour tissues compared with samples from adjacent normal ones. TUFT1 expression alters the expression of HIF1-Snail signalling in PDAC (Zhou et al., 2016).

MiRNAs are also involved in EMT in pancreatic cancer. For instance, miR-10a promotes EMT via upregulating Hippo signalling pathway and inhibiting WWC2. A notably decreased in the protein levels of N- cadherin, Vimentin, CD44, CD24, EpCAM, Nanog, OCT, and SOX- 2 alongside with an elevated E-cadherin level is observed upon miR-10a inhibition (C. Wang et al., 2020). In addition, MiR-100 and miR-125b levels proportionally increased with the cell mesenchymal status and antagonizes the CDH1expression. TGF-β treatment induces miR-100 and miR-125 through SMAD2/3 (Ottaviani et al., 2018). ANX2 and TNC high expression is associated with peritoneal recurrence and poor prognosis in PDAC. Mechanistically, ANX2-TNC functional role is to maintain mesenchymal phenotype and fosters neoplasia (Yoneura et al., 2018).

Twist1 is a direct substrate of AURKA. AURKA associates with Twist1 and regulate subcellular localization and levels of N-cadherin, CD44, Slug and Snail levels (Wang et al., 2017). Secretome of mutant p53 cancer cell sparks migration and epithelial-to-mesenchymal transition (Butera et al., 2020). EMT genes expression patterns were altered once MEG3 was knocked down in PANC-1 cell e.g. *cadherin, Vimentin* and *Snail* (Ma et al., 2018). SALL4 is also involved in EMT genes upregulation and therefore enhancement of cancer metastasis. A positive correlation with vimentin, N-cadherin concordant with a negative correlation with E-cadherin and ZO-1 expression have been observed in URG11 (Peng et al., 2014).

1.7. Cancer stem cell and therapy resistance

About 80% of PDAC patients are diagnosed very late and therefore chemotherapy/ radiotherapy is the only treatment option left for them. However, resistance is a major challenge for all types of therapy either adjuvant, after intended curative surgery or the one intended for advanced disease (Van den Broeck et al., 2009). Resistance in pancreatic cancer is due to tumour microenvironment low permeability or efficient efflux mechanism of chemotherapeutic agents (Olive et al., 2009; Olson and Hanahan, 2009). In pancreatic cancer, cells displaying properties of cancer stem cells (CSC) known as side population (Haraguchi et al., 2006). They represent the tumour's subpopulation responsible for therapy resistance and disease recurrence (Clarke et al., 2006). The side population (SP) resistant cells are CD45+ and CD31+ cells and resistant to Gemcitabine. Three significantly upregulated pathways in

the SP involved in chemoresistance conceded by KEGG pathway analysis: stem cell factor; regulation of apoptosis; and epithelial-mesenchymal transition (Van den broeck et al., 2012). Upregulation of stem cell markers (CD24, CD44, CD144 or EpCAM) and associated genes (*PDX1, SHH, CBX7* or *OCT4*) as well as EMT regulator group (SNAI1/Snail, SNAI2/Slug, TWIST) was observed (Quint et al., 2012). Gemcitabine treatment induces the expression of ITGA1, which promotes therapy resistance and metastasis **(Fig.5)**. ITGA1 is required for PDAC cell survival and for collagen induced PDAC cell attachment, which is induced by PEAK1, BMPR2, COL4A1 and ZEB1 as well as ITGA1. ITGA1 also may regulate TGF-β responses in pancreatic cancer, that upregulates ZEB. Overall, the absence of ITGA1 decreases gemcitabine potency by nearly 10-fold (Gharibi et al., 2017).

Epithelial-mesenchymal transition changes induced by gemcitabine are preserved phenotypes in pancreatic cancer (El Amrani et al., 2019). ATP-binding cassette subfamily G member 2 expression is high in pancreatic cancer and a correlation between ALDH1, CD44v9, OCT4, SOX2, and NESTIN was found in $ABCG2^+$ cells but not in $ABCG2^-$ cells (Sasaki et al., 2018). *MSX2,* for instance, is a chemosensitivity determinant factor regulating the function of *ABCG2* gene. *MSX2* controls ABCG2 via SP1 recruitment toward its SP1-binding elements within the *ABCG2* promoter (Hamada et al., 2012). The epithelial-mesenchymal transition phenotype acquisition of gemcitabine-resistant pancreatic cancer cells is combined with notch-signalling pathway activation. Downstream target, NF-KB, and its cascades commit to EMT (Wang et al., 2009). Besides, ZNF32 overexpression reversed inhibitory effect of gemcitabine-resistant pancreatic cancer cells on the malignant progression of persuaded by microRNA-136-5 (Xu et al., 2020). SATB2 is another molecule that is highly expressed in CSCs. SATB2 exerts its stimulatory effect by binding to Bcl-2, Bsp, Nanog, c-Myc, XIAP, Klf4 and Hoxa2 in CSCs.

SATB2 overexpression is in association with Zeb1 and N-cadherin, and inhibition of E-cadherin (Yu et al., 2016). Gemcitabine resistance of pancreatic cancer is enhanced by PVT1, which activates a Wnt/β-catenin and autophagy pathways via miR-619-5p/Pygo2 and miR-619-5p/ATG14 axe **(Fig.5)** inflecting (Zhou et al., 2020). Glycolysis governs DCLK1 expression via downregulating ROS production in GR cells with CSC and EMT features (Zhao et al., 2017). Pancreatic Stellate Cells(PSC) embellished CSC phenotype and radio-resistance of pancreatic cancer cells by promoting relevant

markers (Al-Assar et al., 2014). Therapy-associated cell death activates HMGB1-TLR2 signalling via CD133 and EMT-related genes e.g. more than eight times increase in *Snail* and *Slug* mRNA expression levels were observed (Chen et al., 2018). MiR-210 resolves BxS resistance GEM, by means of exosomes derived from BxR-CSCs (Yang et al., 2020). Pancreatic cancer cells that are resistant to gemcitabine exhibit elevated EMT and CSC characteristics. In pancreatic cancer cell lines with intrinsic gemcitabine resistance, high expression of ZEB1 and vimentin was confirmed, but poor expression of E-cadherin was found. Targets of MiR-145 Linc-DYNC2H1-4 induces upregulation of MMP3, Oct4, Lin28, Nanog, Sox2, and ZEB1 in pancreatic cancer cells (Gao et al., 2017). A phenotype resembling EMT is associated with decreased GPx1 expression in PDAC cells. Abrogation of GPx1 expression increases pancreatic cancer cell resistance to GEM via increased Snail, ZEB 1, and Vimentin level (Meng et al., 2018). Irradiation-induced cell death promotes CD133− dedifferentiation into CSCs. In CD133-cancer cells, HMGB1+ significantly increased the expression of the stem cell markers Oct4, Sox2, and Nanog, as well as their capacity to form spheres(Zhang et al., 2019).

Gemcitabine resistance in pancreatic cancer cells is induced by MiR-365, which baits directly Src Homology 2 Domain Containing 1 (SHC1) and BAX. MiR-365/SHC1/BAX axis has an effect on pancreatic cells survival since the knockdown of SHC1 and BAX increases gemcitabine resistance. In addition, miR-365 upregulates cancer-promoting molecules such as DNA-binding inhibitor 2 and S100P, suggesting the existence of interactions with other cancer-promoting signals. Moreover, miR-365 promotes the expression of molecules that are linked to cancer, including DNA-binding inhibitor 2 and S100P, indicating that it may interact with other cancer-promoting signals (Hamada et al., 2014). Both stromal and tumour cells express CD90, and a high level of CD90 expression is associated with a poor prognosis in PDAC patients. Drug resistance decreased when EMT-ATF was silenced. Drug resistance and survival gene expressions reduced in tandem with the decrease in Snail, Slug, and Twist expressions (Survivin, Muc1, and ABCG2)(Kaşıkcı et al., 2020). In the 5-FU-resistant cells, L1CAM expression is increased. The chemoresistant cells are moderately protected from the 5-FU-induced apoptosis by L1CAM, which is also implicated in the proliferation and invasiveness of the 5-FU resistant clones.(Lund et al., 2015).

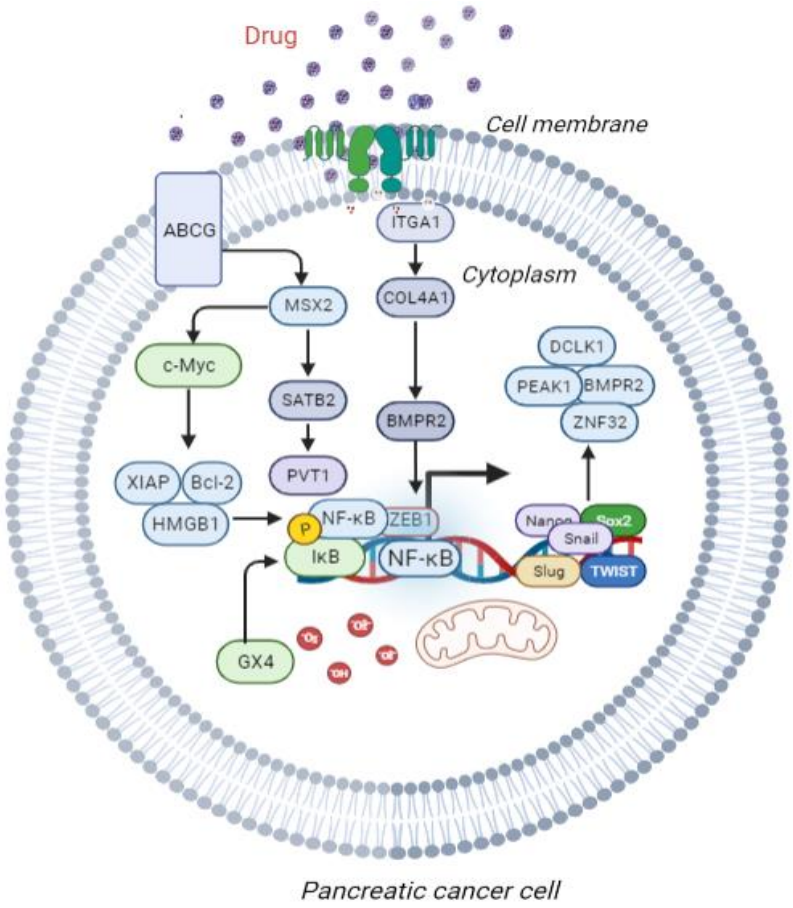

Figure 5. Chemotherapy resistance molecular mechanism in pancreatic cancer stem cells. The drug (e.g., gemcitabine) induced ITGA1, which activation pancreatic stem cell transcriptional machinery.

1.8. Metastasis

A discrete fraction of CD133(+) CXCR4(+) cancer stem cells was found in invasive pancreatic tumours, and these cells control the metastatic phenotype of the particular tumour. The metastatic phenotype of pancreatic tumours was virtually eliminated when the cancer stem cell pool for these migratory cancer stem cells was depleted, but their tumorigenic potential was unaffected. CD133 (+) CXCR4(+) cancer stem cells that migrate are necessary for tumour metastasis (Hermann et al., 2007). LOXL2-positive tumours displayed a considerably greater rate of distant recurrence patterns. Also, there is proof that reactive oxygen species (ROS), which is a hallmark for successful distant metastasis, affect metastasis and self-antioxidant capacity. Researchers have discovered that the Spalt-like protein 4 (SALL4) regulates mitochondrial ROS via the FoxM1/Prx III axis at the molecular level. It's probable that SALL4 primarily promotes ROS and the endothelial-mesenchymal transition (EMT) phenotype in PDAC cells to increase metastatic effectiveness. (Huynh et al., 2018). In pancreatic cancer, the EMT-

activator Zeb1 plays a critical role in cell plasticity and metastasis. Reduced grading, invasion, and distant metastases in PDAC are caused by Zeb1 deletion (Krebs et al., 2017). Besides, LOXL2 overexpression enhanced the migration potential via upregulating Snail expression and phospho-FAK/phospho-SRC and decreasing the expression of CDH1 (Park et al., 2016). Overexpression of SERPINB5 correlated with increased metastasis scores. In contrast, overexpression of AKAP12 was correlated with lower metastasis and invasion scores (Mardin et al., 2010). The expression of Hh molecules in cancer tissues and non-tumour tissues differed noticeably. The PANC-1 cell line, which exhibits poor differentiation and invasiveness, had high GLI1 expression. (Hao et al., 2013). SMARCAD1 enhances migration and invasion in PANC-1 cells. SMARCAD1 overexpression considerably aided PANC-1 cells' capacity to create wounds and migrate (Liu et al., 2019). REST was knocked down, which inhibited the ability of PANC-1 and AsPC-1 cells to proliferate, migrate, and invade, as demonstrated *in vitro* studies (Jin et al., 2019).

Epithelial-mesenchymal transition (EMT), loss of E-cadherin expression, and up-regulation of mesenchymal genes like Snail were brought about by the *in vivo* selection of highly metastatic cancer cells. EMT and metastasis were induced *in vivo* when E-cadherin was genetically inactivated in parental cells. Snail, histone deacetylase 1 (*HDAC1*), and histone deacetylase 2 (*HDAC2*) are all components of a transcriptional repressor complex that controls the expression of E-cadherin in highly metastatic cells. In line, mesenchymal pancreatic cancer tissues and primary cell lines from genetically altered (*KRAS* G12D) mice revealed HDAC-dependent downregulation of E-cadherin and strong metastatic potential. Ultimately, *HDAC* activity is required for EMT and *E-cadherin* silencing by transforming growth factor beta in human pancreatic cancer cells. (von Burstin et al., 2009). As demonstrated in CP and PDAC, low miR-192 expression may help to either start or maintain an EMT phenotype and hence promote metastasis, which reduces patient survival periods (Botla et al., 2016).

Slower growth rates, higher sensitivity to apoptosis-inducing drugs, and reduced motility and invasiveness were all characteristics of the low RAS activity of *KRAS* gene. Clones with lower RAS activity also had a lower propensity to form tumours in mouse xenograft models and had higher survival rates in mouse orthotopic pancreatic cancer models, according to *in vivo* tests (Padavano et al., 2015).

There is a downregulation of MiR-4282 in pancreatic cancer samples. Low levels of miR-4282 indicate a poor prognosis for pancreatic cancer patients as well as a high incidence of lymphatic and

distant metastases. MiR-4282 overexpression significantly reduced the capacity of PANC-1 and BxPC-3 cells to migrate. Via certain binding sites, ABCB5 specifically targeted MiR-4282. In pancreatic cancer tissues, ABCB5 level is inversely associated to that of miR-4282. The inhibitory effects of miR-4282 overexpression on the growth of pancreatic cancer might be eliminated by overexpressing ABCB5. (Li and Hou, 2020). For invasion and metastasis caused by fructose substitution, ST6Gal1 is necessary (Hsieh et al., 2017).

HMGB1 is released from dying cells both *in vitro* and *in vivo* following radiation. The EMT program and TLR2 PI3K/Akt pathway, which are involved in HMGB1-mediated cell invasion, govern tumor cell motility (Chen et al., 2018). The human PDAC-CSCs' *in vivo* tumour growth and metastasis is significantly influenced by the CD95/CD95L system. Via Sck, CD95 activates the PI3K and MAPK/ERK pathways (Teodorczyk et al., 2015). Nodal expression levels were inversely associated to the degree of pancreatic cancer differentiation, with weaker nodal expression observed in pancreatic cancer tissues as opposed to those with high levels of differentiation. As nodal promotes pancreatic cancer cell motility and invasion, nodal signalling in pancreatic cancer cells stimulates the Smad2/3 pathway. Nodal signalling promotes MMP2 and CXCR4 expression and causes EMT. (Duan et al., 2015). Pancreatic ductal adenocarcinoma proliferation, migration and invasion are promoted by PCAT6 via regulating miR-185-5p/CBX2 axis (W. Wang et al., 2020). Compared to nearby normal tissue samples, pancreatic cancer tissues displayed a considerably increased expression of the lncRNA LINP1. Patients with highly expressed lncRNA LINP1 demonstrated a higher frequency of distant metastases, but a poorer overall survival rate. LINP1 knockdown significantly reduced the capacity of pancreatic cancer cells to proliferate, invade, and migrate. (Chen et al., 2020). OCT4 promotes progression of pancreatic cancer. By inhibiting OCT4, MiR-335 prevents pancreatic cancer cells from metastasizing. (Gao et al., 2014). In pancreatic cancer cell lines and tumour tissue samples, MALAT-1 expression is increased.MALAT-1 suppresses cell migration and invasion. MALAT-1 facilitates tumour progression by inducing EMT. Cancer stem-like cell markers are expressed less frequently when *MALAT-1* is knocked down (Jiao et al., 2014). Snail, Slug, and Twist-1 inhibition decreases PC cells' capacity for cellular migration. PC cells become less invasive and less able to adhere to laminin when *Slug***,** *Snail*, and *Twist* are silenced (Kaşıkcı et al., 2020)**.** Pancreatic cancer cell migration, invasion, and EMT are controlled by Linc-ROR (Fu et al., 2017b). Cdk4/6 upregulates the expression of genes promoting

invasion and metastasis. Marked induction of b-catenin, Slug, Twist, N-cadherin, and vimentin (Liu and Korc, 2012). *In vitro*, LIFR adversely controls the invasion and metastasis of PC cells. The PATU-8988/LIFR cells' overexpression of LIFR dramatically reduced the expression of vimentin, beta catenin, and slug while inducing the expression of E-cadherin. (Ma et al., 2016).

1.9. Immunological suppression

PDAC cells population highly expressing CD90 possess significant stemness characteristics and tumorigenicity. A physical contact between CD90 positive cells and monocytes/macrophages is made possible by CD90 acting as an anchor for monocyte/macrophage adherence. In response, immunosuppressive properties of immune cells are promoted by the interaction between CD90 positive cells and monocytes/macrophages, which improves the stemness and epithelial-mesenchymal transition (EMT) of PDAC cells. Moreover, the majority of the CD90 positive population expresses PD-L1, giving these cells another way to elude immune monitoring (Shi et al., 2019). Significant relationships have been found between TNFSF9 and the genes STAT4, STAT1, TNF, IFNG, FOXP3, TGF, CTLA4, and TIM-3, which are markers for T helper 1, Treg, and T cell exhaustion (Wu et al., 2021). As evidenced by the considerably elevated levels of Snail, Slug, Twist, Zeb-1, Nanog, and Oct-4, monocytic myeloid-derived suppressor cells accelerate EMT via activation of STAT3 (Panni et al., 2014).

1.10. Cellular dormancy

Dormancy is the process by which pancreatic cancer cell as a single cell or as micrometastases become nonproliferative (Wikman et al., 2008). The absence of growth factor signalling and the activity of metastatic suppressor genes cause cellular dormancy in pancreatic cancer, whereas the absence of an activated angiogenic switch at the secondary site causes angiogenic dormancy, or the presence of immunological factors causes angiogenic dormancy (immunologic dormancy) (Páez et al., 2012). A wide range of the cell cycle machinery is downregulated when SOX2 is elevated in different types of tumour cells, which suppresses both tumour growth and quiescence. (Metz et al., 2020). The biological underpinning for cancer recurrence upon oncogene reactivation is the survival of dormant pancreatic cancer cells that retain oncogenic KRAS expression. In cancer cells that are quiescent, killing oncogenic

KRAS activates AKT and increases IGF-1R signalling as a form of compensation.(Rajbhandari et al., 2017).

1.11. Tumour secretome

Cellular components of PDAC tissue, such as cancer-associated fibroblasts or immune and tumour cells, release molecular information into the tumour microenvironment as part of their secretome; the relevant proteins also end up in the peripheral blood (Grønborg et al., 2006; Truong and Pauklin, 2021). The protein composition of the secretome is highly sensitive to pathological changes in the tumour and could thus provide an early picture of tumour development (Dowling and Clynes, 2011; Mustafa et al., 2011). Detecting variations in the abundance of cancer-associated proteins in blood may therefore provide a non-invasive process for sensitive and possibly early diagnosis. Another advantage would be that it could be repeated in short intervals as part of the normal follow-up after surgery without much extra burden to the patient (Qi et al., 2018). Quite a few blood-based PDAC markers have been reported overall (Al-Shaheri et al., 2021), only CA19-9 is being utilised on a routine basis for the management of pancreatic cancer patients (Poruk et al., 2013).

1.12. MicroRNAs

Non-coding RNAs called microRNAs (miRNAs) have a length of 22 nucleotides. MiRNAs interact with target mRNA of genes, resulting in their reduced translation or degradation. About 700 miRNAs identified so far representing about 3% of the human genome (Ambros, 2004). MiRNAs control the expression of 60% of the protein-coding genes in humans (Friedman et al., 2009). They serve as gene expression regulators and control a wide range of cellular processes, including growth, differentiation, proliferation, and apoptosis. In cancer, miRNAs play oncogenic or tumour-suppressive functions including pancreatic cancer (Esquela-Kerscher and Slack, 2006). They regulate KRAS family and their downstream genes e.g. miR-96, miR-126, and miR-217 and therefore act as oncogenes (Jiao et al., 2012; Zhao et al., 2010). They also modulate the function of tumour suppressor genes, stemness and epigenetic pathways (Tesfaye et al., 2019). Circulating miRNAs are also attractive potential

molecular markers because of their stability, abundance, and technical ease of their isolation and amplification with inexpensive and non-invasive methods (Visani et al., 2015).

1.13. Early detection of pancreatic cancer recurrence

A blood-based assay for the detection of PDAC recurrence would be advantageous since it may yield early diagnosis. Also, it could be repeated in short intervals as part of the normal follow-up after surgery without any extra burden to the patient (Qi et al., 2018). Quite a few blood-based PDAC markers have been reported (Al-Shaheri et al., 2021), except CA19-9 that is currently used on a routine basis for the management of pancreatic cancer patients (Poruk et al., 2013). There have been few publications about blood-based detection or prediction of PDAC recurrence, dealing with different molecule classes. The presence of circulating tumour DNA (ctDNA) was reported as a predictor of decreased recurrence-free survival (Groot et al., 2019b). Since ctDNA concentration is extremely low and varies substantially between patients, it may be missed for technical reasons, however. With respect to protein biomarkers, blood samples from 14 patients were studied and compared to the protein content of organoids derived from patients with early or late recurrence. Only very few samples were analysed in this study. Also, comparison of the protein content of blood and organoids may not be relevant. Last, there was no validation of potential protein biomarkers (Rittmann et al., 2021). Another investigation found six exosomal miRNAs that discriminated patients with recurrence from those without recurrence with an accuracy of 72% to 78% (Nishiwada et al., 2022).

1.14. Dissertation objectives

This dissertation aimed at establishing a blood-based assay that allows diagnosis of PDAC recurrence at high accuracy and also early enough so that the recurrent tumour is small enough to be removed successfully by surgery. In addition, the work defined the frequency and the intervals at which a diagnostic blood analysis must be carried out.

Previous work in the division of functional genome analysis has shown that protein analysis of peripheral blood from patients or healthy individuals – additionally compared to the secretomes of tumour or non-tumour cells – identified protein variations, which distinguish at high sensitivity and

specificity between healthy people and patients with chronic pancreatitis or PDAC (Mustafa et al., 2017). On the basis of these results, I set out to detect tumour recurrence by studying abundance variations of proteins in patient sera. The aim was to detect and predict tumour recurrence in PDAC patients after surgical resection earlier and with better accuracy than currently possible. Since many cancer-associated proteins are circulating in low concentrations, their analysis requires a highly sensitive process (Skalnikova et al., 2011). Also, detection of tumour protein markers in serum is obscured by highly abundant proteins, such as albumin and globulins; their depletion could introduce biases in the abundance measurements of other proteins (Govorukhina et al., 2003). Immunoassays allow circumventing these technical challenges. I utilized a microarray made of 2977 antibodies that detect 2,286 proteins. Also consecutively collected blood samples from the same patients were analysed so as to look at changes over time.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Software

Table 1: Software

Name	Company
Genepix Pro 6.0	Molecular Devices (Sunnyvale, CA, USA)
Power scanner v1.2	Tecan Trading AG (Switzerland)
R Studio	https://rstudio.com/
PubMed	https://pubmed.ncbi.nlm.nih.gov/
Fluostar Galaxy 4.11-0	BMG Labtech (Offenburg, Germany)
Ingenuity pathway analysis	Qiagen (Venlo, Netherlands)
Bioinformatics and evolutionary genomics	VIB/ UGent (Gent, Belgium)
AzureSpot	Azure Biosystems (Dublin, USA)
Uniprot	https://www.uniprot.org
2100 Expert	Agilent (Santa Clara, California)
HUSAR	https://w2h2.dkfz.de/menu/cgi-bin/w2h/w2h.start

2.1.2 Equipment

Table 2: Equipment

Equipment	Name/Reference	Company
Pipettes	Pipetman P1000, No.: FA10006M, Pipetman P200, No.: FA10005M, Pipetman P20, No.: FA10003M, P10, No.: F144802	Gilson Inc. (Middleton, USA)
Pipetting aid (Pipetboy)	-	Integra Bioscience (Hudson, USA)
Multi-channel pippet 5-100 µl	7059567	Biohit (Darmstadt, Germany)
Multichannel pipet 50-1000 µl	-	Gilson Inc. (Middleton, USA)
Microarray spotter	MicroGrid MGII600	BioRobotics (Cambridge, UK)
SMP3B stealth pins	101092-942	Telechem (CA, USA)
Microarray scanner	Power Scanner V1.2	Tecan Group Ltd. (Männedorf, Switzerland)
Epoxysilane-coated slides	-	Nexterion-E Schott (Jena, Germany)
Centrifuge	Centrifuge Sigma 2K15	Sigma (Osterode am Harz, Germany)
Centrifuge	Biofuge	Heraeus (Hanau Germany)

Thermal cycler	685112	Biozyme GmbH (Oldendorf, Germany)
ELISA plate reader	Fluostar Galaxy	BMG Labtech (Offenburg, Germany)
Shaker	BUEH_14003	Edmund Bühler GmbH (Bodelshausen, Germany)
Freezer	-	Bosch GmbH (Gerlingen, Germany)
CO_2-Incubator,	No.: MCO-19AIC	Sanyo (Moriguchi, Japan)
Laminar flow workbench, Class II	-	The Baker Company (Sanford, USA)
Inverted microscope	L-J0202-000-GE	HUND (Wetzlar, Germany)
Vortex	Z258423	Scientific industries Genie-2 (New York, USA)
Quadrichem chamber	-	Vivascience (Hannover, Germany)
Slide booster hybridization station	-	Advalytix (Munich, Germany)
Cylinders and flasks	-	-
Bioanalyzer 2100	G2939BA	Agilent (*Santa Clara, California)*
Qubit flourometer 4	Q33238	Invitrogen (California, USA)
Electrophoresis power supply	-	E-C apparatus corporation (USA)
MultiskanFC microplate reader	51119000	Thermo Fischer Scientific (Waltham, USA)
Block thermostat	CX112016	Eppendorf (Jülich, Germany)

2.1.3 Chemicals

Table 3: Chemicals

Chemical	**Reference**	**Company**
Tris-Hydrochloride	167620010	Fisher Scientific (Schwerte, Germany)
Tris-Base	T1503-1KG	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Sodium Chloride	7647-14-5	Fisher Scientific (Schwerte, Germany)
Sodium Azide	A1430,0100	AppliChem GmbH (Darmstadt, Germany)
Sodium Hydroxide	655104	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Tween 80	P1754-500ML	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Skim milk powder	1602.0500	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Bicine	B3876-250G	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
DMSO	276855	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Glycine	33226	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

NP-40 (20%)	85124	Thermo Fisher Scientific (Waltham, USA)
Na-Cholate (10%)	C1254	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
ASB-14 (5%)	A1346	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
12-Maltoside	D4641	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Glycerol (99%)	G5516	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
EDTA	8040.3	Carl Roth GmbH (Karlsruhe, Germany)
PMSF	1111	Gerbu Biotechnik GmbH (Heidelberg, Germany)
Protease & Phosphatase inhibitor	78440	Thermo Fisher Scientific (Waltham, USA)
Benzonase	70664-3	Merck Life Science GmbH (Eppelheim, Germany)
IMDM growth medium	21980-032	Thermo Fisher Scientific (Waltham, USA)
1x PBS-Buffer (pH 7.4)	10010-015	Thermo Fisher Scientific (Waltham, USA)
0.05% Trypsin-EDTA	25300-062	Thermo Fisher Scientific (Waltham, USA)
Foetal Bovine Serum	26050-070	Thermo Fisher Scientific (Waltham, USA)
Penicillin/Streptomycin mix	15140-122	Thermo Fisher Scientific (Waltham, USA)
2-Propanol	33539-2,5L	Thermo Fisher Scientific (Waltham, USA)
6-Aminocaproic acid	A7824-100G	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
Ammonium persulfate	A1142,0250	AppliChem GmbH (Darmstadt, Germany)
Trypsin	15090046	Gibco/Invitrogen, Karlsruhe, Germany
L-Glutamine	25030149	Gibco/Invitrogen, Karlsruhe, Germany
Foetal Bovine Serum (FBS)	11573397	Gibco/Invitrogen, Karlsruhe, Germany
Methanol	M/4000/PC17	Thermo Fisher Scientific (Waltham, USA)
Agarose for molecular biology EEO	SLCH6024	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

2.1.4 Antibodies and Kits

In total, 2,977 antibodies are used for antibody microarrays production and listed in Suppl. Tab. S1.

Table 4: Kits

Name	Reference	Company
Pierce BCA Protein Assay Kit	23227	Thermo Scientific (Rockford, USA)
Dy-549-NHS	549P1-01	Dyomics (Dresden, Germany)
Dy-649-NHS	649P1-01	Dyomics (Dresden, Germany)

DNAJC3 ELISA Kit	MBS9336160	my BioSource (San Diego, USA)
BRD3 ELISA Kit	MBS9500849	my BioSource (San Diego, USA)
miRNA easy kit	172041013	Qiagen (Hilden, Germany)
RealSeq-Biofluids NGS Library Preparation Kit	600-00048-SOM	(Biosciences, Santa Cruz, USA)
Qubit dsDNA HS assay kit	2174889	Life Technologies (Oregon, USA)
Agilent DNA High Sensitivity Kit	50674620	Agilent Technologies (Waldronn, Germany)
Nucleospin Gel and PCR	740609.50	Machery-Nagel (Düren, Germany)
GeneRule Low range DNA ladder	SM1193	Thermo Fisher Scientific (Waltham, USA)
Qubit RNA HS assay	2159938	Life Technologies (Oregon, USA)

2.1.5 Consumables

Table 5: Consumables

Name	**Reference**	**Company**
Centrifuge tube PP with screw cap PE, 15 mL	02-502-3001	Nerbe plus (Winsen/Luhe, Germany)
Centrifuge tube PP with screw cap PE, 50 mL	02-572-3001	Nerbe plus (Winsen/Luhe, Germany)
Safe-Lock tubes 1.5 mL	2027-12-28	Eppendorf (Hamburg, Germany)
Safe-Lock tubes 0.5 mL amber	2022-01-28	Eppendorf (Hamburg, Germany)
96-well flat bottom microplate	655101	Greiner Bio-One International GmbH (Kremsmünster, Austria)
SurPhob tips reload, 1250 µL	VT0173	Biozym Scientific GmbH (Hessisch-Oldendorf, Germany)
SurPhob tips reload, 200 µL	VT0143	Biozym Scientific GmbH (Hessisch-Oldendorf, Germany)
SurPhob tips reload, 10 µL	VT0113	Biozym Scientific GmbH (Hessisch-Oldendorf, Germany)
T75-Flask	658 175	Greiner Bio-One GmbH (Frickenhausen, Germany)
Serological pipette 5 mL	4487	Corning Incorporated (Corning, USA)
Serological pipette 10 mL	4488	Corning Incorporated (Corning, USA)
Serological pipette 25mL	4489	Corning Incorporated (Corning, USA)
Vivaspin centrifugal Concentrators	VS2091	Sartorius AG (Bretten, Germany)
Coculture 6-well plates with inserts	140640	Thermo Fisher Scientific (Waltham, USA)
RNAZAP spray	SLBQ7780	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)

PCR tubes 0.2 ml thin-welled with flat-cup (RNase free)	60431117	Thermo Fisher Scientific (Waltham, USA)
Feather disposable scalpel	02.001.30015	Feather (Osaka, Japan)

2.1.6 Cell lines

Table 6. Cell lines whose secretome was analysed.

Cell line	Gender	Cell source	Histology	Grade	Patient age (years)
AsPC-1	Female	Ascites	Adenocarcinoma/ Well-moderately differentiated	G2	62
BxPC-3	Female	Primary tumour	Adenocarcinoma/ Moderately differentiated	G2	61
Capan-2	Male	Liver metastasis	-	-	56
MIA PaCa-2	Male	Primary tumour	Adenocarcinoma/ Poor-moderately differentiated	G3	65
PANC-1	Male	Primary tumour	Ductal epithelioid carcinoma/ Poorly differentiated	G3	56
Suit2-020	Male	Liver metastasis	-	-	73
HPDE	Female	Immortalised human pancreatic duct epithelial cell	Acute and chronic pancreatitis	-	51

Table 7: Cell lines whose proteome was used to prepare the antibody microarray common reference.

Description of established cell lines				
Cell line	**Patient gender**	**Cell source**	**Disease/ grade of differention**	**Patient age (years)**
BxPC-3	Female	Primary tumour	Adenocarcinoma / moderately differentiated	61
FAMPAC	Female	Primary tumour	Adenocarcinoma: cystic fibrosis / poorly differentiated	43
IMIM-PC1	Male	Primary tumour	Adenocarcinoma / moderately differentiated	-
MIA PaCa-2	Male	Primary tumour	ductal carcinoma / poor-moderately differentiated	65
PANC-1	Male	Primary tumour	ductal epithelioid carcinoma / poorly differentiated	56
SK-PC-1	Female	Primary tumour	Adenocarcinoma / well differentiated	-

SU.86.86	Female	Primary tumour	Ductal carcinoma / moderately differentiated	57
Capan-1	Male	Liver metastasis	Adenocarcinoma / well differentiated	40
Capan-2	Male	Liver metastasis	Adenocarcinoma / well differentiated	56
CFPAC-1	Male	Liver metastasis	Adenocarcinoma: cystic fibrosis / well differentiated	26
Suit-2	Male	Liver metastasis	Adenocarcinoma / well differentiated	73
Suit-007	Male	Liver metastasis	Adenocarcinoma / moderately differentiated	73
Suit2-020	Male	Liver metastasis	Adenocarcinoma / moderately differentiated	73
Suit-028	Male	Liver metastasis	Adenocarcinoma / moderately differentiated	73
Colo357	-	Lymph node metastasis	Adenocarcinoma / well differentiated	-
T3M4	Male	Lymph node metastasis	- / moderately differentiated	56
A818-1	Female	Ascites	Adenocarcinoma / moderately differentiated	75
A818-4	Female	Ascites	Adenocarcinoma / moderately differentiated	76
A818-7	Female	Ascites	Adenocarcinoma / moderately differentiated	77
AsPC-1	Female	Ascites	Adenocarcinoma / moderate-well differentiated	62
HPAF-II	Male	Ascites	Adenocarcinoma / moderate-well differentiated	44

2.1.7. Buffers and solutions

Table 8. Buffers and Solutions

Buffer or solution	**Components**
3 L 10x TBS	94.56 g Tris HCl 240 g NaCl 3 g NaN3 Dissolve in ddH2O and pH 7.6
1 L 10x TBE	108 g Tris base 55 g Boric acid 7 g EDTA Dissolve in ddH20 and pH: 8.0

20% Tween 80	20 ml of Tween 80 in 80 mL ddH2O Sterile to 100 mL bottle through 0.22µl filter
10x TBST	TBS with 0.1% (v/v) 20% Tween 80
Lysis buffer for protein isolation	500 µl of NP-40 1000 µl of Na-Cholate 1000 µl of ASB-14 1000 µl of 12-maltoside 2000 µl of glycerol (99%) 1000 µl Bicine (0.5M, pH8.5) 1000 µl EDTA.2Na (0.02 M) 1000 µl of NaCl (1.50 M) 50 µl of PMSF (200 mM in isopropanol) 100 µl of protease and phosphatase inhibitor cocktail 0.4 µl of Benzonase (100 U/µl) 1346 µl of ddH2O
Milk blocking buffer for microarray	10% milk powder in 1XTBST
Spotting buffer	100 mM bicine 0.05% Tween-20 0.05% sodium azide 5% trehalose 5 mM magnesium chloride

2.2. Methods

2.2.1. Patients' samples

10 ml tubes containing blood samples were taken from each patient, and centrifuged at 1,600 g for 10 min. at 4°C. The serum was stored immediately at -80°C until use. For biomarker discovery, 149 serum samples from 101 individuals were obtained from the Pancobank repository of the European Pancreas Centre at the Department of Surgery, University Hospital Heidelberg; Pancobank is a member of the Biomaterial Bank Heidelberg. I analysed the serum samples only, whose pathological diagnosis from resected material was confirmed to be PDAC. Serum samples were collected at the time of surgery and during post-operative clinical follow-up every three to six months until the patient died, or the follow-up was stopped for another reason. For validation, an independent cohort of 60 serum samples was collected prospectively from 41 patients to document the diagnostic performance of the biomarker signature at real clinical conditions. Informed consent in writing had been obtained from participants and the ethics committee of Heidelberg University has given the ethical approval (ethics votes 159/2002,

708/2019 and S-508/2022). The work described here was performed in compliance with provisions of the Declaration of Helsinki.

2.2.2. Protein profiling by antibody microarray

2.2.2.1. Production of antibody microarrays

Antibody microarrays were produced using a well-established protocol described earlier (Alhamdani et al., 2012; Mustafa et al., 2017). Briefly, the antibodies were spotted at a concentration of 1 mg/ml antibody in 100 mM bicine buffer (pH 8.5), 0.05% Tween-20, 0.05% sodium azide, 5% trehalose, 5 mM magnesium chloride, 137 mM sodium chloride onto epoxy-coated slides (Nexterion-E; Schott, Jena, Germany) using a MicroGrid MGII600 contact printer (BioRobotics, Cambridge, UK) with SMP3B pins (Telechem, Sunnyvale, USA) at an humidity of 55% to 65%. I spotted each antibody in duplicates, included positional markers and controls and allowed an overnight equilibration at room temperature and 55%-65% humidity followed by a long-term storage in dark and dry conditions at 4C°. Initially, microarrays containing all 2,977 antibodies were produced. For validation, the number of antibodies was reduced to 568, which targeted 355 informative biomarker candidates and 213 non-informative proteins.

2.2.2.2 Preparation of common protein reference sample

A common protein reference sample was studied in parallel to each actual sample, providing an internal control of experimental performance and quality. At the same time, it was serving as a reference for data normalization between the individual experiments. Preparation of the sample has been described in detail before (Alhamdani et al., 2012). In short, 22 pancreatic cancer cell lines (**Tab. 7**) were cultivated for 24-36 hours and then split into six plates. At 80% confluency, all cells were harvested, mixed, and a single protein extraction was performed as described earlier (Alhamdani et al., 2010b). The protein sample was labelled with fluorescent dye (Dyomics, Jena, Germany) and stored in aliquots at -20°C until use. For all experiments described here, aliquots of the very same protein reference sample were used.

2.2.2. 3. Cell culture and secretome collection

The non-cancerous, immortalized human pancreatic duct epithelial cell line HPDE-E6E7 **(Tab. 6)** and the pancreatic cancer cell lines PANC-1, BxPC-3, AsPC-1, Capan-2, MiaPaCa-2, and Suit2-020 were acquired from ATCC and verified by the DKFZ Genomics Core Facility. Regular checks for mycoplasma infection were performed for all cells. I used Iscove's Modified Dulbecco's Medium (IMDM) with 10% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin to cultivate cells (Invitrogen, Darmstadt, Germany) at 37°C in a humidified atmosphere of 5% CO_2 until they reach 85-90% confluency. I used Phosphate-buffered saline (PBS) to wash the cells three times, and serum-free media for additional two washes. Cells were cultivated for a further 48 hours after being incubated in serum-free media for 12 hours to synchronize cell growth. The medium was then taken out, centrifuged for 10 minutes at 3,500 g, filtered through 0.22 M nylon filters, and kept at -80°C.

To study the secretome of cancer-associated fibroblasts, human immortalized pancreatic stellate cells (PSCs) were co-cultivated with PANC-1, BxPC-3, AsPC-1, Capan-2, MiaPaCa-2 and Suit2-020, respectively, using 6-well co-culture inserts (Thermo Fischer Scientific, Waltham, USA) under the same growth conditions described above. The PSC cell line was a kind gift from Ralf Jesnowski from Mannheim University Hospital in Germany (Jesnowski et al., 2005). PSC cell was also co-cultivated with HPDE in the same conditions.

I isolated peripheral blood mononuclear cells (PBMCs) from blood samples taken from healthy donors at Heidelberg Blood Bank. All donors had provided written informed consent, and the local ethics commission had approved the analysis (ethical vote 2016-615N-MA). In short, 15 ml blood were added to the SepMate-15 system (Stemcell Technologies, Cologne, Germany) and diluted with 15 ml PBS. The manufacturer's instructions were followed during the cell purification process. After centrifugation at 1,200 g for 15 min, the top layer of buffy coat was collected. Centrifuged again at 300 g for 8 min, the mononuclear cells were washed with 25 ml PBS twice and resuspended in PBS, 0.5% BSA, 2 mM EDTA. The enriched monocytes were counted and co-cultured with PANC-1, BxPC-3, AsPC-1, Capan-2, MiaPaCa-2, Suit2-020, respectively, as described above. PBMCs was co-cultivated with HPDE in the same co-culture condition described above.

2.2.2.4 Preparation of conditioned media

According to the manufacturer's instructions, I utilized vivaspin-20 tubes with a molecular weight cut-off value of 3 kDa (Sartorius, Göttingen, Germany) for secretome preparation. Each tube was filled with 20 ml of growth medium from each separate cell culture and spun in a swing bucket centrifuge at 5,000 g for two hours at 4 °C (Thermo Fisher Scientific). Using the same centrifugation settings, I used 20 ml of 0.1 M bicine buffer (pH 8.5) for desalting twice. A bicinchoninic acid protein assay reagent kit was used to assess the protein concentration in the final ultrafiltered solution (Thermo Fisher Scientific). SDS gel electrophoresis was used to evaluate the overall protein integrity, and samples were stored at -80°C until use.

2.2.2.5 Antibody microarray analysis

The protein concentration of serum samples was adjusted to 4.0 mg/ml using phosphate buffer (pH 7.2). The proteins in the serum samples or the common reference were labelled with the fluorescent dyes DY649 or DY549 (Dyomics, Jena, Germany), respectively, at a molar protein/dye ratio of 7.5 in PBS (pH 7.2) at 4°C for 2 h. Subsequently, 10% glycine in PBS was used to quench unreacted dye. Microarray analyses were performed as previously described in detail (Alhamdani et al., 2010a). Prior to incubation, the microarrays were equilibrated in 20 mM Tris (pH 7.6), 150 mM NaCl (TBS) and then washed twice with TBS containing 0.05% Tween 80. The microarray surface was blocked with 10% non-fat dry milk (BioRad, Munich, Germany) in TBS, 0.05% Tween 80 for 3 h at room temperature. The blocked slides were incubated with 25 µg/ml each of labelled serum protein and the common reference at 4°C in the dark overnight. The slides were washed four times with TBS, 0.05% Tween 80 for 5 min, rinsed in deionized water and air-dried in a ventilated oven at 37°C. A PowerScanner system (Tecan, Männedorf, Switzerland) was used for image capture at constant laser power and photo-multiplier tube gain. Image analysis was performed with GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, USA) generating numerical values of signal intensities.

2.2.2.7 Enzyme linked immunosorbent assays (ELISAs)

Serum levels of the proteins DNAJC3 and BRD3 were determined by quantitative ELISA using commercially available kits from myBiosource (San Diego, USA) and abbexa (Cambridge, UK),

following the manufacturers' instructions. Optical density was measured at 450 nm using a MultiskanFC microplate reader (Thermo Fischer Scientific).

2.2.2.8 Microarray data analysis

The data from the microarray experiments were analysed using a very well-established bioinformatic pipeline described before (Zhang et al., 2022). In short, LIMMA package in R was utilised to study differential protein abundance (version 4.0.4) (Ritchie et al., 2015). Using the function "backgroundCorrect," the Loess method was used to normalize the data with an offset for the background correction (0.50) (Ritchie et al., 2007). Variations within and between microarrays were corrected by applying the functions 'normalizeWithinArrays,' and 'normalizeBetweenArrays.' Multiple testing was done using the Empirical Bayes test and the Bonferroni-Hochberg adjustment (Smyth, 2004). An adjusted p-value of less than 0.05 was considered significant. For evaluating the diagnostic power of individual proteins, univariate logistic regression combined with ROC analysis was done in R using the pROC package (Robin et al., 2011). Least absolute shrinkage and selection operator (LASSO) regression based on the R package 'glmnet' (v. 4.1-3, 34) (Friedman et al., 2022) and recursive feature elimination (RFE) with 5-fold cross validation of the R package 'caret' (v. 6.0-91) were applied feature selection to enhance predictive accuracy (Kuhn, n.d.). The identified marker molecules were used to train an SVM classifier with the 'e1071' package (version 1.7-9) (Pisner and Schnyer, 2020). Classifier parameters were optimized by an exhaustive search approach to avoid over- or underfitting. Using the SVM decision values, a ROC curve and the respective AUC value were calculated. The classifier was then used with fixed parameters to evaluate its diagnostic performance using information derived from samples of the validation cohort that were prospectively gathered. The Ingenuity Pathways Analysis (IPA) software package (version 6.3; Ingenuity Systems, Redwood City, USA) was used to predict functional characteristics of differentially expressed proteins. With the use of UniProt's web-based Gene Ontology tool, component annotation was mapped (www.uniprot.org).

2.2.3. Serum miRNA profiling by small RNA sequencing

2.2.3.1. MiRNA extraction

Total RNA including miRNAs were extracted from 200µl serum using miRNAeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Extraction efficiency was assessed by qPCR using standard curve obtained from spike in control according to manufacturer's instructions. Also, the quantity of extracted RNA was assessed by Qubit high sensitivity RNA kit (Life Technologies, USA).

2.2.3.2. Library preparation

In the discovery phase, 149 Individual libraries were prepared from 5µl extracted miRNA using Real-Seq Biofluids NGS library preparation Kit (Biosciences, Santa Cruz, USA) according to the manufacturer's instructions. After the PCR amplification, each library PCR products was run in 4% agarose gel electrophoresis for two hours at 80 V. The library band of 140-145 bp were excised using sterile surgical scalpel (Feather, Japan) under UV transilluminator using the 366-nm wavelength. The DNA was then extracted from the gel using Nucleospin Gel and PCR kit (Machery-Nagel, Germany) according to the manufacturer's instruction. Libraries concentration was measured by Qubit DNA high sensitivity kit (Life Technologies, USA), and the concentration in ng/µl was converted into molar concentration. I also checked the size of the miRNA libraries using the Bioanalyzer 2100 and DNA high sensitivity kit (Agilent, USA). Using the molar concentration and library size, I normalized each library concentration to 10 nM/µl. Libraries were pooled at equal concentration (10nMµ) and volume (10 µl) and sent to the DKFZ core facility for small RNA sequencing, where additional quality control of the molar concentration and fragment size were done by Tapstation (Agilent, USA). For prospective validation, I also prepared 76 small RNA sequencing libraries using the same protocol described above.

2.2.3.3. Small RNA sequencing

The 149 libraries generated during at the discovery cohort were multiplexed in 10 lines and sequenced by Hiseq2000 v4 single read 50bp with 51 cycles. Additional 76 libraries, generated for prospective validation, were multiplexed in 4 lines and sequenced utilizing Nextseq 550 single read 75bp.

2.2.3.4. Quantitative PCR (qPCR)

miRNA biomarker candidates emerging from small RNA sequencing were further verified by quantitative PCR (qPCR) testing. Using the miRCURY LNA RT Kit for RNA isolated with the miRNeasy Serum/Plasma kit, reverse transcription was carried out. To convert the results of each qPCR reaction into the number of copies, a standard curve based on cel-miR-39-3p was created. Then, 5µl of the 2x miRCURY SYBR Green Master mix and 1µl of the PCR primer mix were added to 4µl of the cDNA template, which had been diluted 1:20. The PCR procedure includes an initial 95°C heat activation for 2 minutes, followed by 45 cycles of primer annealing and extension at 56°C for 1 minute and denaturation at 95°C for 10 seconds. QPCR reactions were run on a Life Technologies 480 machine based on the miRNA assays guidelines.

2.2.3.5. Small RNA sequencing data analysis

Data in FastQ file format was obtained from DKFZ data management repository, where also reads quality control check was performed. The data was analysed using HUSAR (Heidelberg Unix Sequencing Analysis Resources), which is a very well-established bioinformatics platform at DKFZ (“https://www.dkfz.de/en/forschung/zentrale_einrichtungen/cfomics/login.html?m=1543423542&,” n.d.). In short, sequencing reads were mapped using “sRNAMapper” tool after trimming the sequencing adapter to human genome (Chr38) with default parameters. The clean Fastq files were then annotated for ncRNA using “ncRNAannotator” that utilize bowtie for annotation of *Homo sapiens* precursor miRNAs. Data bases used for the annotation are Ensemble 85, GtRNAadb2011, Rfame 12.1, miRbase 21, piRNA 2012 and piRNABank 2.0. For differential expression analysis of annotated miRNAs, the tool “CompaRNA” in HUSAR was used. The parametric hypothetical test for DeSeq at 0.01 threshold of p value was used with a minimum negative fold change of -1 and a minimum positive fold change of 1 in algorithmic scale. The count reads of differentially expressed miRNA were extracted and converted into logarithmic scale and used for the biomarker selection process. For evaluating the diagnostic power of individual miRNA, univariate logistic regression combined with ROC analysis was done in R using the pROC package (Robin et al., 2011). Least absolute shrinkage and selection operator (LASSO) regression based on the R package ‘glmnet’ (v. 4.1-3, 34) (Friedman et al., 2022) and recursive feature elimination (RFE) with 5-fold cross validation of the R package ‘caret’ (v. 6.0-91) were applied for

feature selection to enhance predictive accuracy (Kuhn, n.d.). The identified marker molecules were used to train an SVM classifier with the 'e1071' package (version 1.7-9; (Pisner and Schnyer, 2020)). Classifier parameters were optimized by an exhaustive search approach to avoid over- or underfitting. Using the SVM decision values, a ROC curve and the respective AUC value were calculated. The classifier was then applied with fixed parameters to assess its diagnostic performance with the data resulting from the prospectively collected samples of the validation cohort.

2.2.4. Combining protein and miRNA biomarkers

Protein and miRNA tested samples were divided randomly into a training cohort and a validation cohort. Empirical Bayes test and Student's t-test were employed, respectively, to confirm significant changes in protein and miRNA abundance. I used Recursive Feature Elimination with 5-fold cross validation to build a SVM classifier using the R package e1071(Pisner and Schnyer, 2020). The classifier with fixed parameters was then applied to the data resulting from the validation samples for the confirmation of the model prediction and classification potential and to determine the diagnostic value of the combined biomarker panel.

3. RESULTS

3.1. Clinical patient information

Biomarker discovery was performed on 149 serum samples obtained from 101 patients, who had undergone curative surgical resection of a primary PDAC tumour at the Surgery Department of Heidelberg University Hospital between April 2008 and March 2018. Adjuvant chemotherapy had been prescribed to 87% of them. The blood samples were collected during routine clinical follow-up visits and stored at the Pancobank repository of the European Pancreas Centre (EPZ) at the surgery department of Heidelberg University Clinics. Patients' demographic characteristics, pre-operative symptoms, radiological findings, laboratory data and pathologic factors were collected; also, results were recorded of pre- and post-operative cross-sectional imaging (CT and MRI) and measurements of CA19-9 or CEA blood levels (**Tab. 9**). Some 65% of the patients had tumour recurrence within two years after surgery, while no evidence of recurrent tumour was detected in the others during this period. The recurrence status was determined by standard diagnostics at Heidelberg University Hospital, in particular CA19-9 levels and CT or MRI imaging, according to the NCCN clinical practice guidelines (Tempero et al., 2017). For validation, I used prospectively collected samples in order to demonstrate the power of the biomarker classifiers in a setting close to the real clinical situation. These samples came from 36 patients of the EPZ, who had tumour resection done between February 2020 and June 2021. Three quarters of the patients underwent adjuvant chemotherapy. The recurrence status was monitored every three months for a period of two years or until recurrence was detected.

3.2. Serum protein profiling

For the identification of protein biomarker candidates, I studied the protein content of serum samples from patients with and without clinically confirmed tumour recurrence; the design of the overall analysis is shown in **Fig. 6**. A microarray made of 2,977 antibodies targeting 2,286 proteins was used for the analysis. First, 149 serum samples were studied. Sixty-seven of them came from patients who had experienced recurrence at the time of blood collection according to radiological and laboratory evidence produced during the clinical follow-up.

Table 9. Clinico-pathological characteristics of PDAC patients.

Variable	Discovery cohort (n = 101)	Prospective cohort (n = 36)
Sex		
Male	46 / 46%	24 / 66%
Female	55 / 54%	12 / 33%
Age at surgery in years		
Median	62,4	63
Range	36-83	39-82
Tumour location		
Head	68 / 67%	22 / 61%
Body	20 / 20%	5 / 14%
Tail	12 / 12%	3 / 8%
Other	1 / 1%	6 / 17%
Neoadjuvant chemotherapy	10 / 10%	4 / 11%
Yes	91 / 90%	32 / 89%
No		
T stage	10 / 10%	6 / 17%
T1	17 / 17%	22 / 61%
T2	71 / 70%	5 / 14%
T3	3 / 3%	3 / 8%
T4		-
N stage		
N0	33 / 32%	13 / 36%
N1	36 / 36%	12 / 33%
N2	32 / 32%	11 / 31%
M stage		
M0	97 / 96%	34 / 94%
M1	4 / 4%	2 / 6%
Tumour recurrence		
Yes		
No	66 / 65%	22 / 61%
	35 / 35%	14 / 39%
Recurrence type		
Local	36 / 54%	5 / 23%
Liver	12 / 18%	4 / 18%
Lung	7 / 11%	2 / 9%
Others	3 / 5%	5 / 23%
Multiple loci (>2)	8 / 12%	6 / 27%
Adjuvant chemotherapy		
Yes	88 / 87%	30 / 83%
No	12 / 12%	6 / 17%
Unknown	1 / 1%	-
R status		
R0	43 / 43%	28 / 78%
R1	54 / 54%	7 / 19%
R2	1 / 1%	1 / 3%
Unknown	3 / 3%	

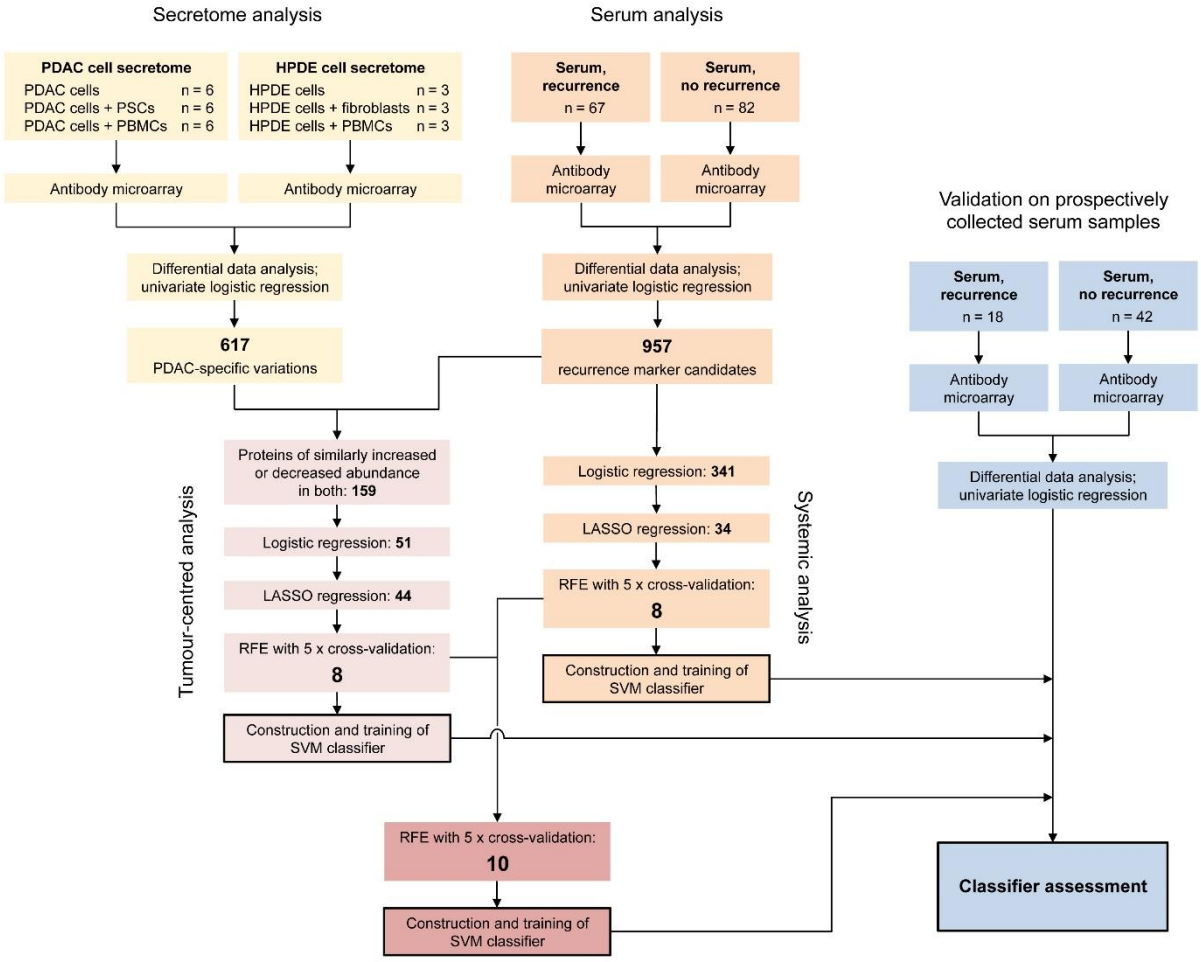

Figure 6. Scheme of the overall protein analysis pipeline. The workflow for the selection and validation of serum protein biomarker classifiers is displayed.

The other 82 samples were grouped into the norecurrence group because of the lack of any such evidence. In the analysis, 957 proteins exhibited a significant difference in abundance between the recurrence and norecurrence group. This relatively high number is not surprising, since many antibodies had been selected based on the likelihood that the targeted proteins could be relevant to pancreatic cancer or at least involved in tumorigenesis overall.

3.2.1. Recurrence classifier made of tumour-associated proteins

To focus on variations in protein abundance that are directly associated with the tumour, I compared the serum results to a list of proteins that were found to be secreted by tumour cells. The rationale was that protein abundance variations in the cell secretome also affect the protein content of the serum. Secretome samples were prepared from individual cultures of the PDAC cell lines AsPC-1,

BxPC-3, Capan-2, MiaPaCa-2, PANC-1 and Suit2-020 as well as co-cultures with either pancreatic stellate cells (PSCs) or peripheral blood mononuclear cells (PBMCs). The cell lines represent tumours that originate from both male and female patients as well as primary tumour and metastasis (**Tab. 6**). The PDAC secretome samples were pooled and protein abundances were compared to those in pooled secretomes isolated from non-cancerous HPDE cells that were also co-cultured with PSCs or PBMCs. Several independent secretome preparations were analysed (**Fig. 6**), yielding 617 proteins that exhibited significant differences between PDAC and HPDE secretomes. Functional annotation of the candidate proteins showed that they are associated with a reduction of apoptosis and a simultaneous increase in cell proliferation and migration (**Tab. 12**). The fact that some proteins are also linked to an increase in immune cell trafficking and inflammatory response to tumour suggests that biomarker candidates may not only originate from tumour cells but might also be released by other cell types within the tumour microenvironment.

There was an overlap of 159 proteins that were detected to be differentially abundant in both serum and secretome analysis; only changes were taken into account that were similar in direction in both studies, namely both significantly increased or decreased, respectively. The likelihood of these molecules to be PDAC-associated is higher than that of the other 798 proteins which also exhibited variations in serum. Receiver operating characteristic (ROC) analysis was individually performed with each of the 159 biomarker candidates and the resulting area under the curve (AUC) value was taken as a measure of performance. No single protein was found to produce an AUC value higher than 90%. Furthermore, enormous variation in the actual performance became apparent once candidates were validated in an independent set of 60 validation samples, which had been collected prospectively during the follow-up after surgical removal of a primary PDAC tumour (**Fig. 7A**).

Given the molecular complexity and heterogeneity of PDAC, a biomarker panel rather than an individual molecular marker is likely to be superior for an accurate and particularly also a robust assay. Therefore, I applied absolute shrinkage and selection operator (LASSO) regression and recursive feature elimination (RFE) with 5-fold cross validation, which are robust procedures for marker selection und deleting unnecessary covariates (Jeon and Oh, 2020; Tibshirani, 1996). The resulting protein panel was used to train a support vector machine (SVM) classifier. The algorithm draws a hyperplane into the

multi-dimensional protein space, by which the samples get separated into two groups in the best possible way (Pisner and Schnyer, 2020). To prevent over- and underfitting, a combination of three parameters (cost, gamma, and epsilon) was tuned during the procedure. The final classifier consisted of 8 proteins – BRD3, CYP3A5, DDI2, GCA, HM13, OSMR, PIGK and RIFK – and distinguished patients with recurrence from those without recurrence with AUC value of 89% (**Tab. 10**). For validating the performance, the classifier was applied to the 60 separate serum samples, yielding an AUC value of 76%; in particular sensitivity was low with 67%.

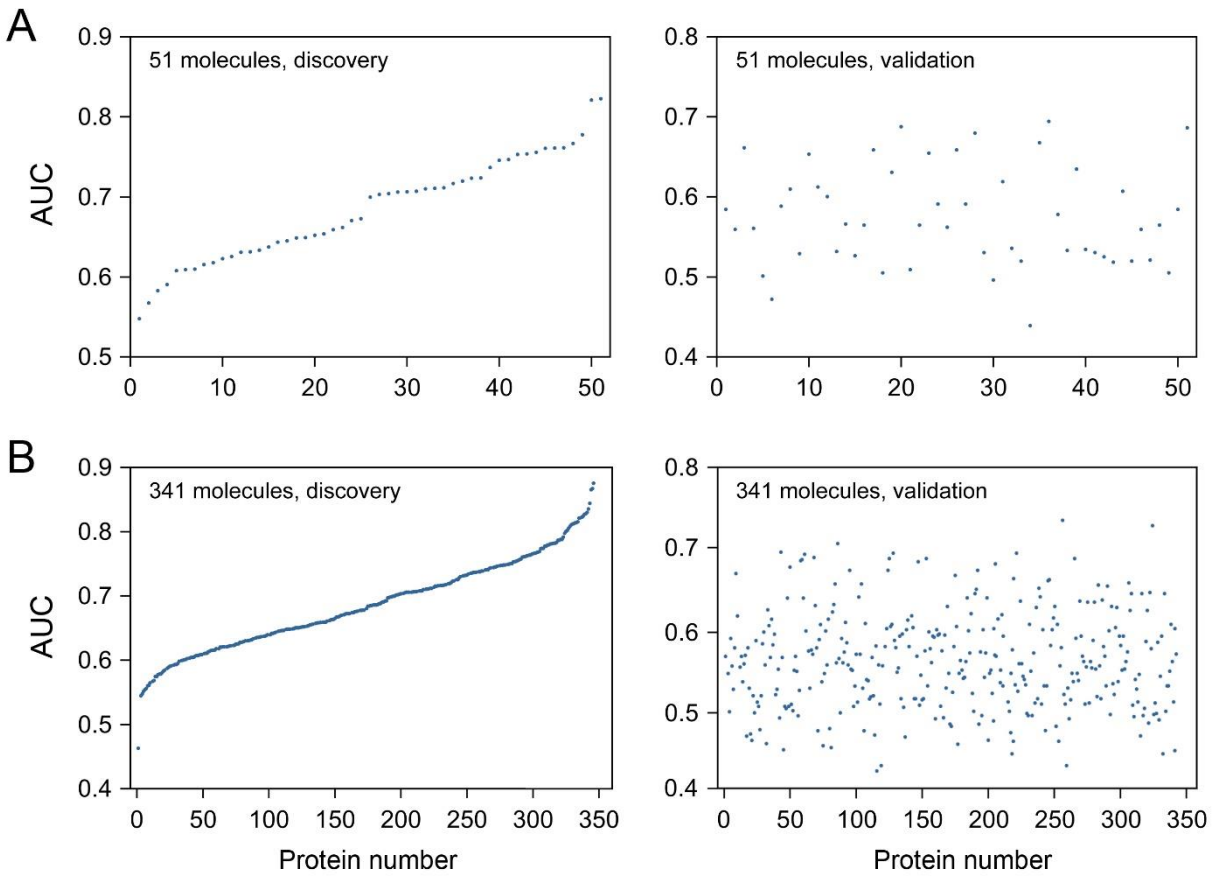

Figure 7. AUC values of individual serum markers. ROC curve analyses were performed for protein markers individually; the respective AUC values are shown. (A) The left panel shows the results calculated from the discovery analysis of the tumour-centred approach. At the right panel, the AUC values are shown that were calculated for the same 51 proteins using the validation samples. The order of proteins is identical to that in the left panel. (B) Identical presentation of the results obtained with 341 proteins that were found by the systemic approach.

Table 10. Protein classifier identified for the diagnosis of PDAC recurrence**.**

Protein classifier	Number of biomarkers	Selection process	Parameters of SVM classifier			Sensitivity		Specificity		AUC	
			Cost	Gamma	Epsilon	Discovery	Validation	Discovery	Validation	Discovery	Validation
BRD3, CYP3A5, DDI2, HM13, OSMR, PIGK, RIFK, GCA	8	Tumour-centred	7.0	0.01	0.30	0.81	0.67	0.88	0.79	89%	76%
ALG2, DNMT1, GCA, IGFBP3, IL23A, SPDL1, TMCC1, VPS4B	8	Systemic	6.0	0.03	0.50	0.84	0.61	0.89	0.71	92%	68%
ALG2, BRD3, DNMT1, HM13, IL23A, OSMR, PIGK, RIFK, SPDL1, VPS4B	**10**	**Combined**	7.0	0.01	0.30	0.90	**0.88**	0.77	**0.78**	91%	**85%**

AUC: area under the curve; SVM: support vector machine.

Table 11. Protein classifiers identified for the prediction of PDAC recurrence.

Protein classifier	Panel size	Predicted time to recurrence	Selection process	Parameters of SVM classifier			Sensitivity		Specificity		AUC	
				Cost	Gamma	Epsilon	Discovery	Validation	Discovery	Validation	Discovery	Validation
FZR1, GFRP, INPP5D, MART, XMRVP12	5	within 3 months	Tumour-centred	8.0	0.10	0.10	0.91	0.91	0.83	0.89	90%	86%
ACTL7A, ACTR10, CD7	3		Systemic	0.5	0.80	0.01	0.87	0.82	1.00	1.00	95%	93%
ACTL7A, ACTR10, CD7, GFRP	4		Combined	0.5	0.50	0.01	0.95	0.91	1.00	1.00	99%	97%
CTPS, HM13, MMD2, NQO1, RPL7A, SCG2, SFXN4	7	within 3 - 6 months	Tumour-centred	0.4	0.02	0.50	0.87	0.66	1.00	0.87	94%	81%
SDCBP, SERPINE1, SLC1A6, TNFAIP1, WNK1, XBP1	6		Systemic	0.1	0.10	0.90	0.89	0.77	0.83	1.00	87%	87%
HM13, SDCBP, SERPINE1, SLC1A6, TNFAIP1, WNK1, XBP1	7		Combined	0.4	0.02	0.50	0.90	0.77	0.83	0.86	85%	85%
IFNGR1, OTUB1, YIPF6	3	within 6 - 12 months	Tumour-centred	7.0	0.20	0.80	0.96	0.91	0.88	1.00	96%	96%
CCNA2, FLRT1, FLT1, NUSAP1, UBE2T, UBE2W, WNK1	7		Systemic	4.0	0.10	0.07	0.96	0.78	0.88	1.00	93%	92%
NUSAP1, OTUB1	2		Combined	10.0	0.20	0.90	0.67	0.73	1.00	0.86	88%	86%
IFNGR1, NUSAP1, OTUB1, YIPF6	4		Combined +	10.0	0.10	0.90	0.96	1.00	0.88	1.00	0.96	100%
IL18, PTPRS, VCAM1	3	later than 12 months	Tumour-centred	7.0	0.40	0.03	0.96	0.77	0.83	1.00	90%	89%
ARHGEF18, CALML5, CDC34	3		Systemic	0.7	0.01	0.90	0.91	1.00	0.83	0.75	92%	89%
CALML5, CDC34, PTPRS, VCAM1	4		Combined	7.0	0.30	0.01	0.98	1.00	1.00	0.75	99%	93%

Table 12. Most frequently predicted functions associated with proteins that differed significantly in abundance in the secretome of pancreatic cancer cell lines compared to non-tumour cells.

Functions annotation	p-value	Predicted activation state	Activation z-score	Molecules	# Molecules
Apoptosis	3.7 E-14	Decreased	-3.390	AKT3, BAX, BCL2L1, BMX, CBX3, CDC25A, CDKN1A, CSTA, CSTB, CYSLTR1, DDIT3, DKK1, DLG1, ECT2, EFEMP1, FUS, FUT4, GCLM, GMNN, GPM6B, GRB10, GSN, HSH2D, IL10, IL6, ITGA1, KRAS, MAD2L1, MDH1, MSN, MYD88, NRAS, ODC1, OGG1, OTUB1, PLK1, PMAIP1, PTPN1, PTPN6, RELT, SALL1, TAF4, THOC1, TNFRSF9, TOP1, YWHAB, ZFAND6	47
Proliferation of tumour cell	1.6 E-12	Increased	2.272	AKT3, BAX, BCL2L1, BMX, CBX3, CDC25A, CDKN1A, DDIT3, DKK1, DLG1, DUSP19, ECT2, EFEMP1, FABP2, FUS, FUT4, GMNN, IL10, IL6, ITGA1, KRAS, MAD2L1, MDH1, MSN, MTA1, MYD88, NRAS, ODC1, OGG1, OTUB1, PLK1, PTPN1, PTPN14, PTPN6, RAD52, THOC1, TOP1, UBE2A, UBE2J1	39
Abdominal neoplasm	8.9 E-08	Increased	2.073	AFDN, AKR1C1/AKR1C2, AKT3, AOC1, BAX, BCAS1, BCL2L1, BMX, CBX3, CD58, CD7, CD8A, CDC25A, CDKN1A, CHN1, CHST4, CLDN16, CSTB, CXCL9, CYSLTR1, DDI2, DDIT3, DKK1, DLG1, DLG2, DUSP19, ECT2, EFEMP1, FABP2, FUS, FUT4, GCLM, GMNN, GPM6B, GRB10, GSN, GTF2E2, HMGN2, IL10, IL1R2, IL6, ITGA1, KRAS, MAD2L1, MDH1, MSN, MTA1, MYD88, NBR1, NRAS, ODC1, OGG1, OTUB1, PLK1, PMAIP1, PTPN1, PTPN14, PTPN6, RAD52, RELT, RPL3, SALL1, STK19, TAF4, TCF25, THOC1, TM4SF4, TNFRSF9, TOP1, UBE2A, UBE2E1, UBE2E3, UBE2J1, UBE2T, YWHAB, ZNF124	76
Pancreatic cancer	5.8 E-06	Increased	2.763	AKR1C1/AKR1C2, BAX, BCL2L1, CD7, CDKN1A, CSTB, CXCL9, DDI2, DKK1, DLG1, ECT2, GRB10, IL10, IL6, KRAS, MAD2L1, MSN, MYD88, NRAS, PLK1, PMAIP1, PTPN1, PTPN14, PTPN6, SALL1, TAF4, TCF25, TOP1, UBE2J1	29
Inflammation	3.9 E-06	Increased	2.068	BAX, BCL2L1, CDKN1A, CYSLTR1, IL10, IL6, KRAS, MYD88, NBR1, PTPN6, TNFRSF9	11
Invasive tumour	3.3 E-06	Increased	2.291	AFDN, AKR1C1/AKR1C2, AKT3, BAX, BCL2L1, CDC25A, CDKN1A, CSTB, CXCL9, DKK1, EFEMP1, FUS, IL6, KRAS, MTA1, NRAS, OGG1, PLK1, PTPN1, PTPN14, RPL3, TAF4, TOP1	23
Quantity of myeloid cells	1.4 E-06	Increased	2.446	BCL2L1, CD8A, CDKN1A, DDIT3, DKK1, FUT4, GSN, IL10, IL6, KRAS, MSN, MYD88, PTPN6, TNFRSF9, UBE2W	15

IPA software was used to construct the prediction z-score (positive for activation, negative for inhibition), as explained in the Materials and Methods section.

3.2.2. Diagnostic performance based on a systemic analysis

Alternatively to the approach of comparing serum and secretome data for the selection of tumour-associated informative proteins, I performed the same analysis process described above without any initial filtering of the 957 proteins that exhibited significantly differential abundance in sera from patients with PDAC recurrence compared to the norecurrence group. The rationale here was that the entire body reacts to the re-emergence of the tumour so that also changes in the abundance of proteins released to the blood stream by cells and tissues other than the actual tumour could be indicative of disease (Hiam-Galvez et al., 2021; Paul, 2020). Also, the body might sense the tumour earlier than diagnostic processes. As for the tumour-associated proteins, no individual molecules exhibited sufficient accuracy (**Fig. 7B**). A functional annotation of the serum biomarker candidates revealed much more immunologically relevant aspects compared to the tumour-associated functions, which is in agreement with the assumption that a more global response to the tumour is represented in serum.

The biomarker selection process resulted in an SVM classifier of again 8 proteins – ALG2, DNMT1, GCA, IGFBP3, IL23A, SPDL1, TMCC1 and VPS4B. Remarkably, only protein GCA was part of this classifier and the one created from the tumour-associated proteins. In the discovery sample cohort, the marker panel produced an AUC value of 92%, slightly better than the tumour-centred classifier. However, discrimination power was worse upon validation with the abundance data from the independent 60 sera, yielding an AUC value of only 68% (**Tab. 10**). Again, sensitivity was particularly low with a value of 61%.

3.2.3 Combined analysis

With both approaches yielding classifiers of limited performance, I hypothesized that a combination of both may actually improve accuracy. I therefore took the total of 15 protein markers and performed another round of RFE with 5-fold cross-validation. The remaining proteins were used for training an SVM classifier that was made of 10 proteins: ALG2, BRD3, DNMT1, HM13, IL23A, OSMR, PIGK, RIFK, SPDL1 and VPS4B. Applying this protein panel resulted in an AUC value of 85% upon validation (**Fig. 8**), much better than the 67% and 76% obtained earlier. This improvement was particularly due to a much-increased sensitivity of 88% (**Tab. 10**). Five proteins each of the two initial

classifiers were selected by the process, suggesting similar relevance of both tumour-specific and systemic factors. Protein GCA was not included although it had been part of both initial classifiers, indicating that a combination of complementary rather than supplementary aspects is critical for improving the quality of diagnosis.

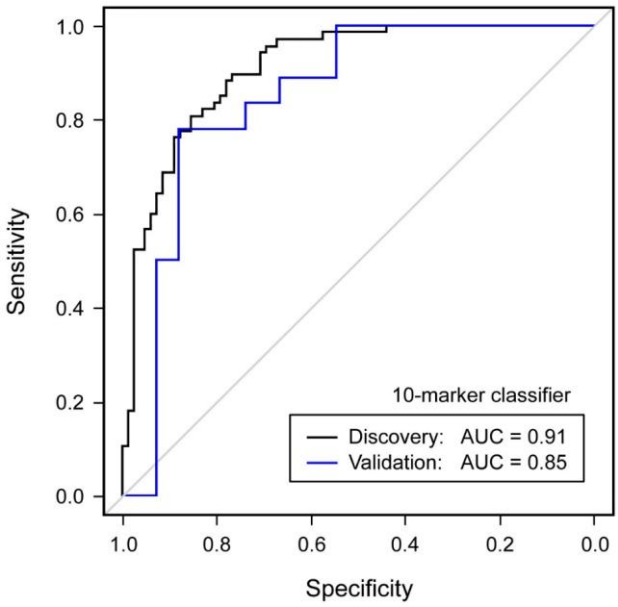

Figure 8. Performance of the 10-protein combined SVM classifier in diagnosing pancreatic cancer recurrence. As determined by the discovery and validation sample cohorts, the results are shown as ROC curves with relevant AUC values.

3.2.4 Early detection of recurrence

Detecting tumour recurrence earlier than currently possible could substantially affect the outcome. If diagnosed early enough, a recurrent tumour could be removed by another surgical intervention resulting in a substantially better prognosis to a patient (Strobel et al., 2013). I used 59 consecutively collected samples from individual patients who had blood samples taken at or close to the dates of their clinical follow-up to see how much earlier the established 10-marker classifier would be able to detect disease compared to clinical diagnosis (**Fig. 9**). An annotation of recurrence was only considered as such, if the following sample or diagnostic analysis confirmed the result. On average, the blood-based classifier detected tumour recurrence nearly 3.5 months earlier than the standard clinical procedures, which included CT imaging.

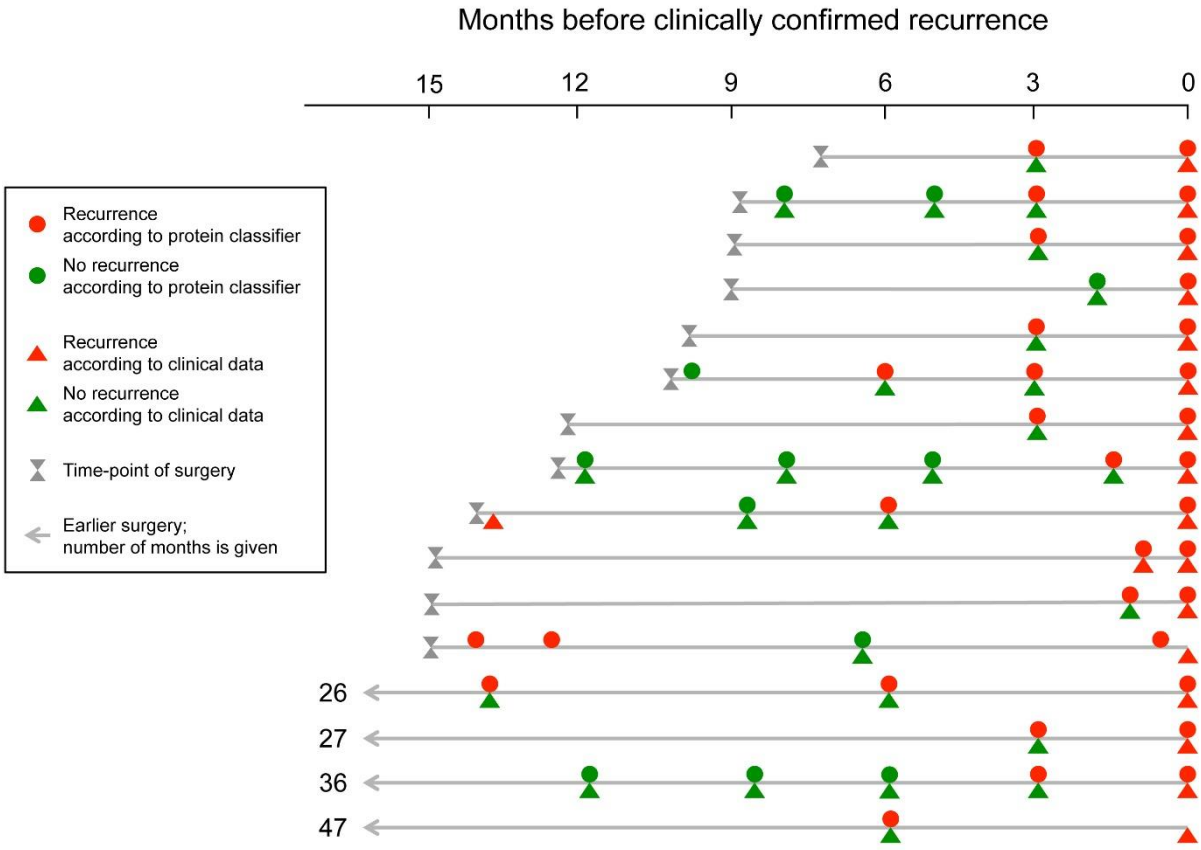

Figure 9. Comparison of detecting recurrence with either the 10-protein classifier or current clinical standard procedures. Several blood samples were drawn from individual patients after tumour resection until recurrence was clinically confirmed. In parallel, standard diagnostics was applied. The time span prior to clinically confirmed recurrence and the respective diagnosis are shown.

3.2.5. Tumour recurrence prediction

Predicting the time of tumour recurrence after surgery could substantially influence PDAC patients' management. It would allow identifying patients at high risk and defining appropriate treatment options (Sugawara et al., 2021). Therefore, in addition to establishing a classifier for diagnosis, I aimed at defining protein signatures that could predict the time to recurrence. To this end, I took again advantage of the consecutively collected samples from individual patients. I defined the date of clinically confirmed recurrence as endpoint and calculated the time from that endpoint backward. Pairwise comparison was performed of serum samples collected at the time of diagnosing recurrence versus those collected prior to recurrence at time intervals of 1 to 3 months, 3 to 6 months, 6 to 12 months, or more than 12 months. The serum analyses yielded 425, 421, 414 and 125 protein biomarker candidates, respectively. As for diagnostics, three approaches were followed, looking for tumour-associated markers, systemic marker molecules or merging the two classifiers for the definition of a combined

predictive protein panel. In most cases, the combined approach produced the best classifier (**Fig. 10**; **Tab. 11**). Only four proteins predicted with high accuracy (AUC values of 99% and 97% in the training and validation analyses) that a tumour could be expected to recur within three months. For the period of 3 to 6 months, a classifier of 7 proteins was required to yield an AUC value of 85%. Only two proteins – NUSAP1 and OTUB1 – were informative as a combined classifier to predict recurrence within 6 to 12 months (**Fig. 10**). However, performance was worse than that of the tumour-centred or the systemic classifier. Most obviously this was due to the very low number of proteins. Therefore, I doubled the number of markers to four by adding the two remaining molecules of the tumour-associated approach (**Tab. 11**) to create a protein panel made of IFNGR1, NUSAP1, OTUB1 and YIPF6, raising the AUC value at validation to 100% (**Fig. 10**). Also, the prediction of a longer-term lack of recurrence (more than 12 months) by a classifier of four proteins was found to be rather accurate with an AUC value of 93% upon validation.

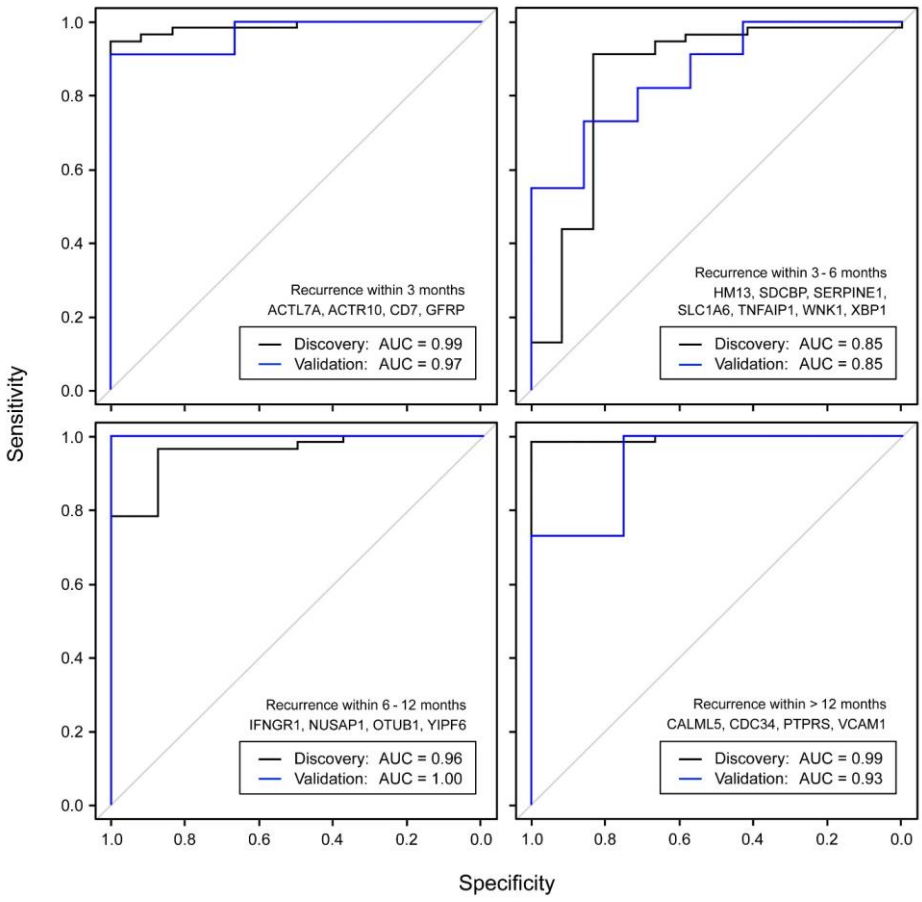

Figure 10. Performance of PDAC recurrence prediction. For the identification of predicting classifiers, pairwise comparisons were done between the protein content of serum samples collected at the time of recurrence versus samples, which had been collected 1-3 months, 3-6 months, 6-12 months, or more than 12 months before. The results, as determined by the discovery and validation sample cohorts, are shown as ROC curves with relevant AUC values.

3.2.6. ELISA validation

No antibody microarray, such as the one used in this study, will be applicable in clinical routine testing. Other immunoassay formats will have to be used instead. ELISA, for example, is well established in clinical diagnostics. To test the robustness of my results, I employed commercially available ELISA kits in order to confirm the variations in serum abundance observed with the antibody microarray analyses. Besides representing a platform that is in widely spread use in clinical diagnostics, the kits also utilised other antibodies targeting the same proteins, thereby checking if bias was introduced that was based on the antibodies. More than 50 individual patient samples were analysed with the respective ELISA. In all cases, high concordance with the data from the antibody microarray analyses was found (e.g., **Fig. 11**). The results nevertheless also exhibited the substantial variation in the abundance of individual markers in different patients, corroborating the need for marker classifiers instead of individual biomarker molecules for an accurate diagnosis. Rather than focussing only on proteins, whose serum levels had been found to vary, I also studied molecules, for which no significant change had been recorded on the microarrays. The ELISAs confirmed these results too (**Fig. 11**), indicating the high degree of reproducibility.

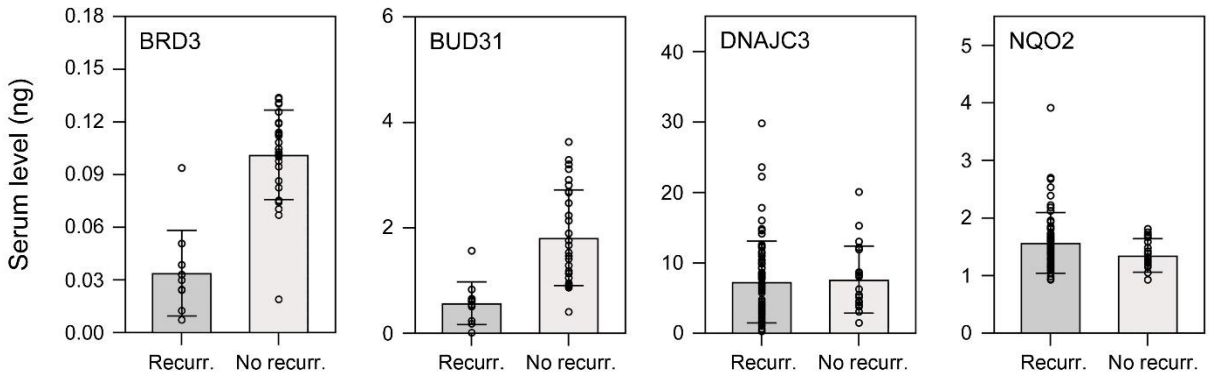

Figure 11. Typical results of validating microarray results with ELISAs. The serum levels of proteins BUD31 and BRD3 were analysed in 16 serum samples from patients with recurrence and 35 samples from patients without recurrence two years after surgery. In addition, the protein levels of DNAJC3 and NQO2 were determined in 35 samples from patients, who had experienced tumour recurrence, and another 21 patients with no recurrent tumour. All analyses were performed with commercially available ELISA kits that used antibodies which are different from the ones used on the antibody microarrays but target the same proteins. In accordance with the microarray results, the variation was highly significant ($p \leq 0.001$) for BUD31 and BRD3, while no significant change could be observed for DNAJC3 and NQO2.

3.3. Serum miRNA analysis

For miRNA analysis, total RNA was isolated from serum samples used for protein analysis. The overall analysis pipeline is shown in **Fig. 12**. Briefly, NGS libraries were prepared using Real Seq Biofluids NGS library Preparation Kit (Biosciences, Santa Cruz, USA). After quality control, the concentration was adjusted to 10 nM/µl. Sequencing was performed in the Core Facility at DKFZ using High-Seq 2000 V4 single read 50 bp. Due to the relatively small number of human miRNAs, 15 samples were sequenced in one lane. The resulting depth of 10 million reads per sample is sufficient for a statistical analysis of the frequency of each miRNA. Sequencing outputs were mapped and annotated to the recent version of miRBase (Kozomara et al., 2019). In total, 1829 precursor miRNAs were detected at least once across all samples. The count reads were filtered for miRNAs that showed at least 10 reads in all samples. Based on these criteria, 850 mature miRNAs were identified across all the samples, which were used for further analysis. To identify miRNA signatures capable of detecting and predicting tumor recurrence, first the miRNA contents of 75 samples was studied and compared serum samples defined with recurrence with those samples without recurrence at respective endpoint. The statistical analysis identified 153 miRNAs that showed a significant difference in the abundance between the two groups (**Fig. 12**).

3.3.1. miRNAs-based recurrence classifier

To the end of defining a PDAC-specific miRNA signature capable to discriminate between samples with recurrence from those without recurrence, the focus was on 100 miRNAs that showed significant P-value on logistic regression analysis **(Fig. 12)**. I first looked at the accuracy performance of individual miRNAs molecules. Although some individual miRNAs showed an accuracy of 90% on the discovery analysis, they accuracy dropped significantly upon validation indicating that the individual information is not sufficient for robust and reproducible diagnosis (**Fig. 13**). Using LASSO and RFE with 5-fold cross validation, a classifier made of 7 miRNAs (hsa-mir-100, hsa-mir-215, hsa-mir-3916, hsa-mir-484, hsa-mir-6752, hsa-mir-6773, hsa-mir-6883) was identified, which were utilized to construct a SVM-based miRNA signature. Interestingly, the 7 miRNAs classifier performance was superior to that of protein signatures achieving 97% accuracy based on the discovery analysis. Upon validation, the classifier accuracy remained at 90% (**Fig. 14 & Tab.12**).

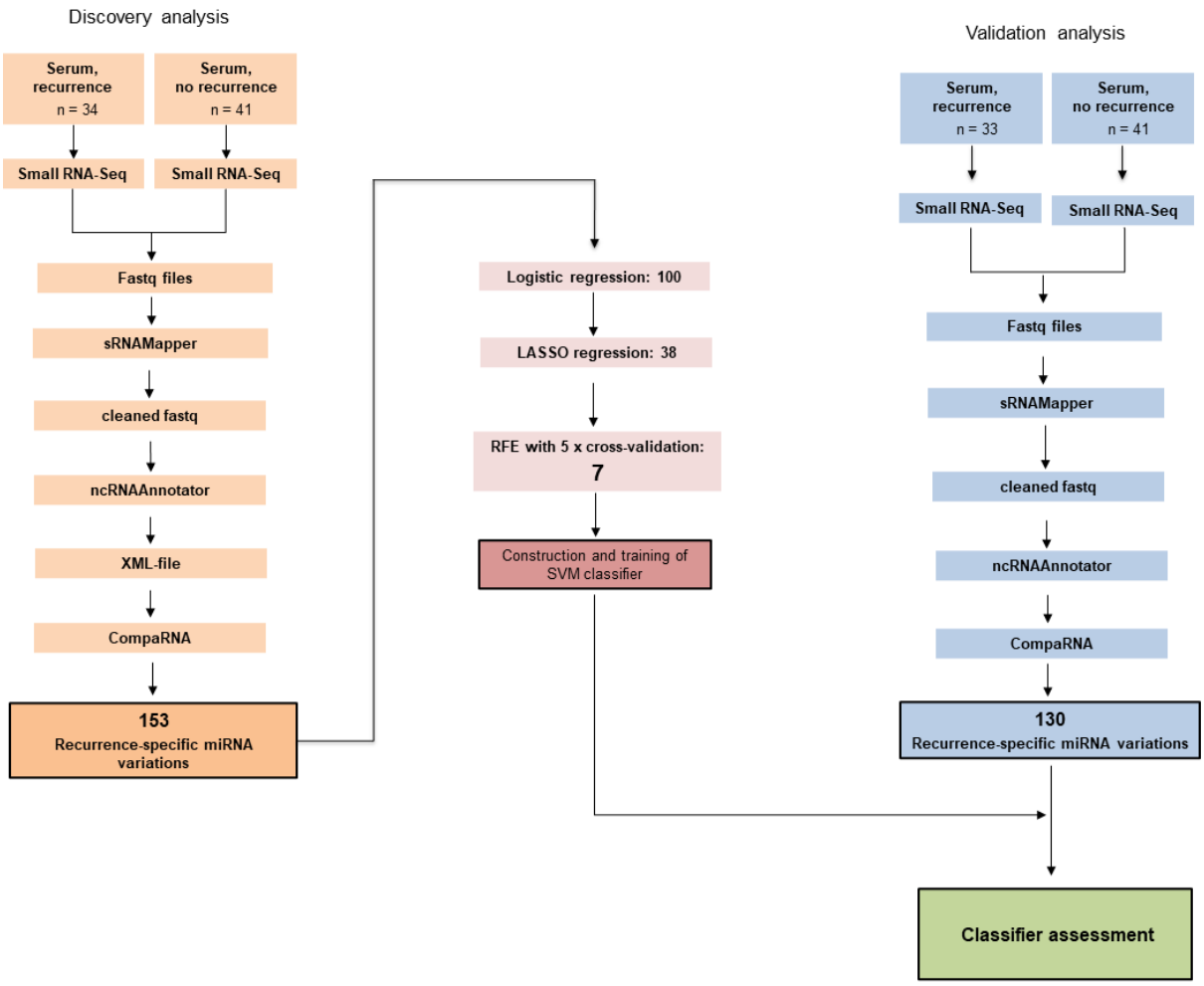

Figure 12. Scheme of the overall miRNAs analysis pipeline. The workflow is shown of the processes for the identification of serum miRNAs classifiers and their validation.

3.3.2. Tumour recurrence prediction using miRNAs

I also aimed to use miRNA information to predict tumour recurrence time taking the advantages of consecutively collected blood samples and detailed clinical information of the patients. The pairwise comparisons of miRNA contents in serum samples collected at the time of recurrence with samples collected in a time frame between 1-3 months, 3-6 months, 6-12 months or > 12 months prior to recurrence yielded 33, 79, 34 and 101 miRNA variations. Using the same approach in **Fig. 12**, a predictive classifier consisted only of four miRNAs (hsa-mir-3620, hsa-mir-3665, hsa-mir-495, hsa-mir-129-1) predicted at robustly reproducible accuracy the recurrence that likely to happen within 3 months' time (**Fig. 15 & Tab.12**). Less accurate but similarly robust and reproducible predictive accuracy was obtained from the model aimed to prognose the recurrence time within 3-6 months. The model consisted of hsa-mir-3614, hsa-mir-184, hsa-mir-218-2, hsa-mir-6752 and yielded 85% accuracy at the discovery

analysis and upon validation (**Fig. 15 & Tab.12**). Out of 34-miRNA variations between samples collected at time of recurrence versus those collected within 6 to 12 months prior to recurrence only three miRNAs namely hsa-mir-3178, hsa-mir-658, hsa-mir-6752 could work together and yielded a moderate accuracy on validation (82% and 70%) (**Fig. 15 & Tab.12**). Predicting tumour recurrence in time-window beyond the 12 months was the most difficult to achieve. Although the 13-miRNA classifier accuracy was 100% in the discovery analysis, the model yielded 80% accuracy upon validation **(Fig.15)**.

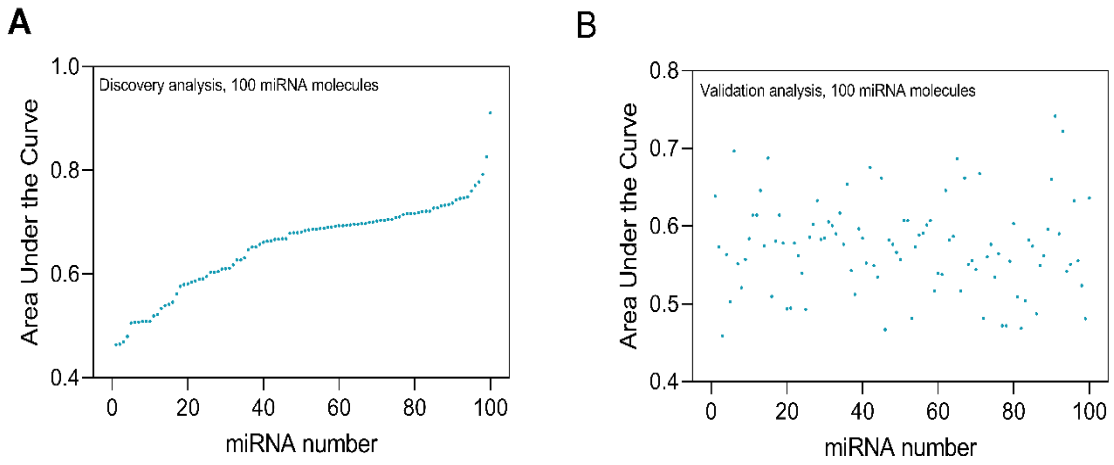

Figure 13. AUC values of individual miRNA markers. ROC curve analyses were performed for miRNA markers individually; the respective AUC values are shown. (A) The left panel (A) shows the results calculated from the discovery analysis. At the right panel (B), the AUC values are shown that were calculated for the same miRNAs using the validation samples. The order of miRNAs is identical to that in the left panel. (B) Identical presentation of the results obtained with 100 miRNAs that showed a significant p value in the logistic regression analysis.

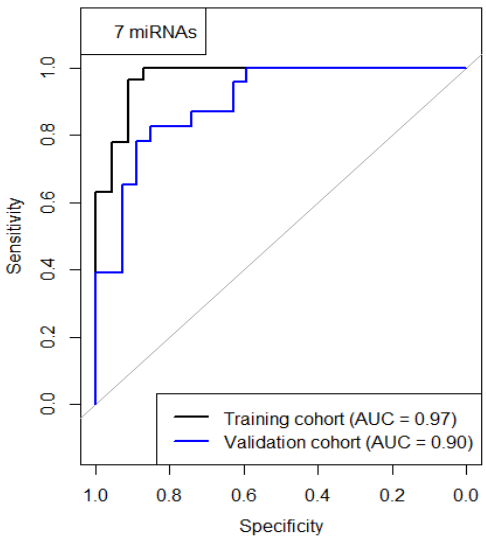

Figure 14. Performance of the 7 miRNAs classifier in diagnosing pancreatic cancer recurrence. Findings are shown as ROC curves and associated AUC values, as determined by the discovery and validation sample cohorts, respectively.

Table 13: List of serum-based miRNA classifiers

Biomarker names	Diagnostic objective	Panel size	SVM classifier parameters	Discovery			Validation		
				Sens	Spec	AUC	Sens	Spec	AUC
hsa-mir-100, hsa-mir-215, hsa-mir-3916, hsa-mir-484, hsa-mir-6752, hsa-mir-6773, hsa-mir-6883-5P	Recurrence vs. nonrecurrence	7	Cost: 3 Gamma: 0.02 Epsilon: 0.08	0.96	0.91	0.97	0.83	0.85	0.90
hsa-mir-3620, hsa-mir-3665, hsa-mir-495, hsa-mir-129-1	Recurrence vs.1- 3 months	4	Cost: 0.1 Gamma: 0.01 Epsilon: 0.9	0.84	1.00	0.90	1.00	0.67	0.90
hsa-mir-3614, hsa-mir-184, hsa-mir-218-2, hsa-mir-6752	Recurrence vs. 3-6 months	4	Cost: 3 Gamma: 0.02 Epsilon: 0.06	0.96	0.67	0.86	0.92	0.68	0.85
hsa-mir-3178, hsa-mir-658, hsa-mir-6752	Recurrence vs. 6-12 months	3	Cost: 0.4 Gamma: 0.04 Epsilon: 0.01	0.67	1.00	0.83	0.60	0.83	0.70
hsa-let-7i, hsa-mir-127, hsa-mir-1273d, hsa-mir-1303, hsa-mir-133a-1, hsa-mir-133a-2, hsa-mir-134, hsa-mir-1976, hsa-mir-486-1, hsa-mir-566, hsa-mir-654, hsa-mir-7108, hsa-mir-7851	Recurrence vs.> 12 months	13	Cost: 9 Gamma: 0.1 Epsilon: 0.8	1.00	1.00	1.00	1.00	0.80	0.82

AUC: area under the curve; ROC: Receiver Operating Characteristics; RFE: Recursive Feature Elimination; SVM: support vector machine.

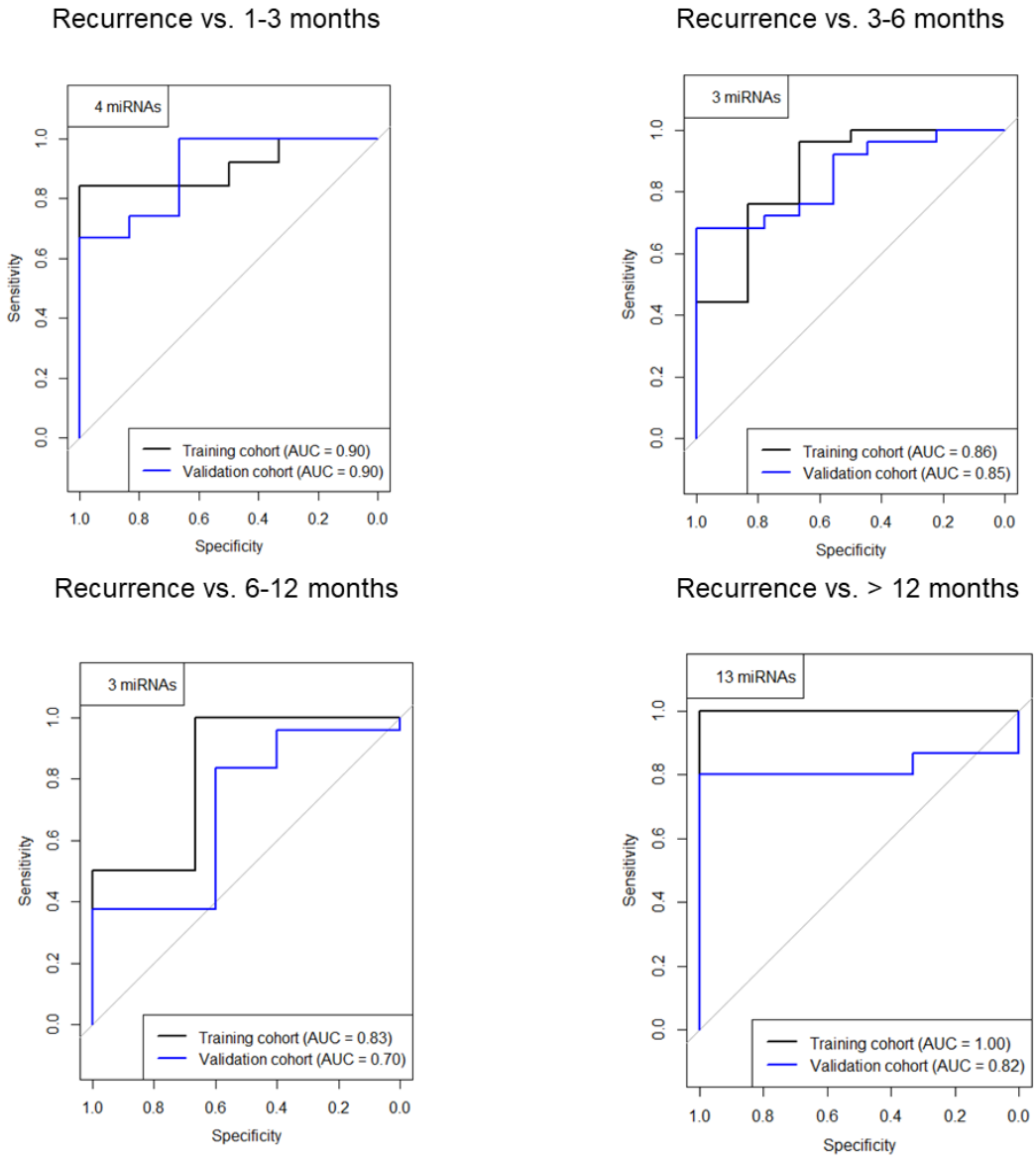

Figure 15. Performance of miRNAs recurrence prediction classifiers. For the identification of predicting classifiers, pairwise comparisons were done between the protein content of serum samples collected at the time of recurrence versus samples, which had been collected 1-3 months, 3-6 months, 6-12 months, or more than 12 months before. The findings are shown as ROC curves and related AUC values that were obtained from the discovery and validation sample cohorts, respectively.

3.3.3. RT-PCR validation

I aimed at confirming the clinical utility of miRNAs signatures described here using quantitive RT-PCR. The rationale was demonstrating the usefulness of simple, cheap, and robust qRT-PCR-based assays that could be easily translated in clinical settings. Also, for the purpose of technical validation of our observations from the small RNA sequencing. I utilized miRCURY LNA to validate the abundance variations of hsa-mir-625 and hsa-mir-6883-5P. Locked Nucleic Acid (LNA) technology allows an

effective and highly accurate discrimination between different miRNA molecules (Rasmussen and Roberts, 2007). In concordance with the data obtained from small RNA sequencing, high variations in the abundance of has-mi-6883-5P between recurrence and non-recurrence was observed while no significant difference was seen in the sera contents of has-mir-625 (**Fig. 16**).

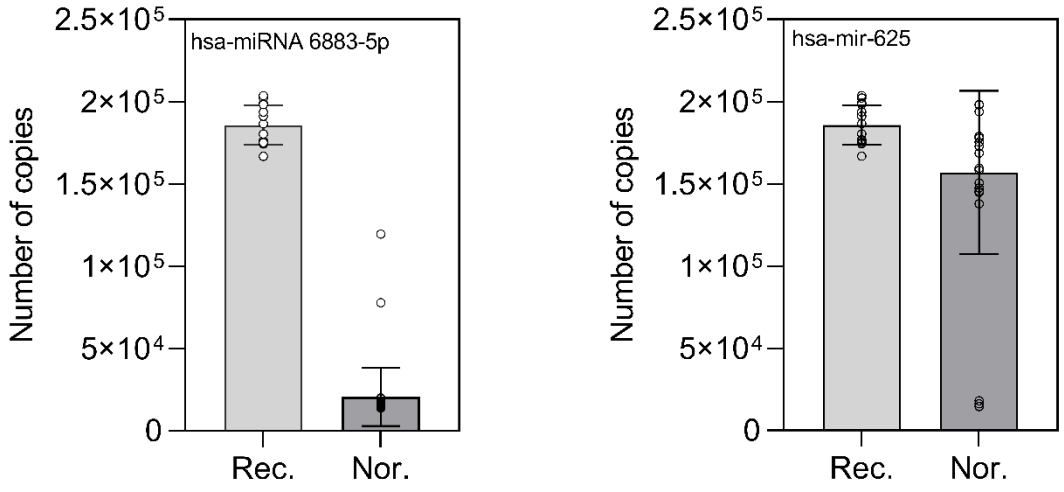

Figure 16. Typical results of validating miRNAs with RT-PCR. The serum levels of has-miRNA 6883-5P and has-miRNA-625 were analysed in 15 serum samples from patients with recurrence and 35 samples from patients without recurrence two years after surgery. In accordance with the small RNA sequencing, the variation was highly significant ($p \leq 0.0001$) for has-miRNA 6883-5P, while no significant change could be observed for has-miRNA 625.

3.4. Combining miRNA and proteins for early detection of tumour recurrence in PDAC

Although the discrimination between recurrence from nonrecurrence samples achieved relatively high accuracy using either a protein or miRNA classifiers, the number of molecules included in the signatures is relatively high (10 for protein signature and 7 for miRNA classifier). Also, for clinical use, it will be easier to analyse less marker molecules assuming that they are sufficiently informative. Our hypothesis was that a multi-parametric assay might improve further the accuracy of the assay assuming that the information from miRNAs would supplement or complement the diagnostic information obtained from the protein biomarkers. Therefore, I tried to develop a classifier that incorporates differentially abundant proteins and miRNAs to further enhance the performance. The 17 marker molecules were combined and Recursive Feature Elimination with 5-fold validation was used to find the best combination. A four markers classifier made of proteins BRD3, PIGK in combination with

hsa-mir-6773, hsa-mir-6883-5P yielded an accuracy of 91% and 83% on the training and validation cohort respectively **(Fig. 17).**

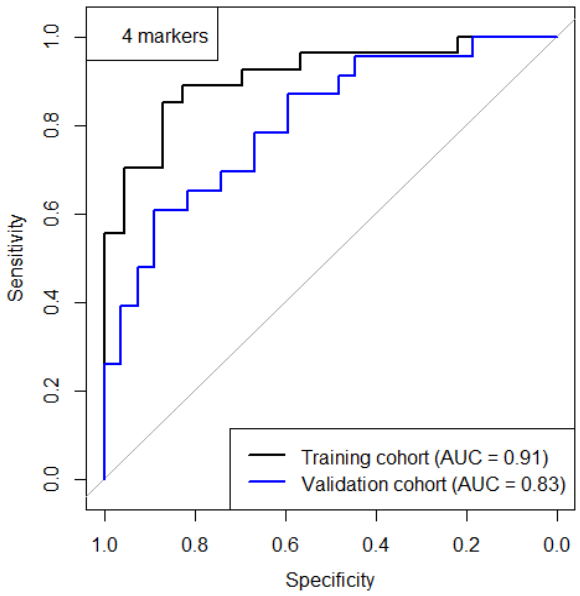

Figure 17. Diagnostic ability to distinguish between samples with recurrence and samples without recurrence using a panel of two proteins and two miRNA biomarkers. As determined by the training and validation cohorts, the results are shown as ROC curves and related AUC values.

4. DISCUSSION

Tumour resection is the best treatment option for PDAC patients at tumour stages I or II. Since the majority of patients experience a relapse in the following two years, it is crucial to have robust and effective diagnostic means to detect recurrence accurately and while the tumour is still small enough to be surgically removed again with then high chances of good outcome (Strobel et al., 2013). Post-operative monitoring of PDAC patients currently includes CT scans and testing of CA19-9 levels (Tempero et al., 2017) but frequently fails to detect a recurrent tumour early (Daamen et al., 2018a, 2018b). Moreover, homozygous mutations in the gene FUT3 result in a lack of fructosyltransferase activity, which prevents CA19-9 from being detected in 5–10% of patients. (Yue et al., 2011). Due to its accessibility and durability, blood is a desirable source for biomarkers. As a result, over the past ten years, a tremendous amount of liquid biopsy research has been conducted on blood-based biomarkers(Mattox et al., 2019). Serological biomarkers also have the benefit of being able to be longitudinally evaluated over the course of an illness. Yet, a challenge could be that not all molecular markers react right away to the excision of the main tumour or reappear upon recurrence.

Here, I firstly investigated the diagnostic utility of changes in protein abundance in serum. The non-invasiveness of blood-based diagnosis ensures minimal impact on the patient and thereby permits its repeated application and thus a close monitoring of disease progress. The analysis would fit into the existing clinical diagnostic environment. Two approaches were executed and evaluated – analysis in a tumour-centred or a more systemic manner – for their potential to identify relevant protein biomarkers. Both yielded similarly effective protein classifiers. By combination of the two, better performance was achieved even with only 10 of the 15 proteins of the original classifiers, indicating the value of combining complementary rather than supplementary factors for enhancing and refining diagnostic performance. Similar effects have been reported by combining biomarkers of different molecular classes toward the same end (Al-Shaheri et al., 2021). The point was further emphasized by the fact that the one protein contained in both the tumour-centred and the systemic classifiers was not part of the final, combined protein marker signature.

A validated AUC value of 85% would not be sufficient for randomly screening individuals with no risk or strong suspicion of disease. However, the specifications for diagnostic precision are strongly dependent on the overall setting and diagnostic objectives. Basically, all PDAC patients, who undergo resection of the primary tumour, will experience recurrence and usually within some two years after surgery. In consideration of the dire consequences if recurrence is diagnosed too late, picking it up with the accuracy achieved by this assay could make a big difference in patient survival. Of a more tentative nature is the average time span of about 3.5 months by which the protein classifier detected tumours earlier than current processes. While the result is encouraging, more analyses have to be performed in order to refine and confirm it. The time gained by the protein classifier seems to differ substantially between patients. Many factors and parameters, such as size and location of the resected tumour, were not taken into consideration but may make a significant difference. Additionally, the presence of co-existing clinical diseases such as diabetes and obstructive jaundice could have an effect. For confirmation, a study is required that consists of more samples so as to check for any co-founding factors.

The studies described here were done with serum samples from a single clinical source: the EPZ at the surgery department of Heidelberg University Clinics. For classifier development, I used selected, well-annotated samples that had been collected and stored for research purposes over several years. On purpose, however, I used blood samples for validation that were collected during ambulant outpatient visits that are part of the routine follow-up at EPZ after tumour resection, lacking prior knowledge about sample quality or patient outcome. These samples represent the clinical situation in which any classifier has to perform. This may have contributed to the slightly lower AUC values upon validation. All variations due to reasons other than biology, such as sample handling, should be minimal since only samples from EPZ were used. Prior to any clinical application, the classifiers need to be validated in a wider, multicentre study in order to accommodate also aspects such as different handling procedures. Nevertheless, the study results are likely to be robust since the data was evaluated on an adequate number of samples for both marker identification and validation. In addition, the technical confirmation with ELISA using other antibodies in a different assay format confirmed the solidity of the results.

The annotation of samples as “recurrent” or “non-recurrent” was based on the standard diagnostic processes applied in clinical routine. We cannot exclude that samples may have been falsely annotated as “non-recurrent”, for example, since the tumour was missed for technical limitations of routine diagnostics. This would introduce a bias insofar as the distinction of the samples into two groups, which were used to define the protein signatures, would be less stringent and thus affect the discrimination power of the resulting classifiers. This possible bias, however, would reduce the apparent accuracy of our analysis, so the actual accuracy would be higher than what is reported here.

Besides diagnosis, the protein abundance variations also allowed at high accuracy to predict the period until recurrence is likely to happen. In combination with the ability of detecting actual recurrence, this could have substantial consequences on patient management. For example, blood samples could be collected at longer or shorter intervals, which are adapted to the time-frame predicted for the respective patient. Thus, the results could have an immediate impact on individual patients. Besides using the scheme of training and validation in the assay development, I tested four markers molecules using ELISA that utilized independent antibodies. The significant and non-significant observations I have seen in the antibody microarray were confirmed by ELISA illustrating high degree of reproducibility. Furthermore, ELISA is established technique in clinical laboratories, and this could facilitate the translation of the results into clinical practice.

Next to analysing serum proteins, I used Next Generation Sequencing for profiling the miRNAs variations in order to define recurrence-specific miRNA signatures. Although NGS is powerful technology for miRNA expression profiling, poor accuracy is likely to happen due to biases that stem from sequence-dependent variability in the enzymatic ligation reactions (Sorefan et al., 2012). To reduce the bias risk, miRNA molecules were ligated using a single adapter, circularized, and circular fragments were selected using magnetic beads. This process greatly reduced biases in library preparation and sequencing, allowing the identification of a large variety of miRNAs from only 200 µl serum and increased the sequencing output 1,000-fold (Barberán-Soler et al., 2018). I improved the protocol further to get rid of adapter dimers as well as other fragments that may affect the sequencing depth or introduce sequencing bias. To this end, I ran libraries for 2 hrs on 4% agarose gel and the libraries corresponding to miRNAs (143bp) only were excised and extracted from the agarose gel. Libraries qualities were

checked by two independent methods: First, at the division of functional genome analysis utilising the Bioanalyzer 2100 and DNA high sensitivity kit and second, at the sequencing core facility of the DKFZ using tap-station. Only libraries of high quality were quantified and pooled. Since it is unlikely that a single marker would be enough for accurate diagnosis looking at the AUC values of individual miRNAs markers, a combinatory of several molecules is a better alternative. A classifier consisted of 7 miRNAs was constructed and trained with all parameters being optimized to avoid under or over fitting. To confirm the utility and reproducibility of the data generated in the discovery cohort, I analysed the miRNA contents of serum samples from independent using the same protocol. The 7-marker classifier was tested with all parameters being fixed. Remarkably, the classifier achieved high accuracy upon validation indicating that the classifier is reproducible and robust and that there was no under or overfitting problem in the SVM model. The classifier sensitivity and specificity are very high that warrant clinical application. Since miRNAs are stable marker molecules, testing can be easily performed. I confirmed the utility and applicability of RT-PCR based-testing as well.

Using longitudinally collected serum samples together with detailed monitoring information, I established miRNA-based algorithms for predicting the time when recurrence is likely to happen. The prognostic information derived from these classifiers would substantially guide oncologists to draw appropriate personalised treatment plans for individual patients. The prediction models developed in this dissertation would improve substantially the risk assessment and survival of PDAC patients. Patients could be classified as high risk, if recurrence is predicted to likely happen within the next 3-6 months. In this case, a closer follow up and treatment plans should be adjusted to the risk level. In contrast, patients in whom non-recurrence is predicted, unnecessary invasive procedures should be avoided. In comparison to other clinical (e. g NCCN guidelines) and pathological process, the classifiers are more accurate in the recurrence risk assessment providing prognostic information about the disease progression in the next 2 years. In other words, the classifiers would provide optimal indication for surgical re-resection of the tumour while it is still small, substantially improving the patients' survival. They could also help oncologists to make decisions to continue chemotherapy or not and reduce ineffective and invasive surgeries performed for occult metastases. Since the panels can be used at multiple time points, this will allow clinicians to continuously evaluate the patients' clinical course.

A review published in the journal Cancer Treatment Review comprehensively summarized and compared biomolecules in many investigations (Al-Shaheri et al., 2021). Global processes for biomarker discovery processes, such as mass spectrometry or antibody-microarrays, analysed few thousand molecules (Hoheisel et al., 2013; Liu et al., 2017), which is only a small percentage of the total number of produced proteins assuming that one gene only encodes one protein, which is not a true assumption in fact. Therefore, many studies have no or only little overlap with others. Furthermore, it is likely that different sample preparation protocols and detection methodologies introduced discrepancies between studies, although it is difficult quantify this. Comparing miRNA content in whole-blood samples with that of plasma (Johansen et al., 2016; Schultz et al., 2014) highlights that effect this can have. Blood circulation throughout the body is a biological, not a technical, difficulty, since biomarker levels changes seen in the cancer secretome are more difficult to detect in blood as many other cells besides the tumour cells release the same chemicals, masking the result. This influence could also be different from one person to the other. Blood collection via the portal vein may solve the issue, however there is currently little evidence for this. (Chapman et al., 2019) and such approach would be even more difficult and invasive.

Another barrier to clinical utility is a concrete definition of biomarkers. The use of diagnostics that are more binary in nature, such as those that are present in cancer but absent in healthy or vice versa, or that look for specific mutations, methylation variations in DNA or RNA, or the presence of circulating tumour cells or protein isoforms, may improve their accuracy. Moreover, a significant fluctuation in a certain molecule's concentration could be employed as a marker. (Brosseau, 2018). As DNA methylation has proven to be an effective diagnostic tool for identifying other tumour entities, it may potentially be a good candidate for recurrence although there isn't much information accessible right now. It is challenging to make an accurate diagnosis using ctDNA and CTC analyses because of insufficient sensitivity and specificity. Nonetheless, they may be beneficial for monitoring recurrent diseases, prognosis, and risk stratification(Pécuchet et al., 2016). Such molecules may also be suitable for reliable diagnosis once a profiling procedure by alternative ways that decreases the degree of freedom for the specific patient that is being looked at (Neumann et al., 2018). CTCs have mostly been employed in cell-based analysis for recurring tumour identification, prognosis, and treatment monitoring. Yet,

numerous cell types both inside the microenvironment and far outside the histological tumour boundaries undergo significant molecular changes (Bauer et al., 2018). Once procedures for their enrichment alongside that of tumour cells are in place, they might also be used for diagnostic purposes, giving a much more detailed and individualized image of the particular tumour.

Exploring the molecular underpinnings of PDAC recurrent development is a critical first step in the identification and evaluation of biomarkers. Instead of concentrating on internal pathways and processes, attention should be paid to intercellular communication and substances that affect a recipient cell after uptake in the development of blood-based molecular diagnostics. For discriminatory detection, the relevant molecules are more likely to end up in the blood when present in appropriate quantities. Since PDAC is a highly aggressive, detecting these changes early in the carcinogenesis process- possibly before the tumour relapses—is essential for successfully catching recurrent tumours. Also, more emphasis should be given to how co-occurring clinical diseases like diabetes and obstructive jaundice affect the performance of biomarkers (Botla et al., 2016; Rhim et al., 2012).

Molecular signature will be adopted in clinical routine diagnosis only, if its performances is obviously superior to the existing procedures, particularly CA19-9, and if it is available in a manner that matches the current testing processes. Given the apparent molecular diversity of PDAC, a marker panel is probably necessary for such accuracy and robustness, creating a new challenge. However, it could be helpful to make incremental progress in the direction of the requested precision for clinical value. To do this, it is important to define the diagnostic objectives clearly, moving from the simplest ones—like recurrence—through the more challenging ones, such early primary tumour diagnosis.

5. CONCLUSIONS

For detecting tumour recurrence following surgical excision we deal only with a very narrowly defined patient population, thus tumour specificity is not a concern. Moreover, most patients experience recurrence within two years of surgery. Hence, even a relatively unreliable diagnosis of recurrence can be enough to justify clinical action. The analysis of the protein and miRNA contents of blood permits prediction and detection of tumour recurrence in PDAC patients after curative surgery with an accuracy that substantially surpasses the performance of currently used processes, in particular CA19-9 testing. Serum proteins analysis indicated the existence of changes that are either due to the tumour's presence or based on the body's systemic reaction to it. Combining both miRNA and proteins reduced the number of molecules required to achieve an accurate and robust diagnosis. The results could have a direct and immediate benefit for patients with pancreatic cancer and could be translated to clinical practice quickly. In addition, the process could proof the applicability of the signatures for early diagnosis of the primary tumour. Thus, the results could be applied to screening individuals who are at high risk of pancreatic cancer, potentially having a clinical impact beyond the detection of tumour relapse. In addition, the established assays could serve as a means for monitoring disease progression during chemotherapeutic treatment. Further studies are required to confirm the clinical utility of the biomarkers taking into the account other cofounding factors. Prior to any clinical application, the classifiers need to be validated in a wider, multicentre study in order to accommodate also aspects such as different handling procedures.

I propose a pipeline consisting of triple-meta-analysis approach for transition from laboratory-based research toward translational investigations that are focused on the actual requirements in clinical settings: The best markers within each molecular category are first chosen. Second, all marker types are integrated to define the overall most informative markers; for example, they might eventually just include a small number of marker types, one molecular class, such proteins and miRNAs, or a combination of both classes. Thirdly, the data is contrasted with comparable information received from different tumour entities. The outcome of this procedure should then be verified in a multi-centre study using a huge variety of distinct samples that are obtained under standard clinical circumstances. These particular processes should be standardised using procedures that are already established in routine diagnostics. However, technical improvements should be taken into consideration to improve

performance. However, the current scientific environment is not set up to make it simple for this method to exist halfway between research and clinic. Also, a study of this nature is typically viewed as being quite unimportant and unfancy enough to receive the scalable funding required for such accomplishment. Yet, without such a procedure, it is highly improbable that the very numerous marker molecules discovered in research projects can be successfully chosen and translated into a practical diagnostic procedure that can be used on patients.

ZUSAMMENFASSUNG

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Title der Dissertation: Early detection of tumour recurrence in pancreatic cancer patients by the detection of specific microRNA and protein signatures in peripheral blood

Fach: Experimental Chirurgie, Humangenetik und Molekular Pathologie

Doktorvater: Prof. Dr. med. Thilo Hackert

Ziel der Dissertation war eine blutbasierte Vorhersage und Früherkennung eines Tumorrezidivs bei Patienten zu etablieren, die sich einer Resektion des duktalen Adenokarzinoms der Bauchspeicheldrüse (PDAC) unterzogen hatten. Unter Verwendung eines Mikroarrays mit 2.977 Antikörpern Variationen im Proteingehalt von Serumproben festgestellt, die von 101 Patienten entnommen wurden, bei denen ein Tumorrezidiv aufgetreten war oder nicht, einschließlich nacheinander entnommener Proben derselben Patienten. Sekretomanalysen von nicht-tumorösen und Krebszellen zeigten tumorbedingte Variationen. Ausgewählte Biomarker wurden verwendet, um Support-Vektor-Maschinen-Klassifikatoren zu trainieren. Sie wurden an neuen, prospektiv gesammelten Proben von 36 Patienten validiert, um die Anwendbarkeit zu dokumentieren. Durch die Kombination von Biomarkern, die sowohl durch einen fokussierten tumorzentrierten Ansatz als auch durch eine breite angelegte systemische Analyse ausgewählt wurden, wurde ein Klassifikator von 10 Proteinen definiert, der Patienten mit Rezidiv von Patienten ohne Rezidiv mit einer Genauigkeit von 91 % unterscheidet. Die Validierung an prospektiv gesammelten Proben erreichte eine Genauigkeit von 85 %. Die Rezidiv Erkennung erfolgte im Durchschnitt 3,5 Monate früher als bei aktuellen Verfahren. Neben der Diagnose wurden Proteinsignaturen ermittelt, die es erlauben, den Zeitraum vorherzusagen, nach dem eine Rezidiv des Tumors wahrscheinlich ist.

Ich untersuchte auch den miRNA-Gehalt von 149 Serumproben mittels Small-RNA-Sequenzierung. Für die Entdeckungsphase wurden 75 Serumproben analysiert. Sie wurden in der Sequencing Core Facility des DKFZ sequenziert. Insgesamt wurden 135 miRNA-Variationen zwischen Rezidiv- und Nicht-Rezidiv und mit Logistik Regression, LASSO-Regression und RFE mit 5-Fold Kreuzvalidierung zur Auswahl informativer miRNA-Biomarker und zum Entfernen unnötiger

Kovariaten getestet. MiRNA-Klassifikator von hsa-mir-100, hsa-mir-215, hsa-mir-3916, hsa-mir-484, hsa-mir-6752, hsa-mir-6773, hsa-mir-6883-5P wurde konstruiert und trainiert. Die Algorithmus-Parameter wurden optimiert, um eine Über- oder Unteranpassung zu vermeiden. Ich habe die Signatur in einer unabhängigen Kohorte validiert, wobei alle Parameter festgelegt wurden. Bemerkenswerterweise konnte der miRNA-Klassifikator in der Entdeckungs- bzw. Validierungskohorte mit einer Genauigkeit von 97 % bzw. 91 % zwischen Rezidiv und Nicht-Rezidiv Proben unterscheiden.

Die Kombination von miRNAs und Proteinen erfolgte unter Verwendung der Proben, die gleichzeitig durch Antikörper-Microarray und Small-RNA-Sequenzierung getestet wurden. Die Daten wurden zufällig in eine Trainings- und Validierungskohorte aufgeteilt und RFE mit 5-Fold Kreuzvalidierung wurde auf 17 miRNA- und Proteinmarker angewendet. Unter Verwendung von nur vier Marker Signaturen, zwei miRNAs und zwei Proteinen konnte ich das Rezidiv der Bauchspeicheldrüsenkrebs mit einer Genauigkeit von 91 % nachweisen. Die Signaturgenauigkeit betrug bei der Validierung 83 %.

Die Analyse des Protein- und miRNA-Gehalts im Blut erlaubt die Vorhersage und den Nachweis eines Tumorrezidivs bei PDAC-Patienten nach kurativer Operation mit einer Genauigkeit, die die Leistung derzeit verwendeter Verfahren, insbesondere des CA19-9-Tests, deutlich übertrifft. Die Analyse zeigte auch das Vorhandensein von Veränderungen, die entweder direkt auf das Vorhandensein des Tumors zurückzuführen sind oder auf der systemischen Reaktion des Körpers darauf beruhen. Die Kombination von miRNA und Proteinen reduzierte die Anzahl der Moleküle, die für eine genaue und robuste Diagnose erforderlich sind. Die Ergebnisse könnten einen direkten und unmittelbaren Nutzen für Patienten mit Bauchspeicheldrüsenkrebs haben und schnell in die klinische Praxis übertragen werden. Darüber hinaus konnte das Verfahren die Anwendbarkeit der Signaturen für die Früherkennung des Primärtumors belegen somit könnten die Ergebnisse auf das Screening von Personen angewendet werden, die ein hohes Risiko für Bauchspeicheldrüsenkrebs haben, was möglicherweise über die Erkennung eines Tumorrezidivs hinaus klinische Auswirkungen haben könnte. Darüber hinaus könnten die etablierten Assays als Mittel zur Überwachung des Krankheitsverlaufs während einer chemotherapeutischen Behandlung dienen.

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Zhu, H., Wang, D., Zhang, L., Xie, X., Wu, Y., Liu, Y., Shao, G., Su, Z., 2014. Upregulation of autophagy by hypoxia-inducible factor-1α promotes EMT and metastatic ability of CD133+ pancreatic cancer stem-like cells during intermittent hypoxia. Oncol. Rep. 32, 935–942. https://doi.org/10.3892/or.2014.3298

PERSONAL CONTRIBUTION TO DATA ACQUISITION

At the beginning of my Ph.D., I did a systemic analysis of the literature on blood-based PDAC diagnosis. As a result, the review article has been published in *Cancer Treatment Reviews*.

FN Al-Shaheri, MSS Alhamdani, AS Bauer, N Giese, MW Büchler, T Hackert, JD Hoheisel. **Blood biomarkers for differential diagnosis and early detection of pancreatic cancer.** *Cancer Treatment Reviews*. 2021. 96, 102193.

Furthermore, an original research article manuscript based on the results **3.1** and **3.2** is currently under review at *Gastroeneterology*. I conceptualized and planned this work, performed 90% of the experimental work, did 90% of the bioinformatic and statistical analysis and about 80% of data assessment and interpretation. Writing the initial manuscripts was also done by me.

FN Al-Shaheri, T Colbatzky, C Zhang, MSS Alhamdani, AS Bauer, H Boekhoff, L Xu, MW Büchler, T Hackert, L Sperling, M Schenk, C Tjaden, N Giese, JD Hoheisel , U Heger. **Blood-based protein classifier for predicting and early detecting recurrence of pancreatic ductal adenocarcinoma after tumour resection – combining a tumour-centred and a systemic approach.** *Gastroeneterology*, under-review.

The results of chapter **3.1** and **3.3** and **3.4** is the basis of another original article manuscript. My task here was again project conceptualization and planning besides designing and performing 95% of the experimental work and 90% of the bioinformatic and statistical analysis. I am also writing the initial manuscript.

FN Al-Shaheri, A Hotz-Wagenblatt, C Zhang, T Colbatzky, AS Bauer, MW Büchler, T. Hackert, N. Giese, U. Heger, and JD. Hoheisel. **MicroRNAs as liquid biopsies for early detection and prediction of tumour recurrence in pancreatic cancer after surgery**. *In preparation.*

I published the following first-author paper during my Ph.D. time:

FN Al-Shaheri, KM Al-Shami, EH Gamal, AA Mahasneh, NM Ayoub. **Association of DNA repair gene polymorphisms with colorectal cancer risk and treatment outcomes.** *Experimental and Molecular Pathology*.2020. 113, 104364.

I also have co-authored the following publication during my Ph.D. time:

C Zhang, **FN Al-Shaheri**, MSS Alhamdani, AS Bauer, JD Hoheisel, M Schenk, U Hinz, P Goedecke, K Al-Halabi, MW Büchler, N Giese, T Hackert, S Roth; **Blood-based diagnosis and risk stratification of patients with pancreatic intraductal papillary mucinous neoplasm (IPMN)**. *Clin Cancer Res*. 2023. doi: 10.1158/1078-0432.CCR-22-2531.

In addition, I presented my work in the following national and international conferences in the following formats:

Talks:

FN Al-Shaheri, MSS Alhamdani, AS Bauer, N Giese, MW Büchler, T Hackert, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. *International Ph.D. Student Conference*, Glasgow, UK, 2021.

FN Al-Shaheri, MSS Alhamdani, AS Bauer, N Giese, MW Büchler, T Hackert, JD Hoheisel. Early detection of tumor recurrence PDAC patients by specific protein signature in peripheral blood. DKFZ *Ph.D. Students Retreat*, Heidelberg, Germany, 2021.

Posters Presentations**:**

FN Al-Shaheri, T. Colbatzky, MSS Alhamdani, AS Bauer, T Hackert, N Giese, MW Büchler, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. *EACR Liquid Biopsies*. Conference, Bergamo, Italy, 2022.

FN Al-Shaheri, MSS Alhamdani, MW Büchler, AS Bauer, N Giese, T Hackert, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. ***International Ph.D. Student Conference***, Heidelberg, Germany, 2022.

FN Al-Shaheri, MSS Alhamdani, N Giese, AS Bauer, MW Büchler, T Hackert, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. *DKFZ Poster Presentation*, Heidelberg, Germany, 2021.

FN Al-Shaheri, T. Colbatzky, AS Bauer, N Giese, MW Büchler, MSS Alhamdani, T Hackert, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. *EACR Liquid Biopsies Conference*, Online, Germany, 2021.

FN Al-Shaheri, MSS Alhamdani, AS Bauer, N Giese, MW Büchler, T Hackert, JD Hoheisel. Early detection of tumour recurrence PDAC patients by specific protein signature in peripheral blood. *Functional and Structural Genomic Retreat*, Schöntal, Germany, 2020.

APENDICES

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Geschäftsleitung:
Dr. med. Verena Pfeilschifter

24.10.2019
ts-cd/bw

BERUFSRECHTLICHE BERATUNG

Unser Zeichen: **S-708/2019 (Bitte stets angeben)**
Titel: **Aufbau einer Blutzellen-, Serum- und Gewebebank für molekularbiologische Untersuchungen und zur Etablierung diagnostischer und prognostischer Marker benigner und maligner Erkrankungen des Gastrointestinaltraktes und der endokrinen Organe**

Sehr geehrter Herr Professor Büchler,

die Ethikkommission hat Ihr Forschungsvorhaben in der Sitzung am 21.10.2019 beraten und hat keine Bedenken gegen die Durchführung der Studie.

Sie gibt jedoch folgende Empfehlungen bzw. Hinweise:

<u>Allgemein:</u>

1. In diese neue Biomaterialbank soll die Gewebebank (S-301/2001) und die Blutzellen und Serumbank (S-159/2002) integriert werden. Es wird darauf hingewiesen, dass diese bestehenden Biomaterialien nur in dem Umfang verwendet werden dürfen, wie es in der damaligen Informationsschrift und Einwilligungserklärung beschrieben wurde. Auch die Aufbewahrungszeit ist zu beachten.
2. Die angegebene Aufbewahrungsdauer der Daten und Proben in der Biobank auf *„unbestimmte Zeit"* ist sehr ungenau. Wenn möglich, ist eine konkrete Angabe zur Aufbewahrungsdauer anzugeben (oder ggf. Kriterien für die Festlegung der Aufbewahrungsdauer (vgl. Art. 13 Abs. 2 lit. a DSGVO).

<u>Patienteninformation:</u>

3. Es ist den Patienten deutlicher zu erläutern, dass es sich um eine Gewebe- und Serumbank handelt. Daher sollte nur eine Bezeichnung verwendet werden, um die Patienten nicht zu verunsichern. Alle anderen Bezeichnungen wie „neue Bank", nur „Gewebebank" oder „Pancobank" sind zu streichen.

Wir wünschen Ihnen bei der Durchführung der Studie viel Erfolg.

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22.03.2021
ts-bw/al

BERUFSRECHTLICHE BERATUNG
Zweitschrift – ersetzt die Berufsrechtliche Beratung vom 19.03.2021; Änderung des Titels

Unser Zeichen: **S-083/2021 (Bitte stets angeben)**

Titel: Alter Titel: **Retrospektive Korrelation von klinischen und molekularen Parametern zur Identifizierung von neuen Biomarkern und therapeutischen Targets beim Pankreaskarzinom und deren Validierung mittels Organoid-Kulturen**
Neuer Titel: **Korrelation von klinischen und molekularen Parametern zur Identifizierung von neuen Biomarkern und therapeutischen Targets beim Pankreaskarzinom und deren Validierung mittels Organoid-Kulturen**

Sehr geehrter Herr Professor Hackert,

die Ethikkommission hat Ihr Forschungsvorhaben in der Sitzung am 15.02.2021 beraten. Die zusätzlich angeforderten Informationen sind am 01.03.2021 und 18.03.2021 eingegangen.

Die Ethikkommission hat keine Bedenken gegen die Durchführung der Studie.

Sie gibt jedoch folgende Empfehlungen bzw. Hinweise:

Allgemein:

1. Gemäß der aktuellen Beschreibung im Studienprotokoll (z.B. S. 6 f., Abschnitt „5. Ziele der Studie": „...Medikamenteneinnahme...") handelt es sich bei der geplanten Studie um eine Untersuchung, die ggf. auch dazu bestimmt ist, Erkenntnisse zur Anwendung von Arzneimitteln im Rahmen ihrer Zulassung zu sammeln (Anwendungsbeobachtung). Die Kommission weist darauf hin, dass gemäß § 67 Abs. 6 AMG für Anwendungsbeobachtungen eine unverzügliche Anzeigepflicht gegenüber der zuständigen Bundesoberbehörde, der Kassenärztlichen Bundesvereinigung, dem Spitzenverband Bund der Krankenkassen und dem Verband der Privaten Krankenversicherung e.V. besteht.
2. Es wird darauf hingewiesen, dass zukünftige Fragestellungen/Studien auf Grundlage der im Rahmen dieser Studie verwendeten Proben/Daten als Neuanträge bei der Ethikkommission einzureichen sind, sofern der aktuelle Antrag diese Aspekte nicht beinhaltet.

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25.08.2022
ts-sn

BERUFSRECHTLICHE BERATUNG

Unser Zeichen: **S-508/2022 (Bitte stets angeben)**
Titel: **Analyse von Proteinen, miRNA und ctDNA im Serum und Plasma von Patient:innen in der Nachsorge nach Resektion eines Pankreaskarzinoms**

Sehr geehrte Frau Dr. Heger,

die Ethikkommission hat Ihr Forschungsvorhaben in der Sitzung am 15.08.2022 beraten. Die zusätzlich angeforderten Informationen sind am 22.08.2022 eingegangen.

Die Ethikkommission hat keine Bedenken gegen die Durchführung der Studie.

Wir wünschen Ihnen bei der Durchführung der Studie viel Erfolg.

Bitte leiten Sie das Ergebnis der berufsrechtlichen Beratung und die studienrelevante Korrespondenz allen teilnehmenden Ärzten in unserem Zuständigkeitsbereich weiter.

Mit freundlichen Grüßen

Prof. Dr. med. Dr. h.c. Thomas Strowitzki
Vorsitzender

Anlagen
Anhang

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CURRICULUM VITAE

Name: **Fawaz Naji Saleh Al-Shaheri**

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Birth: 29.12.1984 in Yemen

Citizenship: Yemeni

Education	1999-2002	High school certificate, Al-Shaab high school, Sanaa, Yemen
	2007-2012	Bachelor's studies at Thamar University
	2014-2017	Master studies at Jordan University of Science and Technology, Jordan
	2018-2023	Thesis work at the department of functional genome analysis, Deutsches Krebsforschungszentrum (DKFZ)
Profession:	2002 -2007	Medical Laboratory Technologist, IBB General Hospital
	2012 -2014	Lecturer at the Faculty of Medicine, Thamar University
	2015 -2018	Human Genetics Scientist at Princess Haya Biotechnology Centre
Languages		Arabic, English, and German
Interests		Reading, football playing

ACKNOWLEDGEMENTS

This work would not succeeded without the great support from many people, particularly my inspiring supervisor Dr. Jörg Hoheisel. With Dr. Hoheisel science was a mix of fun and hard work. From him I learned how to do real science and research becomes a hobby and part of my daily life. This feeling kept me standing up every day at 05:00 a.m. and go to the laboratory even if the experiment in previous days failed. I worked independently but interactively with colleagues from the laboratory at DKFZ or beyond. Dr. Hoheisel was also easily accessible whenever I needed an advice. He encouraged me to make my scientific mark and pursue my own ideas, which is a unique opportunity, at least for a Ph.D. student. I was very lucky to work with Dr. Hoheisel and satisfied with the performance I made at the Division of Functional Genome Analysis irrespective of the personal achievements.

The Ph.D. time I spent at DKFZ is the very best time in my life. I had very outstanding colleagues with whom I shared my scientific and personal thoughts. One example I used to talk to is Dr. Andrea Bauer about issues in my project and even in personal matters. Dr. Bauer was always supportive and honest to me along the way. Another exponent of science at the division is my colleague Mr. Henning Boekhoff, with whom I got along from the first day of our Ph.D. and continued to support each other in everything that concern us. Many thanks also to Dr. Mohamed Alhmadani, who supported me very much by shaping the project technically and scientifically to the right direction. Another highly appreciated source of support comes from Mr. Chaoyang Zhang during the bioinformatic analysis of the data or from Mr. Liang Xu during the microarray spotting or the coculture .For them I am grateful.

I am grateful for the TAC member and academic advisor Prof. Dr. Thilo Hackert for the constructive feedbacks in my Thesis. Also many thanks to Prof. Stefan Wiemann for his constructive comments along the time of my Ph.D. I also would like to thank very much Dr. med. Ulrike Heger for her constructive comments and for organizing the prospective analysis. I am grateful for PD. Dr. Natalia Giese and EPZ team for organizing the discovery cohort and clinical data. And of course for this great collaboration between the DKFZ and the Surgery Department at the Heidelberg University Hospital, I would like to thank Prof. Dr. med. Markus Büchler.

I received immense support from Mrs. Anke Mehler, Mrs. Sandara Widder, Mr. Ralf Zimbelmann and Mrs. Melanie Bier during my work at the division of functional genome analysis. For this, I am very thankful. I had also great pleasure to teach and supervise highly motivated and enthusiastic students, from whom I was deeply inspired. In particular, Ms. Teresa Cobatzky, Mr. Justin Brandt, Mr. Thomas Braun, Ms. Harriet Smith and Mr. Nico Hartmann.

EIDESSTATTLICHE VERSICHERUNG

Bei der eingereichten Dissertation zu dem Thema

Early detection of tumour recurrence in pancreatic cancer patients by the detection of specific microRNA and protein signatures in peripheral blood

handelt es sich um meine eigenständig erbrachte Leistung.

1. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

2. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.

3. . Die Richtigkeit der vorstehenden Erklärungen bestätige ich.

4. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Place, Date…… doctoral candidate’s signature…….