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Means and measures to improve treatment efficacy in pancreatic cancer

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vorgelegt von
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List of Abbreviations

5FU	5-Fluorouracil
5FU-EGFR-aptamers	5-Fluorouracil conjugated EGFR aptamers
AuNPs	Gold nanoparticles
CAFs	Cancer associated fibroblasts
CatD	Cathepsin D
CP	Chronic pancreatitis
CTAB	Cetyl-trimethylammonium bromide
EGFR	Epiderman growth factor receptors
EPPT1	Peptides capable of specific targeting of uMUC1
EPR	Enhanced permeability and retention effect
ESPAC	European study group of pancreatic cancer
FA	Folic acid
GEMM	Genetically engineered mouse models
LCM	Laser capture microdissection
MPAP	Myristoylated polyarginine peptide
MPS	Mononuclear phagocyte system
MRI	Magnetic resonance imaging
NTDDS	Novel targeted drug delivery systems
OS	Overall survival
PDAC	Pancreatic ductal adenocarcinoma
PFS	Progression free survival
PLK1, Plk1	Polo-like kinase 1
PNB	Plasmonic nanobubbles
PPTT	Photothermal treatment
REMARK	Reporting recommendations for tumor marker prognostic studies
siRNAs	small interference RNAs
SPIONs	Superparamagnetic iron oxide nanoparticles
TILs	Tumor infiltrating leukocytes
TMAs	Tissue microarrays
uMUC1	underglycosylated mucin-1
WGCNA	Weighted correlation network analysis

1. List of Publications

This cumulative habilitation is primarily based on the following peer-reviewed scientific contributions (in the order of appearance in this thesis):

a) **U. M. Mahajan**, S. Teller, M. Sendler, R. Palankar, C. van den Brandt, T. Schwaiger, J.-P. Kühn, S. Ribback, G. Glöckl, M. Evert, W. Weitschies, N. Hosten, F. Dombrowski, M. Delcea, F.-U. Weiss, M. M. Lerch, J. Mayerle, Tumour-specific delivery of siRNA-coupled superparamagnetic iron oxide nanoparticles, targeted against PLK1, stops progression of pancreatic cancer, ***Gut*** **65**, 1838–1849 (2016).
DOI: 10.1136/gutjnl-2016-311393

b) T. Patino, **U. M. Mahajan**, R. Palankar, N. Medvedev, J. Walowski, M. Münzenberg, J. Mayerle, M. Delcea, Multifunctional gold nanorods for selective plasmonic photothermal therapy in pancreatic cancer cells using ultra-short pulse near-infrared laser irradiation, ***Nanoscale*** **7**, 5328–5337 (2015).
DOI: 10.1039/c5nr00114e

c) **U. M. Mahajan***, Q. Li*, A. Alnatsha, J. Maas, M. Orth, S. H. Maier, J. Peterhansl, I. Regel, M. Sendler, P. R. Wagh, N. Mishra, Y. Xue, P. Allawadhi, G. Beyer, J.-P. Kühn, T. Marshall, B. Appel, F. Lämmerhirt, C. Belka, S. Müller, F.-U. Weiss, K. Lauber, M. M. Lerch, J. Mayerle, Tumor-Specific Delivery of 5-Fluorouracil-Incorporated Epidermal Growth Factor Receptor-Targeted Aptamers as an Efficient Treatment in Pancreatic Ductal Adenocarcinoma Models, ***Gastroenterology*** , S0016-5085(21)03081-X (2021). (Shared first authors).
DOI: 10.1053/j.gastro.2021.05.055

d) **U. M. Mahajan**, E. Langhoff, E. Goni, E. Costello, W. Greenhalf, C. Halloran, S. Ormanns, S. Kruger, S. Boeck, S. Ribback, G. Beyer, F. Dombroswki, F.-U. Weiss, J. P. Neoptolemos, J. Werner, J. G. D'Haese, A. Bazhin, J. Peterhansl, S. Pichlmeier, M. W. Büchler, J. Kleeff, P. Ganeh, M. Sendler, D. H. Palmer, T. Kohlmann, R. Rad, I. Regel, M. M. Lerch, J. Mayerle, Immune Cell and Stromal Signature Associated With Progression-Free Survival of Patients With Resected Pancreatic Ductal Adenocarcinoma, ***Gastroenterology*** **155**, 1625-1639.e2 (2018).
DOI: 10.1053/j.gastro.2018.08.009

e) **U. M. Mahajan**, E. Goni, E. Langhoff, Q. Li, E. Costello, W. Greenhalf, S. Kruger, S. Ormanns, C. Halloran, P. Ganeh, M. Marron, F. Lämmerhirt, Y. Zhao, G. Beyer, F.-U. Weiss, M. Sendler, C. J. Bruns, T. Kohlmann, T. Kirchner, J. Werner, J. G. D'Haese, M. von Bergwelt-Baildon, V. Heinemann, J. P. Neoptolemos, M. W. Büchler, C. Belka, S. Boeck, M. M. Lerch, J. Mayerle,

Cathepsin D Expression and Gemcitabine Resistance in Pancreatic Cancer, ***JNCI Cancer Spectr* 4**, pkz060 (2020).
DOI: 10.1093/jncics/pkz060

f) **U. M. Mahajan***, A. Alnatsha, Q. Li, B. Oehrle, F.-U. Weiss, M. Sendler, M. Distler, W. Uhl, T. Fahlbusch, E. Goni, G. Beyer, A. Chromik, M. Bahra, F. Klein, C. Pilarsky, R. Grützmann, M. M. Lerch, K. Lauber, N. Christiansen, B. Kamlage, I. Regel, J. Mayerle, Plasma Metabolome Profiling Identifies Metabolic Subtypes of Pancreatic Ductal Adenocarcinoma, ***Cells* 10**, 1821 (2021). (*Corresponding author)
DOI: 10.3390/cells10071821

g) J. Mayerle, H. Kalthoff, R. Reszka, B. Kamlage, E. Peter, B. Schniewind, S. González Maldonado, C. Pilarsky, C.-D. Heidecke, P. Schatz, M. Distler, J. A. Scheiber, **U. M. Mahajan**, F. U. Weiss, R. Grützmann, M. M. Lerch, Metabolic biomarker signature to differentiate pancreatic ductal adenocarcinoma from chronic pancreatitis, ***Gut* 67**, 128–137 (2018).
DOI: 10.1136/gutjnl-2016-312432

Major contributions not listed in cumulative habilitation:

h) C. E. M. Jakob*, **U. M. Mahajan***, M. Oswald*, M. Stecher, M. Schons, J. Mayerle, S. Rieg, M. Pletz, U. Merle, K. Wille, S. Borgmann, C. D. Spinner, S. Dolff, C. Scherer, L. Pilgram, M. Rüthrich, F. Hanses, M. Hower, R. Strauß, S. Massberg, A. G. Er, N. Jung, J. J. Vehreschild, H. Stubbe, L. Tometten, R. König, LEOSS Study group, Prediction of COVID-19 deterioration in high-risk patients at diagnosis: an early warning score for advanced COVID-19 developed by machine learning, ***Infection*** (2021), doi:10.1007/s15010-021-01656-z. (Shared first authors).
DOI: 10.1007/s15010-021-01656-z

i) G. Beyer*, **U. M. Mahajan***, C. Budde*, T. J. Bulla, T. Kohlmann, L. Kuhlmann, K. Schütte, A. A. Aghdassi, E. Weber, F. U. Weiss, A. M. Drewes, S. S. Olesen, M. M. Lerch, J. Mayerle, Development and Validation of a Chronic Pancreatitis Prognosis Score in 2 Independent Cohorts, ***Gastroenterology* 153**, 1544-1554.e2 (2017). (Shared first authors).
DOI: 10.1053/j.gastro.2017.08.073

j) **U. M. Mahajan**, C. Gupta, P. R. Wagh, P. A. Karpe, K. Tikoo, Alteration in inflammatory/apoptotic pathway and histone modifications by nordihydroguaiaretic acid prevents acute pancreatitis in swiss albino mice, ***Apoptosis* 16**, 1138–1149 (2011).
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2. Summary

Patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) suffer from one of the lowest 5-year relative survival rates of 10% among all cancers, while incidence is rising by approximately 1% per year (Siegel et al., 2018). Once established the tumor grows aggressively, often without specific symptoms. Hence, the disease is detected at locally advanced or metastasized tumor stages, when surgical intervention is no longer an option (Kleeff et al., 2016). Though surgical intervention offer improved survival in patients with resectable disease, post-operative morbidity can be as high as 45% (Klein et al., 2014; Mintziras et al., 2021). Consistent efforts have been made focusing on early tumor detection and novel drug development. Various strategies aim at increasing target specificity or local enrichment of chemotherapeutics as well as imaging agents in tumor tissue. Several chemotherapeutic agents have been restricted to phase II and phase III trials, owing to their non-specificity, chemoresistance and toxicity, thus limiting their clinical effectiveness. Two important factors contribute to this ineffectiveness. First, heterogeneity of the tumor, in which tumor cells are surrounded by non-cancerous stromal cells, immune cells, lymphatic cells and extra-cellular matrix. These subpopulations of cells differ widely in their response to cytotoxic drugs and other therapeutic modalities and therapy is only successful if this diversity can be considered. Second, the lack of selectivity of most anticancer drugs for tumor cells posing the risk of significant toxicity to normal tissues resulting in suboptimal dosage (Poste and Kirsh, 1983). The efficacy of anticancer drugs can also be limited by poor diffusion to certain areas of the tumors, because of a number of penetration barriers related to the abnormal neovasculature and altered composition of tumor tissue. Therefore, strategies aimed at increasing the selectivity and amounts of chemotherapeutics delivered to tumor areas could increase the therapeutic index of these class of compounds (Corti et al., 2012; Tannock, 2001). The existence of heterogeneity caused by a mixture of tumor cells and stromal cells produces chemoresistance and limits the targeted therapy of PDAC. Advances in precision medicine for PDACs according to the genetics and molecular biology of this disease may represent the next alternative approach to overcome the heterogeneity of different patients and improve survival outcomes for this poor prognostic disease (Hayashi et al., 2021).

Here, taking all these limitations in consideration, we delineated approaches for targeted delivery of chemotherapeutics in PDAC by means of superparamagnetic iron oxide nanoparticles (SPIONs) (Mahajan et al., 2016; Patino et al., 2015) and oligonucleotide aptamers (Li et al., 2020; Mahajan et al., 2021a). These evaluated platforms can serve as companion diagnostics. We further evaluated spatiotemporal correlation of immunofibrotic microenvironment with survival (Mahajan et al., 2018) and elucidated prognostic biomarkers

for treatment response (Mahajan et al., 2020). We further uncovers potential of metabolomics for differential diagnosis and stratification of PDAC (Mahajan et al., 2021b; Mayerle et al., 2018). I believe, approaches outlined in the present dissertation offer more individualized treatment strategies and may lead to an overall improved prognosis of patients with PDAC and pave the way for future active targeted aided novel targeted drug delivery systems and precision medicine.

3. Introduction

Despite the growing knowledge and understanding of biological properties and genetic aberrations of malignant cells, therapeutic options of pancreatic ductal adenocarcinoma (PDAC) remain incompletely effective and transitory. Several chemotherapeutic agents have been restricted to phase II and phase III trials, owing to their non-specificity, chemoresistance, and toxicity, thus limiting their clinical effectiveness. Two important factors contribute to this ineffectiveness. First, heterogeneity of the tumor, in which tumor cells are surrounded by non-cancerous stromal cells, immune cells, lymphatic cells and extra-cellular matrix. These subpopulations of cells differ widely in their response to cytotoxic drugs and other therapeutic modalities and therapy is only successful if this diversity is considered. Second, the lack of selectivity of most anticancer drugs for tumor cells posing the risk of significant toxicity to normal tissue resulting in suboptimal dosage (Poste and Kirsh, 1983). The efficacy of anticancer drugs can also be limited due to poor diffusion to certain areas of the tumors, because of several penetration barriers related to the abnormal neovasculature and altered cell composition of tumor tissue. Therefore, strategies aimed at increasing the selectivity and amounts of chemotherapeutics delivered to tumor areas could increase the therapeutic index of these class of compounds (Corti et al., 2012; Tannock, 2001).

Novel targeted drug delivery systems (NTDDS) allow clinical use of new therapeutics, permit anticancer therapy with significantly reduced side effects, and enable new and better chemotherapeutic regimens using existing pharmaceuticals (Moses et al., 2003).

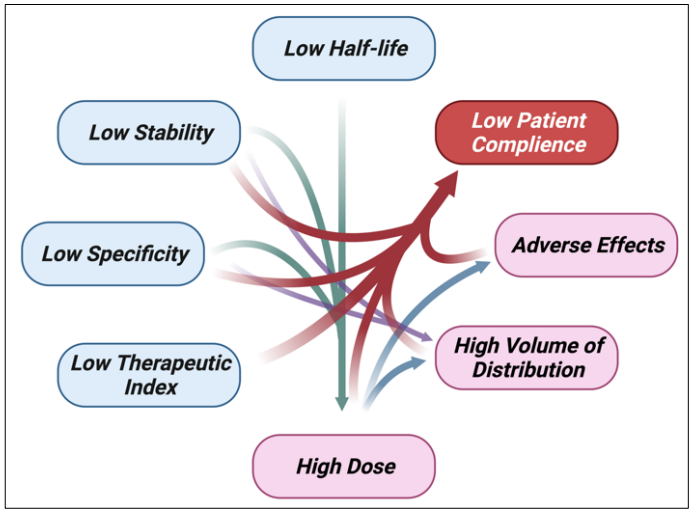

Figure 1: Need for Targeted drug delivery for cancer therapeutics. *Limitations of conventional drug delivery systems are due to pharmacokinetics (low plasma half-life, high volume of distribution), pharmacodynamics (low specificity, narrow therapeutic index) and pharmaceutical properties (low stability and solubility). All these factors lead to high dosage, adverse effects and this in turn contributes to a lowered patients' complience.*

The need for NTDDS over conventional drug delivery formulations stems from with discontented implementation of drugs in conjunction to pharmacodynamic, pharmacokinetic, pharmaceutical and pharmacotherapeutic features **(Figure 1)**. Drug targeting specifically to the site of tumors is not only important to enhance therapeutic effectiveness but also to reduce

toxicity associated with a narrow therapeutic index and high doses. To emphasize, drug targeting results in increased efficacy, modulated pharmacokinetics and biodistribution, increased specificity of localization, decreased toxicity, reduced dose, and improved patient compliance (Bae and Park, 2011).

Active target aided NTDDS centers on recognition of overexpressed receptors or antigens by modifying specific ligands and conjugating it to the surface of the formulation (Blanco et al., 2015; Jiang et al., 2019). The efficacy and potency of these active target aided NTDDS is primarily determined by the activity of the payload, while their safety is dictated by the specificity of the targeting ligand to the insult (Rosenblum et al., 2018; Srinivasarao and Low, 2017). Active target aided NTDDS have several advantages over their non-targeted counterparts. (1) Active target aided NTDDS can deliver their therapeutic moiety selectively into diseased cells and thus avoid nonspecific uptake and the associated toxicity to healthy cells. (2) Active target aided NTDDS can be simultaneously used as a companion diagnostic agent and act as theranostic (Srinivasarao and Low, 2017; Vargason et al., 2021; Zhao et al., 2020). The ultimate goal of novel cancer drug discovery is to allow monitoring drug delivery to the diseased tissue, to understand delivery kinetics with concurrent imaging. Theranostics are compounds that combine these modalities of therapy and diagnostic imaging such that it delivers therapeutic drugs as well as diagnostic imaging agents concurrently within same compound (Bertrand et al., 2014; Kelkar and Reineke, 2011).

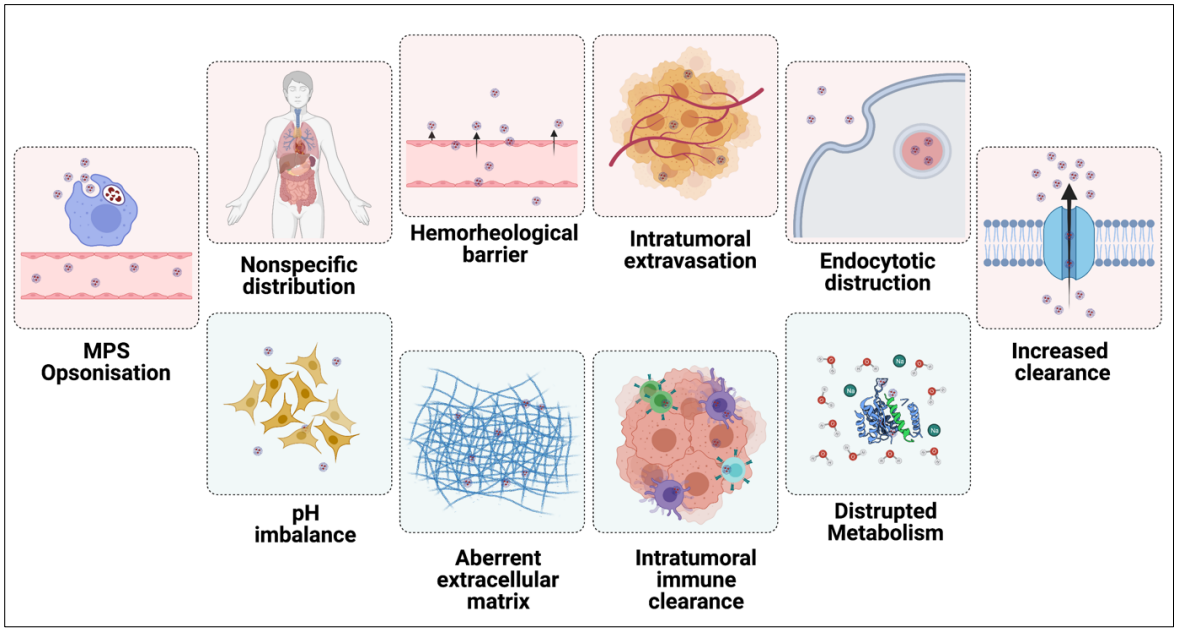

Figure 2: Framework of sequential biological and pathological barriers to nanoparticle drug delivery. *Biological barriers are highlighted in red whereas pathological barriers are highlighted in light blue.*

Though recent development and advances in NTDDS lead to improvement in patient safety and morbidity, these platforms offer only marginal benefits over conventional formulations

(Gradishar et al., 2005; O'Brien et al., 2004). Despite their potential for improving a drug's propensity to accumulate at sites of insult and manipulating plasma half-life, the NTDDS face a complex series of biological and pathological barriers that severely limit site-specific bioavailability, preventing proper therapeutic outcomes. The biological barriers include opsonization and subsequent sequestration by the mononuclear phagocyte system (MPS), nonspecific distribution, hemorheological flow limitations, pressure gradients, cellular internalization, escape from endosomal and lysosomal compartments and increased drug efflux pumps (Blanco et al., 2015; Ferrari, 2010) **(Figure 2, red)**. In addition to the substantial challenges disease stage (early-versus late-stage cancers) and its pathological barriers which include, pH imbalance, aberrant extracellular matrix, hyperactive immune infiltration and altered metabolic changes **(Figure 2, blue)** pose additional challenges. The minimal therapeutic impact observed following NTDDS is a direct consequence of the their inability to overcome these barriers (Blanco et al., 2015).

Within this thesis, we evaluated the performance of different active targeting aided NTDDS and their strategies to overcome barriers associated with suboptimal delivery and efficacy of NTDDS. We also focused on the landscape of targeting moieties with an emphasis on the uniqueness of ligand biology, emerging concepts, clinical advances and provide an overview of targeted delivery strategies including guiding design principles to support informed ligand selection.

4. Objectives

While developing active targeting aided NTDDS, various design and biological considerations were taken into consideration along with building of a suitable experimental system to

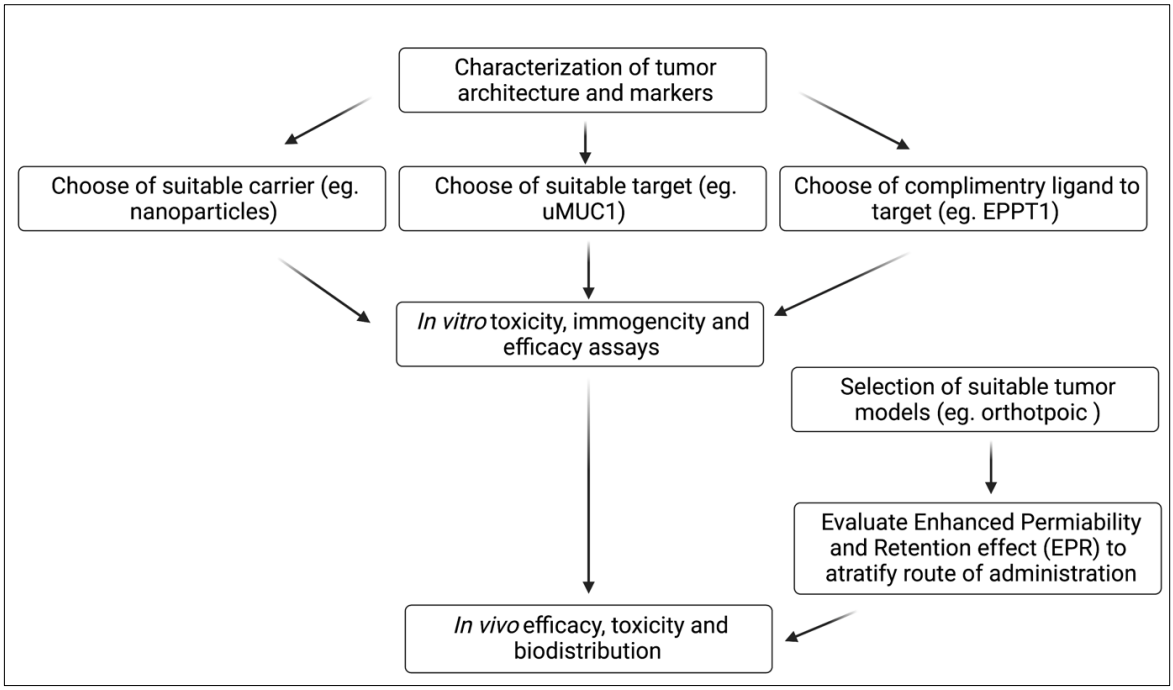

Figure 3: Schematic illustration of the proposed workflow in the development of actively targeted NCs.

evaluate the efficacy of NTDDS **(Figure 3)**. With the aim of active targeting aided NTDDS intended for use in PDAC we chose a model that recapitulates human PDAC in mice. When choosing the target receptor and targeting moiety depending on the cargo of NTDDS the following parameters were taken into consideration.

(1) *Suitable carrier:* Carrier for NTDDS should be selected based on its combinatorial properties such as modifiable surfaces, nanometric size for radial clearance, high surface area to volume ratios, high loading capacity, and ease of synthesis, lack of immunogenicity, and versatile chemistry.

(2) *Suitable target:* The safety profile of a targeted drug lies in the ratio of targeted receptor expression with its absolute levels in tumor cells compared to normal cells. For intracellular targets, receptors that internalize via pathways that bypass the destructive endocytic pathways are to be preferred.

(3) *Suitable ligand:* Ligands are selected based on high binding affinity and specificity for the selected target and display optimal size to allow passive retention via an EPR effect in the tumor. Ligands should exert low immunogenicity, plasma stability and are easy to conjugate to the carriers.

Taking into consideration the framework for a workflow and consideration, we explored active targeting aided NTDDS approaches using superparamagnetic iron oxide nanoparticles (SPIONs) with (i) the synthetic peptide EPPT1, which binds underglycosylated MUC1 (uMUC1), a widely and strongly expressed membrane mucin on PDACs (Hinoda et al., 2003) and (ii) MPAP (myristoylated polyarginine peptides), which enhances cellular uptake and endosomal release (Lange et al., 2016; Medarova et al., 2007) for targeted delivery of siRNA targeted against Polo-like kinase 1 (*Plk1*) (**Mahajan et al., 2016**). Further on, we employed an active targeted delivery approach using gold nanoparticles conjugated with EPPT1 and MPAP for targeted plasmonic photothermal therapy using ultra-short near infrared laser pulses (**Patino & Mahajan et al., 2015**). To overcome limitations of metallic nanoparticles, such as anaphylactic reactions and high clearance by MPS opsonization, we explored EGFR targeted 5-fluorouracil (5FU) delivery using 5FU incorporated aptamers **(Mahajan et al., 2021a**). Aptamers are small oligonucleotide sequences that serve as ligands to target molecules and gaining popularity as a target/carrier due to their advantages of higher tissue penetration, rapid production, low synthesis cost, less immunogenicity, thermal stability, and ease of labeling (**Li et al., 2020**).

PDAC is known for its desmoplastic stroma reaction comprised of activated myofibroblasts, leukocytes and extracellular matrix (Rhim et al., 2014). PDAC desmoplasia is thought to confer biological aggressiveness (Olive et al., 2009), influences PDAC initiation, progression, and relapse (Sherman et al., 2017). Furthermore, evidence in recent years has repeatedly highlighted the diverse role tumor stroma plays in maintaining cancer invasiveness in PDAC, hence serving as the Achilles heel for this malignancy by promoting therapeutic resistance to currently available treatment modalities (Gore and Korc, 2014). To address the role of the tumor stroma which along with regulating the malignancy, poses a challenge for the development of more effective therapeutic options, we delineated histological signatures of immune infiltration and stroma composition that offer more individualized treatment strategies and may lead to an overall improved prognosis of patients with PDAC (**Mahajan et al., 2018**).

In PDAC, biomarkers are lacking, with treatment predominantly determined by stage of disease, performance status, and therapy dominated by cytotoxic agents. Specifically, FOLFIRINOX [5-fluorouracil (5-FU), leucovorin, oxaliplatin, irinotecan], gemcitabine/nab-paclitaxel, and liposomal irinotecan/5-FU combinations have collectively increased survival in the advanced-disease setting (Krantz and O'Reilly, 2018). To address the potential role of the proteolytic enzyme, Cathepsin D, as prognostic and predictive markers of treatment response, we quantified Cathepsin D expression on tumor-tissue microarrays of 403 resected pancreatic

cancer patients from the ESPAC-Tplus trial and evaluated the role of Cathepsin D as a predictive biomarkers for adjuvant gemcitabine treatment (**Mahajan et al., 2020**). Subjected to prospective validation, this study could provide option for treatment stratification of PDAC.

In addition to cell autonomous factors, PDAC show also great heterogeneity in terms of metabolic altercations (Daemen et al., 2015). To understand the causes and functional consequences of metabolic heterogeneity and its implications for treatment response and resistance as well as for the development of personalized PDAC therapies, and to overcome a misleading transcriptome based classification because of intratumoral heterogeneity, we delineated three metabolic PDAC subtypes associated with distinct complex lipid patterns with their relevance to survival in the plasma metabolome (**Mahajan et al., 2021b**).

Undoubtedly, one of the greatest biomarker-related challenges in this field is finding biomarkers that accurately distinguish PDAC from other diseases of the pancreas, where overlapping signs and symptoms make differential clinical diagnosis difficult. To overcome these challenges, we delineated and prospectively validated a metabolic signature (9 metabolites plus CA19.9) that stratify PDAC from CP (**Mayerle et al., 2018**). To improve the metabolite assay, we improved and modified this metabolite signature (mMetabolic signature) in a second multicenter independent prospective validation (**Mahajan et al., in submission**). We believe, all these approaches offer more individualized treatment strategies and may lead to an overall improved prognosis of patients with PDAC and pave the way for future active targeted aided NTDDS.

5. Own Scientific Contributions

5.1. Tumour-specific delivery of siRNA-coupled superparamagnetic iron oxide nanoparticles, targeted against PLK1, stops progression of pancreatic cancer (Mahajan et al., 2016).

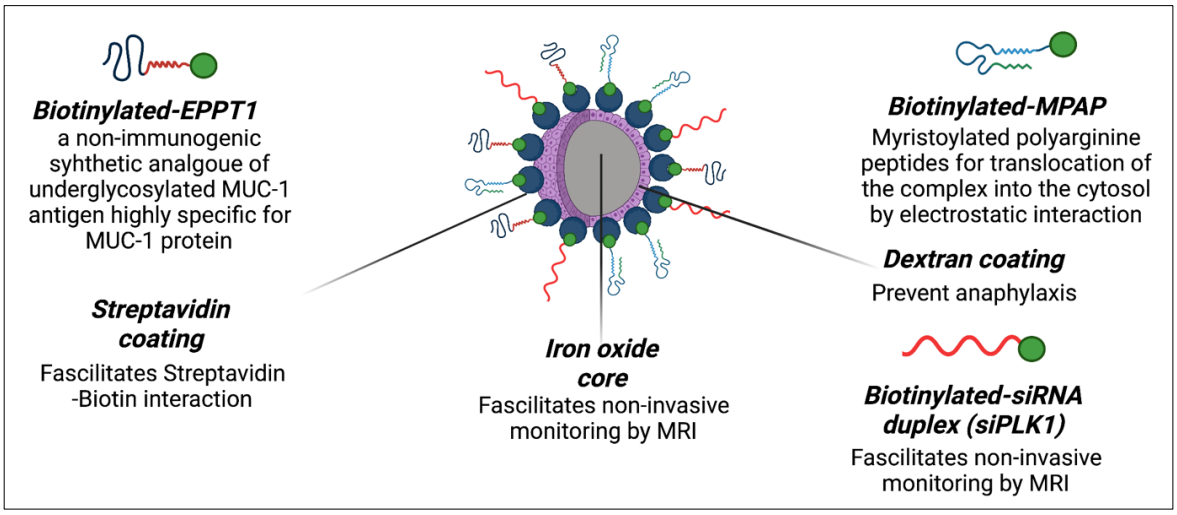

Figure 4: schematic representation of siPLK1 coupled streptavidin conjugated dextran coated superparamagnetic iron oxide nanoparticles (siPLK1-StAv-SPIONs) conjugated to the membrane translocation peptide(MPAP), the underglycosylated MUC1 specific peptide (EPPT1) and siRNA molecules targeting PLK1 (siPLK1).

Considering of the regulation of the cell division cycle and its high-fidelity execution to organismal homeostasis, it is unsurprising that alteration of the cell cycle is a hallmark of the pathogenesis human malignancies (Zhang et al., 2009). It has been now known that many of the genes encoding key regulators of the cell cycles are mutated in PDAC and associated with increased cell cycle progression (Connor et al., 2019). Polo-like kinase 1 (PLK1) is critical regulator in centrosome cycle and plays role in centrosome maturation, sister chromatid segregation and cytokinesis (Raab et al., 2011). Dysregulated levels of PLK1 are associated with PDAC progression (Zhang et al., 2013). Several PLK1 specific small molecule inhibitors have been restricted to phase II and phase III trials owing to its non-specificity, chemoresistance and toxicity (Febrile neutropenia, myelosuppression and neuropathies) (Raab et al., 2011).

The use of siRNAs that allow specific intervention at single molecular stages of the disease may provide a potential therapeutic agent. siRNAs virtually can be used to inhibit the expression of any gene. Major hurdles of *in vivo* delivery of siRNAs are the adequate and delivery to the specific site of interest, stability of siRNAs in serum and non-invasive monitoring of the influence of siRNAs.

The objective of the present study is to evaluate the potential of siRNA conjugated to dual purpose superparamagnetic iron-oxide-nanoparticles (SPIONs) for the therapeutic intervention of PDAC functioning as theranostics. Although the feasibility of using SPIONs for cancer detection and drug delivery has been demonstrated, a major obstacle limiting their clinical application is that non-targeted nanoparticles are unable to reach sufficient concentrations in the tumor site to either produce a strong signal for tumor imaging or to carry optimal amounts of therapeutic agents into tumor cells.

Keeping the limitations of classical PDAC therapy, *in vivo* delivery of siRNA and drug delivery properties of SPIONs in mind, we have developed NTDDS that not only deliver the siRNAs adequately and specifically to the tumor site but also allow non-invasive imaging of the uptake of our therapeutic agent. To accomplish this, we designed dual purpose functional probes with dextran coated superparamagnetic nanoparticles as a backbone which can be detected by magnetic resonance imaging (MRI), and which are further conjugated to streptavidin. Reflecting the fact of streptavidin and biotin as the strongest covalent binding, we coupled biotin conjugated myristoylated polyarginine peptides (MPAPs) for translocation of the complex into the cytosol by electrostatic interaction as well as biotin conjugated EPPT1, a non-immunogenic underglycosylated MUC1 antigen highly specific for MUC1 protein, (a trans-membrane protein, highly expressed in pancreatic ductal adenocarcinoma) which allows tumor specific uptake. Furthermore, we linked biotin conjugated siRNAs directed against PLK1 to the streptavidin anchor as a therapeutic target. siPLK1-StAv-SPIONs found to be efficacious silencing PLK1 as well as decreasing proliferation *in vitro*. To these nanoparticles are selectively taken up by cancer cells by clathrin dependent endocytosis and uptake is uMUC1 dependent. siPLK1-StAv-SPIONs found to evade endosomes to be therapeutically active by proton sponge effect. Administrations of these nanoparticles in tumor-bearing mice (syngeneic orthotopic tumor model as well as Genetically engineered PDAC tumor models (GEMM, KPC mice)) allowed monitoring of delivery of the agent to the tumor and metastasis by MRI imaging and results in efficient silencing of the target gene, *PLK1*. To summarize, this approach can significantly advance the therapeutic potential of siRNAs by providing a way not only to effectively shuttle siRNA to target sites but also to noninvasively access the bioavailability and efficacy of the siRNA at tumor site.

5.2. Multifunctional gold nanorods for selective plasmonic photothermal therapy in pancreatic cancer cells using ultra-short pulse near-infrared laser irradiation (Patino & Mahajan et al., 2015).

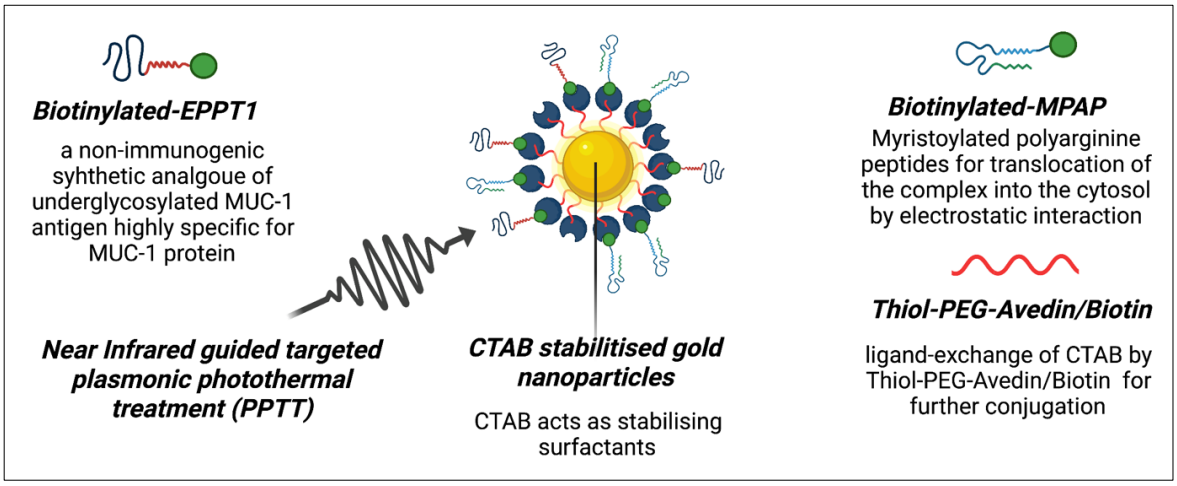

Figure 5: Schematic representation of functionalized gold nanoparticles. *Gold nanoparticles are stabilized by a bilayer of the stabilizing surfactant cetyl trimethylammonium bromide (CTAB), followed by ligand exchange displacing CTAB with thiolated PEG-Biotin. Biotinylated EPPT1 and MPAP can be further conjugated to the gold nanoparticles through biotin–avidin binding. These functionalized nanoparticles can be used for targeted photothermal treatment.*

Gold nanoparticles (AuNPs) have been gaining popularity in targeted therapy due to their low cytotoxicity, the ease of surface functionalization and their unique optical properties related to the localized surface plasmon resonance and its implication in plasmonic photothermal treatment (PPTT) (Delcea et al., 2012; Lukianova-Hleb et al., 2014). In the present study, we developed an efficient multi-functional NTDDS with gold AuNPs as a core that is linked to peptides capable of specific targeting (EPPT1) and enhanced intracellular delivery using MPAP to PDAC cells and their selective destruction through PPTT using ultra-short laser pulse irradiation. One of the bottlenecks in functionalization of AuNPs for specific targeting to cells is the complete removal of the highly cytotoxic stabilizer CTAB from the AuNPs and maintenance of colloidal stability to prevent aggregation. To achieve this, we used ligand exchange method using heterobifunctional thiol-PEG-biotin which replaced CTAB with thiolated-PEG-Biotin. To achieve targeting through specific recognition of PDAC cells, we modify a AuNPs with previously used a synthetic non-immunogenic antitumor-antibody-derived peptide based on the Glu-Pro-Pro-Thr (EPPT1) amino acid sequence that specifically recognizes the membrane-tethered mucin rich underglycosylated glycoprotein (uMUC-1). Further, we incorporated myristoylated polyarginine decapeptide (MPAP) for enhanced cell surface binding, facilitated translocation and enhanced uptake of the dual peptide functionalized AuNPs.

We further tested cellular targeting and uptake efficient of the dual peptide functionalized AuNPs in PDAC cells and observed efficient uptake of dual peptide functionalized AuNPs *in vitro*. Using an ultrashort pulse near-infrared laser, we generated transiently vapor nanobubbles, known as Plasmonic nanobubbles (PNB), resulting in the selective destruction of previously dual peptide functionalized AuNPs enriched PDAC cells by localized cavitation and plasma expansion around AuNPs. Thus, from these localized excitation of small volumes, plasmonic photothermal effects allow an extremely destructive environments around each AuNPs within a target cell in a small target volume laser focus. We observed significant reduction in cancer cell proliferation following dual functionalized AuNPs enrichment and targeted photothermal therapy.

In summary, our approach using a multifunctional AuNPs is promising for the development of novel anticancer therapies that make use of molecular recognition combined with remote photophysical manipulation for plasmonic photothermal guided cancer cells ablation.

5.3. Tumor-Specific Delivery of 5-Fluorouracil-Incorporated Epidermal Growth Factor Receptor-Targeted Aptamers as an Efficient Treatment in Pancreatic Ductal Adenocarcinoma Models (Mahajan et al., 2021a).

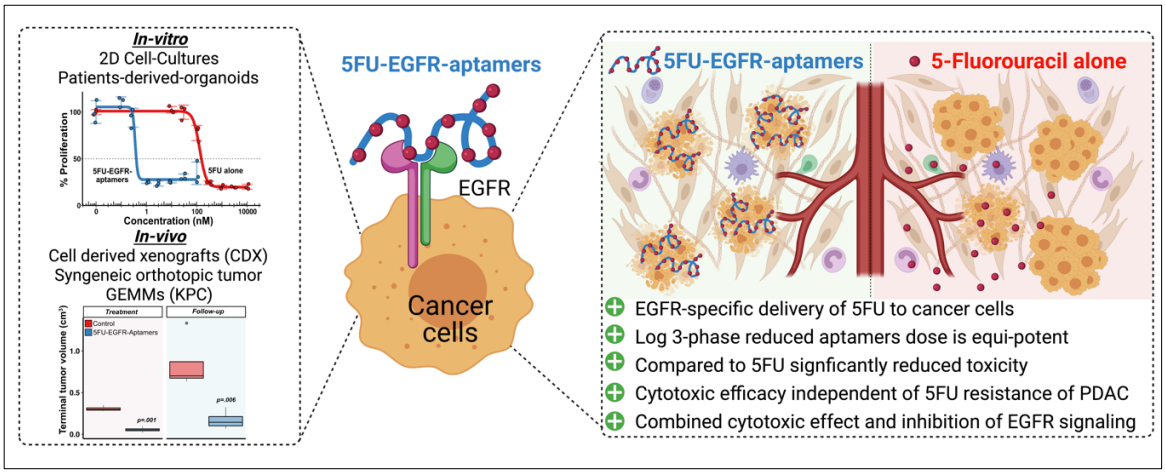

Figure 6: Graphical presentation of EGFR guided 5Fluorouracil delivery using oligonuclotide aptamers (5FU-EGFR-aptamers).

Since its discovery in 1990 by Ellington and Szostak (Ellington and Szostak, 1990), oligonucleotide aptamers provided broad application prospected in fundamental research, drug development, clinical diagnosis and targeted therapy. Several properties such as binding affinity at low nanomolar or picomolar range, in-vitro chemical process of selection, variety of chemical modifications to molecular platforms for diverse function, non-immunoreactivity, modification of bioavailability and pharmacokinetics manipulation, make aptamers attractive target compared to conventional cell-specific targets (Nimjee et al., 2005). Aptamers targeting are not only useful in modulating and blocking physiological response, but also function as carrier for therapeutic and diagnostic agents and conjugated to a variety of molecules including gold nanoparticles (Javier et al., 2008), siRNA (Chu et al., 2006; Dassie et al., 2009; McNamara et al., 2006), and drug encapsulated polymer particles for specific delivery (Farokhzad et al., 2004). With many desirable properties such as ease of synthesis, small size, lack of immunogenicity, and versatile chemistry, aptamers represent a class of targeting ligands that possess tremendous potential in molecular imaging applications. These findings indicate the use of aptamers as an alternative path for the generation of more effective and safer therapeutics in oncology. Given the high versatility of aptamers, the results offer a blueprint for the design of theranostic molecules able to selectively target cancer cells and their metastases in advanced stages of tumors.

Given the overexpression of epidermal growth factor receptor (EGFR) in PDAC, its plasma membrane localization, and its correlation with poor prognosis and disease progression (Moore et al., 2007; Ueda et al., 2004; Xiong, 2004), we expected EGFR to be an attractive target

structure for developing a novel compound that delivers 5FU adequately and precisely to the tumor site. To accomplish this, we designed, synthesized, and modified EGFR-targeting aptamers incorporating pyrimidine antimetabolites (5FU) instead of uracil (5FU-EGFR-aptamers) to test their use and efficacy *in vitro* and *in vivo.*

We observed that 5FU-EGFR-aptamers are taken up specifically by clathrin dependent endocytosis and is EGFR dependent. 5FU-EGFR-aptamers treatment shown to reduce proliferation in a concentration-dependent manner in murine and human PDAC cells. The 5FU-aptamer treatment was equally effective in 5FU-sensitive and 5FU-refractory PDAC cells. 5FU-EGFR-aptamers elicited reduced proliferation in patients derived organoids *in vitro*. We evidenced reduced tumor burden in a syngeneic orthotopic transplantation model of PDAC, in an autochthonously growing GEMMs model and in athymic mice xenografts model with human PDAC cells, following biweekly treatment with 5FU-EGFR-aptamers.

In summary, Tumor-specific targeted delivery of 5FU using 5FU-EGFR-aptamers as the carrier achieved high target specificity; overcame 5FU resistance; and proved to be effective in three different PDAC mouse models and represents a promising backbone for pancreatic cancer chemotherapy in patients. Furthermore, our approach has the potential to target virtually any cancer entity sensitive to 5FU treatment by incorporating 5FU into cancer cell-targeting aptamers as the delivery platform.

5.4. Immune Cell and Stromal Signature Associated with Progression-Free Survival of Patients with Resected Pancreatic Ductal Adenocarcinoma (Mahajan et al., 2018).

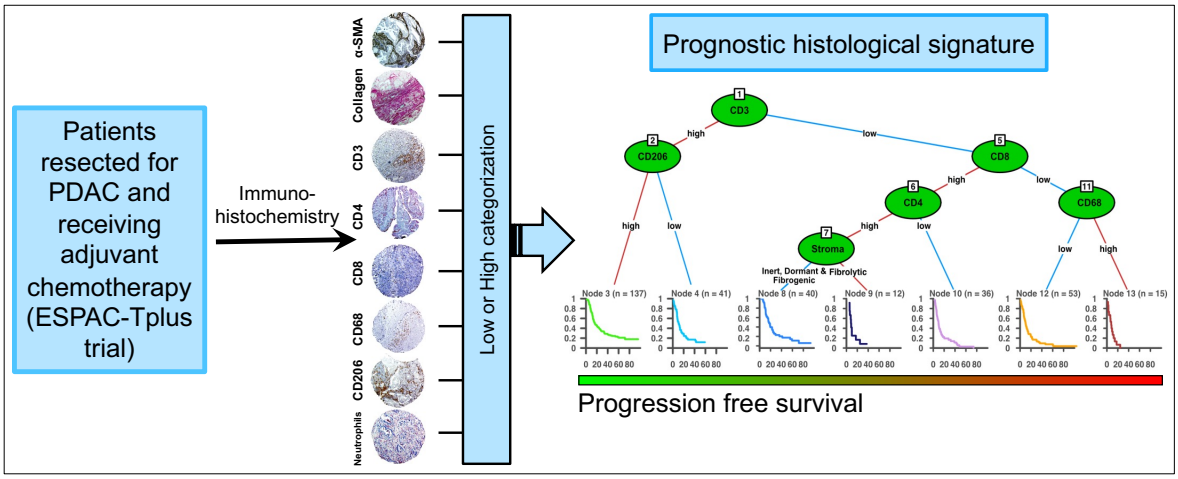

Figure 7: Graphical illustration of immunofibtoic histological signature in stratification of patients progression free survival.

PDAC is known for its desmoplastic stroma reaction comprised of activated myofibroblasts, leukocytes and extracellular matrix (Rhim et al., 2014). PDAC desmoplasia is thought to confer biological aggressiveness (Olive et al., 2009), influences PDAC initiation, progression, and relapse (Sherman et al., 2017). The function of the desmoplastic stroma is likely dynamic during cancer progression and its heterogeneous cellular and acellular constituents change in relation to the prognostic landscapes of cancers (Özdemir et al., 2014; Sherman et al., 2017). There are several studies depicting differential expression of α-smooth muscle actin (α-SMA) and collagen I, as a tumor-promoter or -suppressor depending on stromal turn-over serving as an independent prognostic marker (Erkan et al., 2008; Özdemir et al., 2014; Rhim et al., 2014). Cancer-associated fibroblasts (CAFs) are key players in the tumor microenvironment and its heterogeneity drives cancer phenotypes, notably cancer cell proliferation and invasion, neo-angiogenesis, inflammation, extracellular matrix remodeling and tumor infiltrating leukocytes infiltration.

Recently, in resected PDAC tissue specimens obtained from a cohort of patients enrolled in the ESPAC-1 and -3 clinical trials, we delineated a complex pattern of differential expression profiling of leukocyte subpopulations with an orthogonal behavior with respect to stromal subtypes (combination of α-SMA and Collagen-I) for most tumor infiltrating leukocytes (TILs) subtype (Mahajan et al., 2018). We observed distinct stromal subtypes being responsible for aggressiveness and associated with differential infiltration of immune cells (Mahajan et al., 2018). The presence of TILs within the tumor microenvironment is considered to be an indication of the host immune response to the tumor and reflects the dynamic process of

cancer immunoediting (Sato et al., 2005). Exploiting fundamental principles of PDAC biology and surrounding stromal evolution, we developed a prognostic tree for progression-free survival (PFS) and classified patients into 7 subtypes based on the expression of CD3, CD4, CD8, CD68, and CD206, as well as stromal compositions. Intriguingly, patients with $CD3_{high}CD206_{high}$ signature had the longest PFS, whereas patients with $CD3_{low}CD8_{low}CD68_{high}$ signature harbored the shortest PFS. These results were successfully validated further retrospective study. Subject to prospective studies, this study has significant clinical implications for a more informed risk assessment and improved prognostication of patients with PDAC.

To add, we observed that stromal subtypes not only differ about the expression of alpha-SMA and collagen I, but invariably in terms of unique patterns of leukocytic subpopulations that can robustly predict PFS in patients with PDAC. Hence, this study provides a novel insight that a combination of both stromal and immunologic signatures offers a superior prognostic tool for patients with PDAC.

To summarize, as immunotherapies continue to be refined, these findings may become more significant in paving the path toward more individualized and precision treatments in PDAC patients. Such an integrated prognostic signature incorporating immune infiltrates and stromal components may eventually also permit selection of patients for appropriate treatment regimens, as well as identification of novel therapeutic targets.

5.5. Cathepsin D Expression and Gemcitabine Resistance in Pancreatic Cancer (Mahajan et al., 2020).

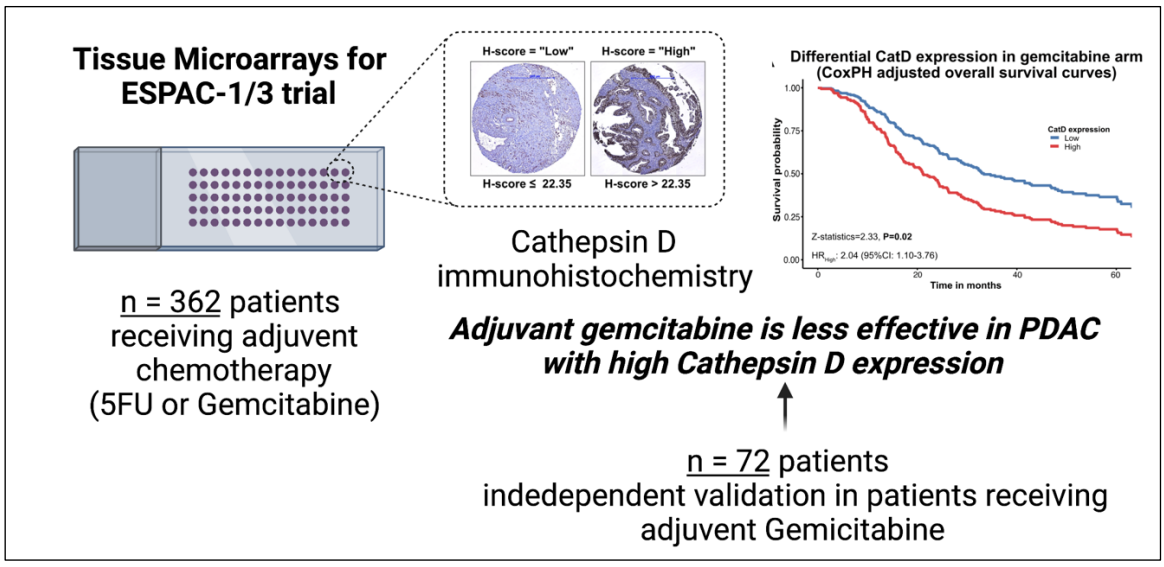

Figure 8: Graphical illustration representing Cathepsin D as independent predictive marker in patients receiving adjuvant gemcitabine.

Biomarker driven treatment strategies are urgently needed for PDAC, but their successful development requires studies according to Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines to reduce bias (Biankin et al., 2015). To identify a relevant biomarker, we used archival material from randomized, controlled trials, European Study Group for Pancreatic Cancer (ESPAC-1 and 3) (Greenhalf et al., 2014; Neoptolemos et al., 2010), balanced for treatment arms, and stratified for cancer stage in resected pancreatic cancer patients.

As a candidate biomarker, we chose the lysosomal aspartic protease cathepsin D (CatD). CatD is overexpressed and hypersecreted in some epithelial cancers. In addition to its ubiquitous role in lysosomes, two biologic activities of the secreted precursor have been demonstrated *in vitro*: mitogenic activity and proteolytic activity for various substrates including proteoglycans and basement membranes in an acidic milieu. Both the growth-promoting activity and its extracellular proteolytic activity suggest that CatD may be of prognostic relevance in PDAC.

Here, we determined whether tumor cells CatD expression predicts overall survival and progression free survival in patients receiving adjuvant gemcitabine or 5FU/FA in the ESPAC-Tplus trial. We validated our findings in an independent, prospectively recruited cohort and used tumor cell culture studies to identify the underlying cellular mechanisms. For this study, we included only patients treated in the ESPAC-1 and ESPAC-3 trials. With this approach, we aimed for a reduced bias by recruiting a stage-corrected group of patients receiving mono-hemotherapy within a randomized trial to test expression levels of a single biomarker, CatD,

in tissues harvested under standardized conditions and externally monitored, quality controlled clinical data. Furthermore, utmost care was taken in generating the tissue microarray (TMA) to reduce bias of tumor heterogeneity as well as increasing sensitivity and specificity of labeling. The bias of nonrandomized studies precludes separating a predictive therapy-specific effect from a disease prognosis-specific effect. Here, we demonstrate the prognostic capacity of CatD expression levels for overall survival in resected PDAC and its potential to predict the efficacy of adjuvant gemcitabine. We could not delineate a correlation between 5FU treatment and CatD expression. In addition to associations with survival outcomes, our study detected an association between CatD expression and gemcitabine responsiveness. One potential explanation regarding how increased CatD expression can influence gemcitabine responsiveness is the crosstalk of CatD with acid sphingomyelinase to maintain sphingolipid rheostat.

To summarize, these data were confirmed in an independent validation cohort. Results from both cohorts imply that adjuvant gemcitabine monotherapy might not be considered in patients with high tumor cell CatD expression. Because adjuvant gemcitabine-treated patients with low CatD levels display a survival benefit, low CatD can be an effective predictive marker of efficacy of gemcitabine. Furthermore, in vitro high CatD expression reflected gemcitabine resistance.

5.6. Plasma Metabolome Profiling Identifies Metabolic Subtypes of Pancreatic Ductal Adenocarcinoma (Mahajan et al., 2021b)

Recent transcriptome approaches uncovered two to four molecular tumor signatures (Bailey et al., 2016; Chan-Seng-Yue et al., 2020; Collisson et al., 2011; Moffitt et al., 2015), with a consensus of a classical and basal-like PDAC subtypes (Collisson et al., 2019; Regel et al., 2020). The studies used whole tissue or laser capture microdissected (LCM) material from resected tumors or from needle biopsies of advanced cases. Examining clinical features, the classical PDAC subtype is associated with a better overall survival rate and is found more frequently in stage I/II PDAC samples, whereas the basal-like subtype is associated with a more aggressive phenotype, culminating in a worse prognosis and, in part, in chemotherapy resistance (Collisson et al., 2011). In contrast, a study from Moffitt et al. indicated that the basal-like subtype responded better to adjuvant chemotherapy (Moffitt et al., 2015). Several initiatives tried to clarify the conflicting results and investigated whether molecular signatures or other biomarkers are able to predict chemotherapy response (Regel et al., 2020). However, novel single-cell RNA sequencing data of human PDAC samples revealed intratumoral molecular heterogeneity, making prediction even more complex. A study from Chan-Seng-Yue et al. demonstrated that classical and basal-like subtypes existed, juxtaposing most of their analyzed PDAC tissue samples (Chan-Seng-Yue et al., 2020). In addition to cell autonomous factors, PDAC show also great heterogeneity in terms of metabolic altercation (Daemen et al., 2015).

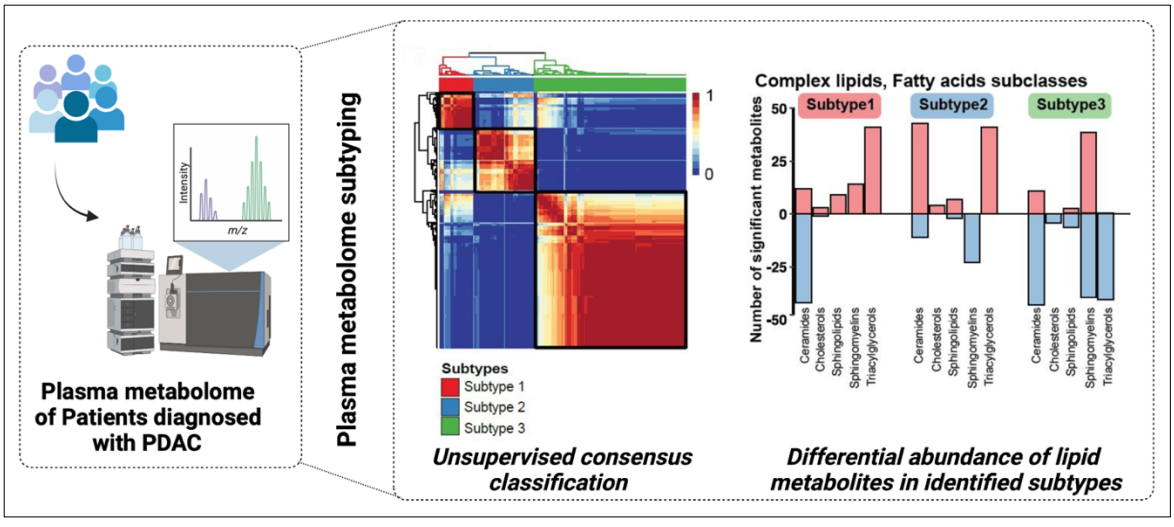

Figure 9: Graphical representation plasma metabolome subtypes showing differential abundance of lipid metabolites.

In the present study, we used plasma metabolome profiles of 361 PDAC patients who we recruited previously to identify blood-derived biomarkers for tumor diagnosis (Mayerle et al., 2018). In our comprehensive approach, we identified different PDAC subtypes based on

plasma metabolite levels. The metabolic rewiring of tumor cells, which is reflected in the plasma of PDAC patients, provides great opportunities in defining cancer characteristics and clinically relevant PDAC subtypes. To understand the causes and functional consequences of sphingolipids heterogeneity and its implications for treatment response and resistance as well as for the development of personalized PDAC therapies, we identified three metabolic PDAC subtypes that were associated with distinct complex lipid patterns. Subtype 1 was associated with reduced ceramide levels and a strong enrichment of triacylglycerols. Subtype 2 demonstrated increased abundance of ceramides, sphingomyelins, and other complex sphingolipids, whereas subtype 3 showed decreased levels of sphingolipid metabolites in plasma. Pathway enrichment analysis revealed that sphingolipid-related pathways differ most among subtypes. Weighted correlation network analysis (WGCNA) implied PDAC subtypes differed in their metabolic programs. Interestingly, a reduced expression among sphingolipid related pathway genes in tumor tissue was associated with the lowest survival rate. These findings suggest that the influence of cellular sphingolipid make-up and its composition have profound context-specific effects on pancreatic oncogenesis.

5.7. Independent Validation And Assay Standardization Of Improved Metabolic Biomarker Signature To Differentiate Pancreatic Ductal Adenocarcinoma From Chronic Pancreatitis (Mayerle et al., 2018 and Mahajan et al., in submission).

The early detection of PDAC without the use of invasive methods is challenging but establishing a cost-effective biomarker with high specificity and sensitivity could significantly improve the treatment and survival in these patients. Unfortunately, routine cancer markers (such as CA19-9) do not seem to be reliable in prediction and detection of early PDAC stages (Hidalgo, 2010; Mayerle et al., 2018). Undoubtedly, one of the greatest biomarker-related challenges in this field is finding biomarkers that accurately distinguish PDAC from other diseases of the pancreas, where overlapping signs and symptoms make differential clinical diagnosis difficult. Indeed, many promising candidate biomarkers, which are excellent at differentiating healthy individuals from patients with PDAC, show disappointing specificity when challenged to discriminate patients with PDAC from patients with chronic pancreatitis (CP) (Costello, 2018).

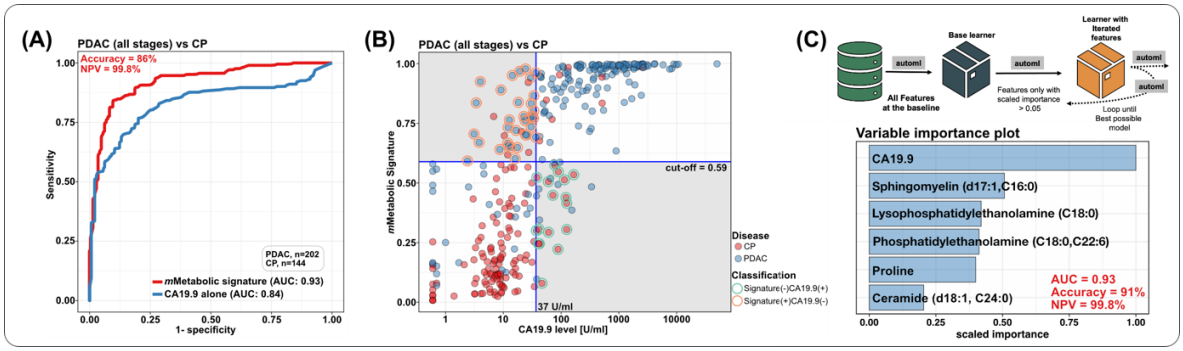

Figure 10: Improved metabolic signature stratifying PDAC from CP. (A) *ROC curves of the improved metabolic biomarker signature (mMetabolic signature) stratifying PDAC vs CP. The ROC curve of CA19.9 demonstrated area under curve of 0.84 compared to 0.93 for the mMetabolic signature.* ***(B)*** *Scatter plot for graphical representation of the mMetabolic signature score. mMETABOLIC signature with the cut-off of 0.59 and CA19-9 on the X axis with the cut-off 37 U/mL. Encircled dots depict subjects that benefit from the mMetabolic signature compared to CA19.9 alone, by preventing miscalssification.* ***(C)*** *Schematic workflow illustrating the machine learning driver iterative reduction of number of metabolites from mMetabolic signature. First, the best performing predictor was selected based on all metabolites in mMetabolic signature (base learner). Next, metabolites were removed following an iterative optimization procedure leading to the minimalistic learner. Ranking of the metabolites for the minimalistic learner by their scaled importance.*

Employing metabolomics, the 'omics technique' that represents a dynamic portrait of metabolic profiles, we delineated a metabolic signature discriminating between chronic pancreatitis and pancreatic cancer which in an unsupervised analysis suggested sphingolipids to be the best discriminating group of metabolites. The metabolic signature distinguishing PDAC from chronic pancreatitis in conjunction with CA19-9 to detect PDAC with a much higher diagnostic accuracy than CA19-9 alone (Mayerle et al., 2018). This study was conducted in 914

subjects which were recruited from three German centres and samples were divided into exploratory, training and test sets. Following metabolome analysis, 477 significantly altered metabolites from 10 ontology classes were identified. A total of 29 metabolites were significantly altered between PDAC and CP in the training set. Subsequently, an Elastic Net algorithm, identified nine metabolite signatures plus CA19-9 with AUC of 0.96 in discriminating PDAC from CP and NPV of 99.9%, assuming a cumulative incidence of 1.95% of PDAC in the CP population. The performance of the biomarker signature was validated in independent test cohort. Thus, the obtained biomarker signature is effective at a time point in the disease pathway when surgical intervention is an effective treatment option.

The metabolic signature was further tested prospectively in an independent multi-centric case-control study (PDAC versus CP) and modified and improved (mMetabolic signature, 12 metabolites) to achieve accuracy of 86% with negative predictive value of 0.998. Further, to improve robustness and interpretability, metabolites with low impact were iteratively removed using machine learning based feature reduction strategy to delineate minimalistic learner that stratify PDAC from CP with accuracy matching to that of mMetabolic signature with minimal number of metabolites. Obtained minimalistic learner consists of 5 metabolites in conjugation with CA19.9 with accuracy of 91%. The performance of identified mMetabolic signature was tested in routine sample handling conditions such as EDTA blood shipping temperature, type of collection tubes. Assay validation revealed that mMetabolic signature should be accessed in plasma of overnight fasted patients and lipemic and icteric plasma samples should be accessed with caution. The results of the improved and updated mMetabolic signature are in submission (Mahajan et. al., in submission).

In summary, following extensive metabolome analysis, we trained and validated biomarker signature that accurately discriminates PDAC from CP. This study uncovers the potential of metabolomics for the diagnosis of PDAC.

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8. Declaration

Hiermit versichere ich an Eides statt, dass ich meine Habilitationsleistung selbständig und ohne andere als die angegebenen Hilfsmittel angefertigt habe, zudem die Herkunft des verwendeten und zitierten Materials ordnungsgemäß kenntlich gemacht habe.

Des weiteren erkläre ich, dass ich mich weder anderweitig habilitiert noch bereits Habilitationsversuche unternommen habe und dass mir kein akademischer Grad entzogen wurde oder ein Verfahren gegen mich anhängig ist, welches zur Entziehung eines akademischen Grades führen könnte.

München, 28. April 2022

Dr. rer. med Ujwal Mukund Mahajan

9. Facsimile of relevant scientific contributions

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