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Evaluation of FAM3C as a potential serum biomarker in ALDH1A3-positive pancreatic ductal adenocarcinoma

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Für meine Familie
Benim ailem için

Danke!
Teşekkürler!

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1. Introduction

1.1. Pancreatic cancer

1.1.1. Epidemiology and etiology

Pancreatic cancer is a deadly disease and the fourth leading cause of cancer-related deaths worldwide[1,2]. In Europe, the incidence of pancreatic cancer and pancreatic cancer-related deaths has continuously risen over the past years[3]. Pancreatic cancer is a disease which is typically seen in older patients, and its incidence is higher in men than in women[4].

Risk factors for pancreatic cancer include smoking[5,6], chronic pancreatitis[7], O-type blood group[8,9] and diabetes[10]. Furthermore, an estimated 10% of pancreatic cancer might be inherited. Although the genetic alterations that cause pancreatic cancer are not yet defined, patients with a family history of pancreatic cancer have a significantly increased risk[11,12].

1.1.2. Histopathology

Even though there are many different types of pancreatic cancer such as acinar cell carcinoma or endocrine tumors, the pancreatic ductal adenocarcinoma (PDAC) is by far the most common and clinically relevant form[13]. Macroscopically, PDAC is a small tumor that tends to infiltrate its surroundings and thus often obstructs the pancreatic and the common bile duct depending on its location within the pancreas[14,15]. The extensive reaction of the surrounding pancreatic tissue and the ensuing fibrosis make it hard to distinguish between invasive PDAC and chronic pancreatitis[15]. In addition, PDAC and chronic pancreatitis are often seen simultaneously in pancreata[15].

Microscopically, PDAC is characterized by a strong desmoplastic reaction of the surrounding pancreas consisting of fibroblastic and inflammatory tissue. Furthermore, the neoplastic glands are often highly differentiated which adds to the difficulty of distinguishing invasive PDAC from pancreatitis[15]. This is why it is crucial to define evident microscopic characteristics of PDAC as defined by Hruban, Pitman and Klimstra in "Tumors of the Pancreas"[14]: (1) haphazard arrangement of the glands, (2) perineural invasion, (3) vascular invasion, (4) a gland immediately adjacent to a muscular artery, (5) luminal necrosis, (6) incomplete lumina, (7) variation in the size of nuclei in a gland by more than four to one, and (8) "naked" glands in fat[14].

There are multiple noninvasive lesions thought to be pancreatic cancer precursors: pancreatic intraepithelial neoplasias (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasms (MCN)[14]. PanIN's are classified into three groups (1-3) according to the extent of histological changes in cell morphology and polarity[16]. PanIN's and IPMN's share some characteristics but are ultimately different types of intraductal neoplasia[16]. They both are noninvasive intraductal lesions that often have a papillary form, produce mucin and are considered to be precursor lesions of invasive PDAC[16]. IPMN's, however, produce more mucin and often lead to macroscopically visible cystic dilatations of the larger pancreatic ducts (>1cm), whereas PanIN's are typically smaller in size and are typically seen in the smaller ducts (<0,5cm) [16,17,18]. In addition, IPMN's and PanIN's have different histological features as well as genetic alterations which can serve to distinguish them[16].
Although there is scientific evidence that proposes that PanIN's and IPMN's to be precursor lesions of invasive PDAC, this theory is still being discussed.
The model of invasive PDAC evolving from precursor lesions such as PanIN's and IPMN's is supported by the fact that these noninvasive preliminary stages carry the same molecular alterations. The common mutations that are seen in PDAC are p53, SMAD4, CDKN2A and KRAS[19]. Interestingly, these genetic alterations can also be detected in IPMN's [20,21,22]and PanIN's[23,24,25]. In addition to having the same genetic alterations, PanIN-3's are found at an increased number in resected pancreata with PDAC compared to pancreata without PDAC[26].

1.1.3. Genetics

The formation of cancer often starts with genetic alterations in a precursor cell[27]. These alterations can affect different genes within a cell: tumor suppressor genes have the function of inhibiting uncontrolled growth and transformation of a cell and inducing apoptosis if required. Oncogenes are genes that play a role in cell growth and differentiation and can cause fast and uncontrolled cell growth when mutated.

Pancreatic cancer has four dominant driver genes: KRAS, CDKN2A, p53 and SMAD4[19]. Accumulation of mutations in these genes eventually leads to a pancreatic cancer precursor cell forming an invasive tumor[28]. Research has shown that these genes are either tumor suppressor genes or oncogenes and that they mutate at different time points in pancreatic carcinogenesis[24,29,30]. KRAS is an oncogene that is

mutated in more than 90% of PDAC's, and its alteration occurs at a very early stage of pancreatic carcinogenesis[19]. CDKN2A is a tumor suppressor gene which plays a role in cell cycle regulation. Mutated CDKN2A can also be seen in the early stages of pancreatic carcinogenesis[19]. The tumor suppressor gene p53 had first been described by multiple research groups in 1979, and its mutation and function as a cell cycle regulator have been characterised in multiple tumor entities[31,32]. In pancreatic cancer, mutated p53 is seen at later stages of carcinogenesis compared to KRAS and CDKN2A mutations[19]. SMAD4, an essential mediator in TGF-beta signalling, is a tumor suppressor that mutates at a later timepoint, similar to p53, in carcinogenesis of PDAC[19,33].

To improve the prognosis of pancreatic cancer, it is essential to improve the diagnostic methods to detect early stages of invasive PDAC. This is because most patients presenting themselves with pancreatic cancer-specific symptoms are at an advanced stage of the disease[34]. Many studies investigated the relation of invasive PDAC and precursor lesions of the pancreas such as pancreatic intraepithelial neoplasias (PanIN) through a genetical approach. The results showed that genetic alterations in PDAC and PanIN's are very similar[35,36,37]. This further supports the current theory of pancreatic cancer progression which hypothesises that invasive PDAC evolves from PanIN's[34,35,36].

1.1.4. Clinical presentation

A significant factor that contributes to the poor prognosis of PDAC is its late diagnosis, which is partially due to the lack of specific early symptoms of pancreatic cancer. Although early symptoms of pancreatic cancer such as changes in bowel habit, nausea and vomiting, pruritus, back pain, shoulder pain, abdominal pain, jaundice, weight loss, dyspepsia, bloating, new-onset diabetes and lethargy have been described, the diagnosis of PDAC still seems to be made too late[38]. Even though patients went to their general practitioner (median of 3 occasions) with alarm symptoms in the two years prior to their diagnosis[38], most patients who are diagnosed with pancreatic cancer have an advanced stage of the disease that is often not surgically resectable and thus not curable[39,40]. For those patients the disease eventually culminates in organ failure due to direct invasion, metastasis or thromboembolic events. The fact that distant metastasis of a primary tumor to other organs is a late event in pancreatic

carcinogenesis and that there is no curative treatment for a metastasised PDAC emphasizes the need for detection at early stages with therapeutic potential[41].

1.1.5. Diagnosis

As aforementioned, the very limited possibility of surgical resection and other therapies of the disease at a late stage, makes early diagnosis paramount. Because of its retroperitoneal location, changes in form and morphology of the pancreas are almost impossible to detect during a clinical examination of the abdomen.

1.1.5.1. Imaging

The search for the optimal imaging technique for early detection and diagnosis of pancreatic cancer is the subject of current research and standards constantly change as technology progresses. Imaging techniques most commonly used include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), endoscopic ultrasound (EUS) and trans-abdominal ultrasound (TAUS). A study from 2012 which analyzed the data from 67 studies identified MRI and CT as the most suitable and practical tools for detecting pancreatic cancer and suggested that TAUS is an excellent complimentary imaging technique[42]. A meta-analysis from 2017 which included 52 studies from 2004 to 2014 investigated the diagnostic ability of different imaging techniques and concluded that MRI had the highest sensitivity (93%) whereas TAUS had the highest specificity (94%) as well as overall diagnostic accuracy (91%)[43]. Although the results of the two studies are not entirely congruent, their conclusions are similar. An important point that has to be considered when looking at these results is that there are distinct differences between these tools. TAUS, for example, is a diagnostic tool which takes an experienced examiner to reach its full potential and other tools such as EUS offer additional diagnostic possibilities such as tissue biopsies.

1.1.5.2. Biomarkers

Although CA19-9 is being used as a diagnostic and prognostic marker for PDAC, it is not routinely integrated in everyday clinical practice[44]. While perioperative CA19-9 can be used as a prognostic marker and aid in postoperative therapeutical decisions, its significance as a diagnostic tool remains low[45]. CA19-9 has difficulties detecting early stages of pancreatic cancer and does not reliably differentiate between malignant and benign disease of the pancreas[46,47,48]. As these difficulties can be observed with many

other potential serum biomarkers, exploration of new specific biomarkers for PDAC remains essential[49].

Neoplastic diseases have a broad effect on protein expression in both tissue and serum through different processes including inflammation and systemic immune response[50,51]. These observations prompted multiple studies to evaluate proteins other than cancer antigens, such as different inflammatory and acute-phase proteins for their use as tumor biomarkers[52,53]. Although some of these studies show that acute-phase-proteins are elevated in malignant disease, the specificity for malignant disease was so low that it could not be considered a tumor biomarker[54].

Multiple studies showed that a combination of CA19-9 together with inflammatory proteins as well as other secreted proteins previously associated with neoplastic disease was able to increase screening sensitivity and specificity when compared to CA19-9 alone. The study identified a panel of three proteins (CA19-9, ICAM-1, OPG) that increased testing sensitivity from 51,4% to 78% and specificity from 90% to 94,1% in PDAC patients compared to healthy patients[55]. Another more recent study showed that a combination of CA19-9, CA125 and LAMC2 could further increase sensitivity from 69,1% to 82% although specificity was decreased from 80% to 73,8% in PDAC patients compared to healthy patients[56]. Although these recent studies show improvement, the necessity to find more sensitive and specific biomarkers for PDAC remains high.

1.1.6. Therapy

A significant factor that contributes to the poor prognosis of pancreatic cancer is the fact that there are few treatment options and complete surgical resection remains the only curative therapy[57]. Other therapeutic options are limited because of the tumor's resistance to chemotherapy as well as radiotherapy[58]. Before beginning treatment, it is essential to do a thorough staging to understand the extent of the disease. At the time of diagnosis, pancreatic cancer can be limited to the pancreas or be at an advanced stage in which it has metastasised to other organs. Pancreatic cancer which is limited to the pancreas can be classified as resectable, borderline resectable or locally advanced. Therapy guidelines for the different stages of pancreatic cancer are defined by the NCCN[59].

1.1.6.1. Chemotherapy

Chemotherapy as a first-line treatment is recommended in patients with metastatic pancreatic cancer and locally advanced pancreatic cancer as well as adjuvant therapy for patients who underwent surgical resection[60].

Until 2011 the two main chemotherapy options were gemcitabine and fluorouracil which had a similar effect[61,62]. A study which was published in 2011 introduced a new therapy regimen FOLFIRINOX (Folinic Acid, Fluorouracil, Irinotecan and Oxaliplatin), which significantly increased the patient's survival but lead to more severe side effects[63]. Because of the extensive side effects, the decision which chemotherapy to choose is mainly based on the patient's ability to tolerate a more toxic therapy. For patients who are not likely to tolerate the side effects of FOLFIRINOX, a combination of gemcitabine and natural albumin-bound paclitaxel is a viable option as it has been shown to be more effective than gemcitabine alone[64]. In conclusion, the decision on which chemotherapy to choose in metastatic cancer, as well as locally advanced cancer, is highly dependent on the individual patient and its performance status[59].

1.1.6.2. Radiotherapy

Radiotherapy in the treatment of pancreatic cancer is used in the therapy of locally advanced nonresectable pancreatic cancer as well as a neoadjuvant therapy and is often combined with chemotherapy[59,65].

1.1.6.3. Adjuvant therapy

Adjuvant therapy is recommended for all patients that had surgical resection of the pancreas with a curative intent[60]. The typical options for chemotherapy regimens are described above and can be combined with radiotherapy[59].

1.1.6.4. Neoadjuvant therapy

Neoadjuvant therapy is an option for resectable and borderline resectable pancreatic cancer as it serves to shrink a tumor before surgery and potentially facilitate a complete surgical resection[66]. Neoadjuvant therapy consists of chemotherapy, radiotherapy or a combination of both. Although adjuvant therapy plays a much more critical role in the treatment of pancreatic cancer, neoadjuvant therapy is an essential option for a subset of patients with an otherwise non-resectable tumor as it may downstage the disease.

In addition, there are indications that the postoperative tumor bed is less sensitive to chemo- and radiotherapy, thus rendering an adjuvant therapy approach impotent[67,68].

1.1.6.5. Surgery

Surgery remains the only option for the treatment of pancreatic cancer with a curative intent[57]. Although general mortality of surgery is low, it is still recommended that surgical resections of the pancreas should be performed in specialized centres[69,70]. Because of the anatomical location of the pancreas in the retroperitoneum, most surgeries are done laparatomically although, if performed by an experienced surgeon, pancreatic tail resections can also be done laparoscopically[71,72].

1.2. Identification of ALDH1A3 as a marker for an aggressive PDAC subtype through a translational approach

As Kras-mediated mTOR activation through the Mek/Erk pathway and to a smaller extent through the PI3K/Akt pathway was shown to be the dominant driving force of pancreatic ductal adenocarcinoma (Figure 1), Kong et al. conducted a more detailed study of these pathways and the mechanisms through which they lead to tumor formation [73] . Also, the role of Tsc1 in mTOR activation was studied as haploinsufficiency of Tsc1 plays a role in Kras mediated tumor formation in the lungs. Two mouse models were created depending on either Mek/Erk ($Kras^{G12D/+}$/$Tsc\text{-}1^{+/-}$) or PI3K/Akt ($Pten^{-/-}$/$Tsc\text{-}1^{+/-}$) pathways for mTOR activation. Interestingly, the two different pathways lead to different macroscopic and microscopic changes of the murine pancreas in Tsc1 haploinsufficient mice: While the Mek/Erk pathways lead to invasive PDAC and extensive metastasis, the PI3K/Akt pathway leads to pancreatic cysts and significantly less metastasis. In concordance with the histological differences of the pancreata, mice depending on Mek/Erk signalling for mTOR activation had a significantly shorter median survival.

To identify genes that contribute to the aggressiveness of the Mek/Erk-dependent pathway, the transcriptional profiles of the $Kras^{G12D/+}$/$Tsc\text{-}1^{+/-}$, $Pten^{-/-}$/$Tsc\text{-}1^{+/-}$ as well as control cell lines were analyzed. The gene that had the lowest p-value and was most specific for the $Kras^{G12D/+}$/$Tsc\text{-}1^{+/-}$ cells was ALDH1A3 (Aldehyde Dehydrogenase Family 1 Member A3). In line with this finding, bioinformatical analysis of the activated pathways in the different cell lines rendered the glucose metabolism as essential to the $Kras^{G12D/+}$/$Tsc\text{-}1^{+/-}$ cells. Further analysis showed that reduced expression of

ALDH1A3 in $Kras^{G12D/+}$/$Tsc\text{-}1^{+/-}$ cells leads to impaired glycolysis in cells and reduced tumor cell growth in transplanted WT mice.

As a next step, the role of ALDH1A3 as a marker for aggressive pancreatic cancer was validated in human PDAC tissue. Interestingly, ALDH1A3 positivity in resected PDAC tissue significantly correlated with shorter postoperative survival (14 months versus 22.8 months) and more lymph nodes positive for cancer cells.

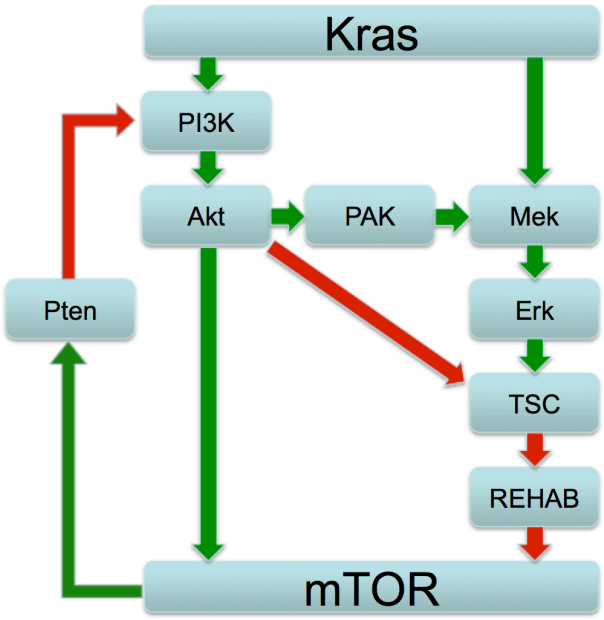

Figure 1: The two major pathways that lead to oncogenic Kras mediated activation of mTOR in pancreatic cancer. Green arrows indicate a stimulatory effect. Red arrows indicate an inhibiting effect.

1.3. The aldehyde dehydrogenase family

1.3.1. General

The aldehyde dehydrogenase (ALDH) family consists of 19 isoforms which have many different physiological functions in the human body including (1) the biosynthesis of retinoic acid[74], (2) metabolization of alcohol[75] and (3) reduction of osmotic, oxidative and chemical stress[76,77,78]. ALDH's carry out their protective and detoxifying function through the oxidation of aldehydes into carboxylic acids[79]. Although aldehydes are physiologically present in the human body, excessive amounts can have a cytotoxic and carcinogenic effect[79]. ALDH's are present in many different organs and tissue types as well as many subcellular compartments of the cell including the cytosol, mitochondria, nucleus, peroxisomes[79].

1.3.1. Cancer stem cell marker

Studies have shown that ALDH is a cancer stem cell (CSC) marker in many different types of cancer and can be used to identify and isolate CSC's from a cancer tissue[80,81]. In addition to being a CSC marker, ALDH seems to play an essential role in the malignant potential and cell function of cancer cells. One of these functions relies on its impact on retinoic acid metabolism which has been described to be altered in cancer cells[82]. As the physiological role of ALDH is to detoxify and protect the cell from various stressors, ALDH has been connected to chemotherapy and radiotherapy resistance in different cancer types[83,84,85]. Many studies have shown that high ALDH expression in the cancer tissue is a marker for metastasis and poor prognosis in different cancer tissues including the pancreas[86,87,88]. Interestingly, ALDH1A3, in particular, has also been identified as a predictor of poor prognosis and metastasis in different cancer tissues including breast and gallbladder cancer[89,90]. In the context of pancreatic cancer, ALDH1A3 was shown to have increased expression in cancer tissue compared to healthy pancreatic tissue[91].

1.4. Family with sequence similarity 3 member C

FAM3C or interleukin-like EMT inducer (ILEI) is a cytokine that has first been described in 2002 and has since been associated with epithelial to mesenchymal transition (EMT) process, metastasis and tumor progression in different cancers[92,93,94,95]. FAM3C is part of the family with sequence similarity three (FAM3) family which consists of four different proteins that are differently expressed in various tissue types and organs[92]. The unprocessed FAM3C molecule consists of a signalling peptide, a propeptide and the FAM3C protein[96].

Upregulated FAM3C expression and cytoplasmic subcellular localization of FAM3C have been associated with increased tumor growth, poor prognosis and metastasis in the invasive front of colorectal cancer, breast cancer and hepatocellular carcinomas[93,94,95]. Also, FAM3C was shown to be upregulated on the protein level in pancreatic cancer cells compared to non-neoplastic pancreatic duct cells[96]. Multiple studies described different staining patterns of FAM3C which were characterised as either granular or cytoplasmic and corresponded with favourable and poor prognosis in human breast and hepatocellular cancer cells[93,94,95].

FAM3C signaling requires oncogenic Ras-mediated MAPK/Erk hyperactivation to carry out its carcinogenic potential[93,94,95]. Although FAM3C was shown to be a

downstream target of the TGF-beta pathway, recombinant FAM3C alone was able to induce EMT in human breast cancer cells[94]. In line with this finding, breast cancer cells that were transfected with FAMC-specific siRNA leading to a transient reduction of FAM3C expression were not able to undergo EMT[94]. Although the signalling pathways of FAM3C have been partially understood, the FAM3C receptor remains unknown.

A study in breast cancer partially described the intra- and extracellular mechanisms required for FAM3C activation[94]. To carry out its tumor-promoting function, FAM3C needs to be cleaved and split from its propeptide by proteases of which plasmin is the most characterized[94]. This process requires a propeptide-dependent connection to structures of the extracellular matrix[94]. Also, the proteolytic processing of FAM3C is dependent on urokinase plasminogen activator (uPA) which is a serine protease[94]. uPA activates plasmin from its inactive propeptide plasminogen by uPA-receptor-dependent proteolytical cleavage[94]. The uPA-system has been extensively studied and has been correlated with many different tumor entities. The overexpression of either uPA or the uPA-receptor in the resected tumor tissue of patients was identified as an independent prognostic marker for a poor outcome in different types of tumors including colorectal cancer and breast cancer[97,98]. In the context of pancreatic cancer, Harris et al. were able to show that high expression of uPA-mRNA in resected pancreatic cancer tissue significantly correlated with poor prognosis[99].

Furthermore, different publications were able to show that elevated serum levels of either uPA or uPA-R correlate with a poor prognosis in soft tissue sarcoma, breast cancer and PDAC[100,101,102]. The authors of the study of uPA expression in the serum of PDAC patients were able to show that patients with PDAC had uPA serum levels which were increased 3-fold compared to the control group. In summary, FAM3C is a downstream target of TGF-beta that is activated by proteolytic cleavage and has been shown to be essential for EMT and tumor progression in different human cancers. Also, FAM3C is connected to the Plasmin/uPA signalling pathway which has been shown to be upregulated in many different cancers including PDAC which makes FAM3C an interesting subject for further research.

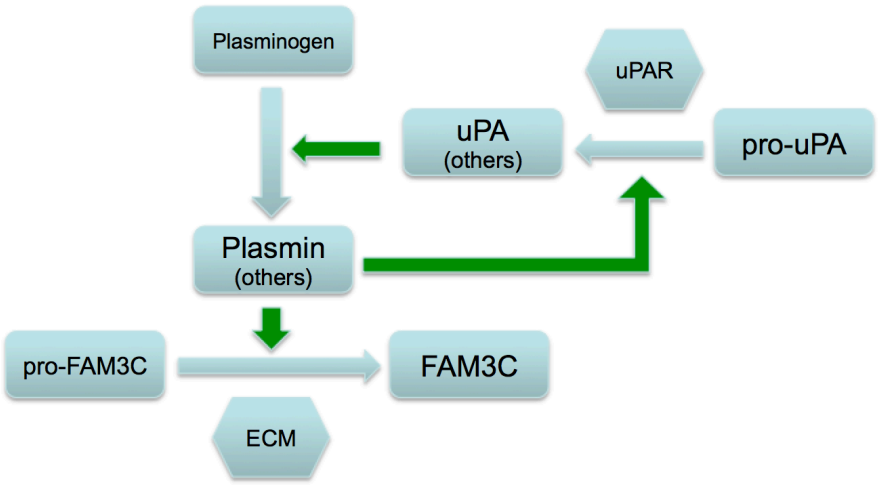

Figure 2: Plasmin-dependent proteolytic activation of FAM3C. Green arrows indicate activation through proteolytic cleavage.

2. Aims of this study

Kong et al. identified the ALDH1A3-positive subtype to be particularly aggressive; a better understanding of this subtype is of great significance to improve its diagnosis and therapy. In particular, this study had the following aims:

To find a serum biomarker that correlates with ALDH1A3 expression in PDAC tissue.

As the postoperative prognosis of patients with the ALDH1A3-positive subtype is inferior, surgical treatment might not be the optimal treatment for this group of patients. As ALDH1A3 is not a secreted protein and is only detectable in resected tissue, a biomarker in the patient's serum needs to be identified before surgery. Patients with the ALDH1A3-positive subtype might benefit from neoadjuvant therapies instead of upfront surgery.

To identify potential druggable targets of ALDH1A3-positive subtype.

To offer the ALDH1A3-positive patients a specific neoadjuvant therapy, putative molecules that play an essential role in the signaling of the ALDH1A3-positive subtype need to be identified.

To clarify if ALDH1A3 is expressed in the early stage of pancreatic carcinogenesis.

If an increased ALDH1A3 expression can be detected in the early stages of pancreatic carcinogenesis, a correlated biomarker might be increasingly expressed in the serum as well. This biomarker might potentially serve as a diagnostic tool in the detection of early stages of PDAC which is currently desperately needed.

3. Materials and methods

3.1 Materials

3.1.1 Laboratory equipment

Alarm Clock	Carl Zeiss, Oberkochen
Analytic Scale A 120 S	Sartorius, Goettingen
Autoclave VX-75	Systec, Wettenberg
Automated Microtome RM2255	Leica Microsystems, Wetzlar
AxioCam MRc5	Carl Zeiss, Oberkochen
Brush	Brunnen, Heilbronn
Centrifuge 5415R	Eppendorf, Hamburg
CO_2 Incubator 37°C Heracell 150i	Thermo Fisher Scientific, Waltham MA (USA)
Computer Hardware	Fujitsu Siemens, Tokyo (Japan)
Cooling Plate COP 30	Medite GmbH, Burgdorf
Embedding Center EG1160	Leica Microsystems, Wetzlar
Film Processor Optimax 1170	Protec, Oberstenfeld
Fridge 4°C	Liebherr, Bulle (CH)
Freezer -20°C	Siemens, Munich
Freezer -80°C HERAfreeze	Thermo Fisher Scientific, Waltham MA (USA)
Glassware	Schott, Mainz
GraphPad Prism 6 Software	GraphPad, San Diego CA (USA)
Hera Cell 150 Incubator	Thermo Fisher Scientific, Waltham MA (USA)
Immunoblotting Equipment	Invitrogen, Carlsbad CA (USA)
Ice-Flaker AF100	Scotsman, Vernon Hills IL (USA)
Magnetic Stirrer	IKA, Staufen
Multichannel Pipet	Eppendorf, Hamburg
GloMax Multimode Reader	Promega, Madison WI (USA)
Microscope Axioskop 40	Carl Zeiss, Oberkochen
Microscope Axiovert 40 CFL	Carl Zeiss, Oberkochen
Microscope Axio Imager M2	Carl Zeiss, Oberkochen
Microscope DME	Leica Microsystems, Wetzlar
Microwave	Siemens, Munich
Multifuge 3SR+ Heraeus	Thermo Fisher Scientific, Waltham MA (USA)
Paraffin Stretching Bath GFL 1052	Julabo, Seelbach

PeqMeter 1.14	Peqlab Biotechnologie, Erlangen
Pipetboy	Integra Bioscience GmbH, Fernwald
Pipet	Eppendorf, Hamburg
Roche LightCycler 480	Roche, Basel (CH)
Rotilabo Stirring Magnet	Carl Roth, Karlsruhe
Thermocycler Doppio	VWR International, Ismaning
Thermomixer	Eppendorf Hamburg
Tissue Cool Plate COP30	Medite GmbH, Burgdorf
Tube Rotator	Greiner-Labortechnik, Frickenhausen
Tube Roller	Stuart, Stone (UK)
Safety Cabinet Herasafe KS 15	Thermo Fisher Scientific, Waltham MA (USA)
Scale HT-500	A&D, Tokyo (Japan)
Scanner	Canon, Tokyo (Japan)
Stereomicroscope	Carl Zeiss, Oberkochen
Stirrer	Labinco, Breda (Netherlands)
Ultrasonic Processor UP100H	Hielscher, Teltow
Uniprotect Air Flow Cabinet	Bioscape GmbH, Castrop-Rauxel
Vacuum Tissue Processor ASP200S	Leica Microsystems, Wetzlar
Vibration Platform	Heidolph Instruments, Schwabach
Vortex Mixer	Neo Lab, Heidelberg
Water bath	GFL, Grossburgwedel
X-Ray Cassette	Rego, Augsburg

3.1.2 Consumables

Blotting Paper	Whatman, Maidstone (UK)
Biocoat Matrigel Invasion Chamber	BD Bioscience, Heidelberg
Cell Scraper	Sarstedt, Nuembrecht
Coverslips	Menzel, Braunschweig
Dako Pen	Dako, Glostrup (DK)
Disposable Scalpell "Feather"	Pfm Medical, Cologne
Disposable Weighing Trays	Carl Roth, Karlsruhe
Embedding Cassettes	Carl Roth, Karlsruhe
Eppendorf Tube (1,5 ml, 2 ml)	Eppendorf, Hamburg
Falcons (15 ml, 50 ml)	Greiner Bio-one, Frickenhausen

Gloves	Ansell, Richmond (Australia)
Hyperfilm	GE Healthcare, Little Chalfont (UK)
Insulin Syringes Micro-Fine	BD Falcon, Franklin Lakes NJ (USA)
Cryotube	Greiner Bio-one, Frickenhausen
Microplate (96-well)	Greiner Bio-one, Frickenhausen
Nitrocellulose Transfer Membrane	Bio-Rad Laboratories, Munich
Parafilm M	Pechiney Packaging, Chiacago IL (USA)
Pipette Tip	Starlab, Hamburg
Tissue Culture Plate (6,24,96-well)	Greiner Bio-one, Frickenhausen
Tissue Culture Dish (100x20 mm)	Sarstedt, Nuembrecht
Tissue Culture Flask (75 cm2)	Greiner Bio-one, Frickenhausen
Serological Pipettes (50,25,10,5 ml)	Greiner Bio-one, Frickenhausen
Superfrost Plus Microscopic Slides	Menzel, Braunschweig
X-Ray Film	AGFA, Munich

3.1.3 Chemicals

0.25% Trypsin/EDTA	Sigma-Aldrich, St. Louis MO (USA)
2-Mercaptoethanol	Sigma-Aldrich, St. Louis MO (USA)
Acetic Acid	Merck Biosciences, Darmstadt
Acrylamide Solution Rotiphorese	Carl Roth, Karlsruhe
Albumin Fraction V	Carl Roth, Karlsruhe
Agarose	Carl Roth, Karlsruhe
Ammonium Persulfate	Sigma-Aldrich, St. Louis MO (USA)
Antibody Diluent	Cell Signaling, Cambridge (UK)
Caerulein	Sigma-Aldrich, St. Louis MO (USA)
Cell Lysis Buffer	Cell Signaling, Cambridge (UK)
Citric Acid Monohydrate	Carl Roth, Karlsruhe
Crystal Violet	Carl Roth, Karlsruhe
DMEM High Glucose No Glutamine	Sigma-Aldrich, St. Louis MO (USA)
ECL Detection Reagent	Amersham, Little Chalfont (UK)
EnVision+ System	Dako, Glostrup (DK)
Eosin Y	Sigma-Aldrich, St. Louis MO (USA)
Ethanol (50%,70%,96%,100%)	Carl Roth, Karlsruhe
Fetal Bovine Serum	Sigma-Aldrich, St. Louis MO (USA)

Glycine	Roche, Basel (CH)
Hematoxylin	Merck Biosciences, Darmstadt
HiPerFect Transfection Reagent	Qiagen, Hilden
Hydrochloric Acid (5M)	Apotheke TU München, Munich
Hydrogen Peroxide (30%)	Carl Roth, Karlsruhe
Isopropanol	Carl Roth, Karlsruhe
Isofluran	CP-Pharma, Burgdorf
LDS Sample Buffer (4x)	Thermo Fisher Scientific, Waltham MA (USA)
Liquid Nitrogen	Tec-Lab, Taunusstein
Liquid DAB+ Sub. Chr. System	Dako, Glostrup (DK)
Methanol	Carl Rothe, Karlsruhe
MOPS	Carl Roth, Karlsruhe
Mounting Medium Vecta Mount	Vector Laboratories, Burlingame CA (USA)
Nitrocellulose Membranes	GE Healthcare, Little Chalfont (UK)
Normal Goat Serum	Dako, Glostrup (DK)
Paraformaldehyde	Apotheke TU München, Munich
Penicillin-Streptomycin	Sigma-Aldrich, St. Louis MO (USA)
Phosphate Buffered Saline (pH 7.4)	Sigma-Aldrich, St. Louis MO (USA)
Phosphatase Inhibitor Cocktail	Roche, Basel (CH)
Powdered Milk	Carl Roth, Karlsruhe
Protease Inhibitor Cocktail	Roche, Basel (CH)
Protein Ladder	Thermo Fisher Scientific, Waltham MA (USA)
Potassium Chloride	Carl Roth, Karlsruhe
RNAse DNAse-free water	Invitrogen, Karlsruhe
Roticlear	Carl Roth, Karlsruhe
Sample Reducing Agent (10x)	Thermo Fisher Scientific, Waltham MA (USA)
SDS Ultra-Pure	Carl Roth, Karlsruhe
Sodium Chloride	Carl Roth, Karlsruhe
Sodium Hydroxide (5M)	Apotheke TU München, Munich
TEMED	Carl Roth, Karlsruhe
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich, St. Louis MO (USA)
Tris Base	Sigma-Aldrich, St. Louis MO (USA)
Tween 20	Carl Roth, Karlsruhe
Trypan Blue Solution (0,4%)	Sigma-Aldrich, St. Louis MO (USA)

3.1.4 Kits

BCA Protein Assay Kit	Thermo Fischer Scientific, Dreieich
Human FAM3C ELISA Kit *Product Code: CSB-EL008228HU*	Cusabio, Wuhan (China)
RevertAid First-strand cDNA Synthesis Kit	Thermo Fischer Scientific, Dreieich
RNeasy Plus Mini Kit	Qiagen, Hilden
SYBR Green 1 Master Kit	Roche, Basel (CH)
Tissue type Plasminogen Activator Human ELISA Kit *Product Code: ab119563*	Abcam, Cambridge (UK)

3.1.5 Buffers and solutions

10x Tris Buffered Saline (TBS)

H2O	800 ml
Tris Base	12.1 g
NaCl	85 g
Adjust pH to 7.4 with	5 M HCl
Constant volume with H2O to	1000 ml

1x TBS

10x TBS	100 ml
H2O	900 ml

Washing Buffer (1x TBS+0.1% BSA)

1x TBS	1000 ml
BSA	1 g

20x Citrate Buffer

H2O	300 ml
Citric Acid	21 g
Adjust pH to 6.0 with	5 M NaOH
Constant volume with H20 to	1000 ml

1x Citrate Buffer

20x Citrate Buffer	50 ml
H2O	950 ml

Electrophoresis Buffer

MOPS	209.2 g
Tris Base	121.2 g
SDS	20 g
EDTA-free acid	6 g
Constant volume with H2O to	1000 ml

Transfer Buffer

Glycine	14.7 g
Tris Base	29.1 g
Methanol	1000 ml
SDS	0.1875 g
Constant volume with H2O to	1000 ml

Washing Buffer

10x TBS	100 ml
Tween 20	0.5 ml
Constant volume with H2O to	1000ml

Blocking Buffer

Powdered Milk	0.5 g
Washing Buffer	10 ml

1x Lysis Buffer

10x Lysis Buffer	1 ml
H20	9 ml
Phosphatase Inhibitor	1 Tablet
Proteinase Inhibitor	1 Tablet

3.1.6 Antibodies and siRNA

Anti-ALDH1A3 Antibody *Product Code: HPA04271*	Sigma-Aldrich, St. Louis MO (USA)
Anti-FAM3C Antibody *Product Code: HPA050548*	Sigma-Aldrich, St. Louis MO (USA)
Anti-IRS2 Antibody *Product-Code: ab134101*	Abcam, Cambridge (UK)
Anti-KRT81 Antibody *Product Code: sc100929*	Santa Cruz Biotech, Dallas TX (USA)
Anti-MCC Antibody *Product Code: HPA037391*	Sigma-Aldrich, St. Louis MO (USA)
Anti-SP100 *Product Code: HPA016707*	Sigma-Aldrich, St. Louis MO (USA)
Anti-β-Actin Antibody *Product Code: sc69879*	Santa Cruz Biotech, Dallas TX (USA)
Anti-E-Cadherin Antibody *Product Code: 14472*	Cell Signaling, Cambridge (UK)
Anti-Mouse IgG HRP Conjugate *Product Code: W402B*	Promega, Madison WI (USA)
Anti-Rabbit IgG HRP Conjugate *Product Code: W401B*	Promega, Madison WI (USA)
HRP Labelled Polymer Anti-Mouse *Product Code: K4003*	Dako, Glostrup (DK)
HRP Labelled Polymer Anti-Rabbit *Product Code: K4003*	Dako, Glostrup (DK)
Hs_FAM3C_FlexiTube siRNA *Product Code: SI00383740* *Target Sequence:* *TACGATGATGGAGCAACCAAA*	Qiagen, Qiagen, Hilden
Negative Control siRNA *Product Code: 1022076*	Qiagen, Qiagen, Hilden

	Immunohistochemistry		Immunoblotting	
Antibody	Primary	Secondary	Primary	Secondary
ALDH1A3	1:100	RTU	1:8000	1:5000
FAM3C	1:500	RTU	1:1000	1:5000
IRS2	1:500	RTU	-	-
KRT81	1:200	RTU	-	-
MCC	1:750	RTU	-	-
SP100	1:500	RTU	-	-
β-Actin	-	-	1:2500	1:5000
E-Cadherin	-	-	1:1000	1:5000

Table 1. Antibody Dilutions for Immunohistochemistry and Immunoblotting. RTU: ready to use.

3.2. Methods

3.2.1. Tissue

3.2.1.1. Pancreatic tissue collection and patient data

Paraformaldehyde
Paraffin
RNAlater

Pancreatic tissue was obtained from patients who had undergone resection surgery for PDAC. Prior to surgery, written informed consent was obtained from all patients. The usage of tissue for further studies was approved by the local Ethics Committee of the Technical University of Munich, Germany. The resected tissue was fixed in 4% paraformaldehyde solution for 24 hours after which it was embedded in paraffin for further histological analysis. In addition, some of the tissue was flash frozen in liquid nitrogen and stored in RNAlater as well. All samples underwent hematoxylin-eosin staining and diagnosis was confirmed histologically.
Patients' data including survival, tumor stage, symptoms and treatment was retrospectively obtained and analyzed from the database of the Technical University of Munich.

3.2.2. Mouse experiments

3.2.2.1. Mice

The caerulein injection experiment, as well as the pancreatic duct ligation experiment, was carried out with KC mice. The genotype, as well as other characteristics, can be seen in the table below. The mice were kept in standard cages and received a standard rodent diet.

Mouse line	Genotype	Number	Age range
KC	$P48^{Cre/+}$; $LSL\text{-}Kras^{G12D/+}$	9	8 weeks

Table 2: Description of genotype, number and age range of the mice subjected to experiments.

3.2.2.2. Caerulein injections

The caerulein injections were carried out on two consecutive days in which the mice were subjected to eight hourly caerulein injections into the peritoneum per day (Figure 3A). Before the beginning of the experiment, a stock solution of 20 µg/ml was prepared.

The mice were then injected with 100 µl of this stock solution containing two µg of caerulein each, rendering a total of 32 µg of caerulein for each mouse. After a waiting period of twelve days, the mice were euthanized and pancreata harvested for further analysis.

3.2.2.3. Pancreatic duct ligation

For pancreatic duct ligation (PDL) surgery, the surgical environment such as instruments and the area of surgery were sterile to minimize the incidence of contamination and infection. The mice were anaesthetised with sevoflurane gas. Appropriate anesthetization was verified by observing the movement of the mice and checking for reflexes by gentle pinching.

Before surgery, the abdomen and thorax were disinfected with an antiseptic solution and the area of surgery was shaved. As the first step of the surgery, a midline incision extending from the sternum to the umbilical area was made using a scalpel. After opening the peritoneum with surgical scissors, the pancreas was located at the posterior wall of the stomach. After gently mobilising and retracting the stomach as well as the duodenum the pancreas was exposed. As a next step, the splenic lobe of the pancreas was located and mobilized by making a small incision and opening the retroperitoneum. The pancreatic duct of the splenic lobe was then ligated with an 8-0 thread while making sure that no blood vessels in anatomical proximity were impaired in the process. After placing the organs back into the abdominal cavity, the midline incision was closed using a 5-0 thread. The mouse was then placed back in its cage and observed.

Two weeks after surgery, the mice were euthanised, and the laparotomy incision was reopened (Figure 3B). After successful ligation of the pancreatic duct, the splenic lobe now appeared to be translucent with little white dots. The pancreas, as well as the spleen, were taken and placed into formaldehyde.

3.2.2.4. Caerulein injection and pancreatic duct ligation

As a third experimental approach, the two procedures explained above were combined (Figure 3C). The mouse was subjected to two consecutive days of caerulein injections and then received pancreatic duct ligation surgery after five more days.

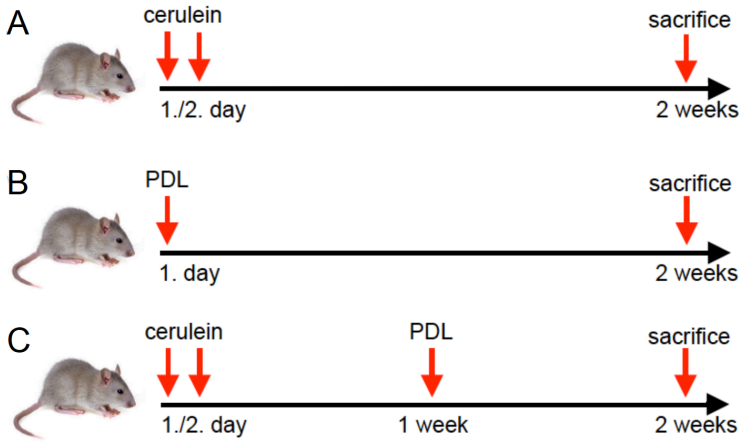

Figure 3: Three different approaches were chosen to induce inflammation in murine pancreata. (A) Mice were subjected to two consecutive days of caerulein injections, and pancreata were harvested after a total of 14 days. (B) Pancreatic duct ligation of the splenic lobe was carried out on the first day of the experiment and pancreata were harvested after a total of 14 days. (C) Mice were subjected to two consecutive days of caerulein injections and additional pancreatic duct ligation surgery after one week. Pancreata were harvested after a total of 14 days.

3.2.3. Staining

3.2.3.1. HE-Staining

Roticlear
Ethanol (100%, 96%, 70%, 50%)
Hematoxylin
Eosin
Vecta Mount Mounting Medium

To render a histological analysis of the structure of tissue possible, the HE-staining is used in which all acidic structures such as the nucleus are stained in blue with haematoxylin, and all basic structures such as collagen are stained in red with eosin. To begin the staining process, the tissue which is fixated onto slides was exposed to Roticlear (3x10 minutes) to remove the paraffin. After rehydrating the tissue in progressively decreasing concentrations of ethanol (100%, 96%, 70%, 50% for 2,5 minutes each) the tissue was stained with hematoxylin and washed with running tap water for 10 minutes. As a next step, the tissue was stained with eosin after which it was shortly washed with water and then dehydrated with progressively increasing concentrations of ethanol (50%, 70%, 96%, 100%). The tissue sections were then exposed to Roticlear (3x10 minutes) and mounted with Vecta mount Mounting Medium.

3.2.3.2. Immunohistochemical analysis

Roticlear
Ethanol (50%, 70%, 96%, 100%)
Citrate Buffer
TBS
BSA
Normal Goat Serum
Methanol
Hydrogen Peroxide
Dako EnVision+ System
Liquid DAB+ Substrate Chromogen System
Hematoxylin
Mounting Medium Vecta Mount
Antibody Diluent

Immunohistochemical staining (IHC) is used to examine the histological localisation of a target protein within a given tissue using a specific antibody through a consecutive colour reaction. Preceding IHC staining, the formalin-fixed and paraffin-embedded pancreatic tissue samples were cut into three µm thick sections and fixated on microscopic slides. At the beginning of the first day, the pancreatic tissue sections were deparaffinized using Roticlear (3x10 minutes) and rehydrated in ethanol with progressively decreasing concentrations (100%, 96%, 70%, 50% for 2,5 minutes each). After washing the tissue with water, antigens were retrieved by boiling the tissue in a microwave oven for 10 minutes using a preheated ten mM citrate buffer (pH=6). After cooling down to room temperature, the tissue was washed with water (1x5 minutes) and TBS containing 0,1% BSA (1x5 minutes). Endogenous peroxidase activity was blocked by exposing the tissue to 3% hydrogen peroxide in 30% methanol for 10 minutes. The tissue was then washed with TBS/0,1% BSA (3x5 minutes). To block the epitopes and prevent nonspecific binding of the primary antibody, the tissue was exposed to TBS containing 10% NGS in a wet chamber for 1 hour at room temperature. Immediately after this step, the primary antibody was applied, and the tissue was incubated overnight at 4°C.

On the second day, the tissue was first washed with TBS/0,1% BSA (3x 5 minutes) after which it was exposed to the secondary antibody in a wet chamber for 1 hour at room temperature. The secondary antibody was either an anti-mouse or anti-rabbit antibody linked to HRPO and binding to the Fc region of the primary antibody. After washing with TBS/0,1% BSA (3x 5 minutes), an amount of 150 µl of DAB chromogen substrate mixture was applied onto the tissue, and the colour reaction was stopped using water. The tissue was then counterstained with hematoxylin after which it was washed with running tap water for 10 minutes and dehydrated in ethanol with

progressively increasing concentrations (50%, 70%, 96%, 100% for 2,5 minutes each). This step was followed by exposure to Roticlear (3x10 minutes). As a final step, the tissue sections were mounted with Vecta Mount Mounting Medium to protect them and make them more resistance to outside factors. Two different researchers evaluated tissue samples. Samples which could not be assessed were evaluated by a pathologist of the Department of Pathology, Klinikum rechts der Isar, Munich.

3.2.4. Cells

3.2.4.1. Cell culture

0,25% Trypsin/EDTA
Cell Lines (AspC-1, MiaPaCa-2, Panc-1, T3M4)
DMEM
FBS
Penicillin
Streptomycin

Human pancreatic cancer cell lines AsPC-1, MiaPaCa-2, PANC-1 and T3M4 were cultivated in 10 cm^2 uncoated Petri dishes. The cells were purchased from the American Type Culture Collection (ATCC). The cells were routinely grown in complete medium in a humid chamber at 37°C containing 95% atmospheric air which was saturated with 5% CO_2. The culture medium was Dulbecco's modified Eagle medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µl/ml streptomycin. After reaching approximately 90% confluency, cells were subcultured by trypsinisation.

3.2.4.2. siRNA transfection

siRNA
HiPerFect Transfection Reagent
AsPC-1 Cells
DMEM + 10 % FBS + 1% Streptomycin & Penicillin
Serum Free DMEM

The transfection of human pancreatic cell lines with small interfering RNA (siRNA) was performed to achieve a transient silencing of mRNA and protein expression of the targeted protein. The experiment was carried out using AsPC-1 cells, a previously validated siRNA molecule as well as a negative control siRNA. The siRNA was purchased from Qiagen and prepared and stored according to the manufacturer's protocol. As a first step, the cells were seeded into 6-well plates with a density of 2 x 10^2 in regular culture medium. After incubating the cells for 24-hours, a transfection

solution containing five µl of siRNA, 15 µl of HiPerFect transfection reagent and 80 µl of serum-free medium adding up to 100 µl per well was prepared. The solution was thoroughly mixed and kept at room temperature for 10 minutes after which it was added dropwise to each well (2.3 ml of culture medium/well). The medium was exchanged entirely after 24 hours, and the protein was collected after 72 hours as described in *Protein Isolation*. Efficiency of transient silencing was checked using immunoblotting analysis. Cells which were used for further experiments were trypsinized and immediately seeded into new 6-well plates. All experimental groups were seeded in duplicates, and the experiment was repeated three times.

3.2.5. Immunoblotting analysis

3.2.5.1. Protein extraction

PBS
Lysis Buffer
BCA Protein Assay Kit

Cells were cultured in 6-well dishes as described in *Cell Culture*. After discarding the culture medium, the cells were washed twice using PBS. Subsequently, the cells were exposed to a cell lysis buffer after which they were scraped off and transferred into tubes. The cells were kept on ice for 20 minutes after which they were homogenised using an ultra sound wave for 10 seconds. Following this step, the tubes containing the cells were centrifuged at 14,000 rpm at 4°C for 10 minutes in a precooled centrifuge. The supernatant was immediately transferred into new tubes. To measure the protein concentration in the supernatant, a BCA Protein Assay Kit was used according to the manufacturer's instructions. As a final step the samples were aliquoted and stored at -80°C.

3.2.5.2. Western blotting

Polyacrylamide Gel
SDS Ultra-Pure
Protein Ladder
Running Buffer
Blotting Buffer
Blotting Module (Sponge-Filter Paper-Gel-Nitrocellulose Membrane-filter Paper-Sponge)
Tween 20
BSA
TBS
Primary/Secondary Antibody
ECL Detection Reagent
Hyperfilm

On the first day of western blotting an amount of 20 µl of protein extract (1 µg protein/ml) was loaded on a polyacrylamide gel with a gradient of 8%-20%. Before this step, the protein was denaturated at 70°C for 10 minutes and size-fractioned in 10% SDS-PAGE. A protein ladder was added to the first and last pocket of the polyacrylamide gel for size reference. The different protein samples were then separated vertically by gel electrophoresis using a tank filled with Running Buffer and the appropriate amount of voltage. The fact that the proteins have a negative charge and different sizes make this separation possible. This process was stopped once the protein ladder approached the lower edge of the polyacrylamide gel. Afterwards, the now separated protein samples were transferred onto a nitrocellulose membrane by electrophoresis using a blotting buffer filled tank and the appropriate voltage. This blotting process required the set-up of a blotting module (sponge-filter paper-gel-nitrocellulose membrane-filter paper-sponge). The nitrocellulose membrane containing the protein was then blocked to prevent unspecific binding by application of TBS containing 0,05% Tween 20 (TBST) and 5% powdered milk. After diluting the primary antibody in TBST/5%milk, the membrane was incubated over night at 4°C on a tube roller.

As the first step of the second day, the membrane was washed with TBST (3x5 minutes) and exposed to the secondary antibody diluted in TBST/5%milk for one hour at room temperature. The membrane was again washed with TBST (3x5 minutes) after which an enhanced chemiluminescence system (ECL) detection reagent was applied onto the membrane. The signal was captured using a hyper film which was then developed in a dark room. As a final step, the hyper film was scanned and digitalised for further evaluation.

3.2.6. Assays

3.2.6.1. Invasion assay

Biocoat Matrigel Invasion Chamber
AsPC-1 Cells (transfected with siRNA and negative control siRNA)
MDEM without FBS
MDEM + 10% FBS + 0.1% Streptomycin/Penicillin
Methanol
Crystal Violet (dissolved in 100% Ethanol)
Vecta Mount Mounting Medium

Cell Invasion was determined using the 8.0 µm pore size Biocoat Matrigel Invasion Chamber according to the manufacturer's instructions. This invasion assay allows for a quantification of the ability of cells to invade a given gel in vitro and compare the results between different experimental groups. Before the experiment, two groups were established: AsPC-1 cells which had been transfected with siRNA and AsPC-1 cells which had been transfected with negative control siRNA. Before using the assay, the matrigel was rehydrated by filling 500 µl of MDEM without serum into each well and chamber and incubating the plate at 37°C for 2 hours. After removing the medium, the wells were filled with 500 µl of fresh culture medium, and the rehydrated chambers were carefully placed inside the wells. Afterwards, an amount of 200 µl of MDEM without serum containing 2 x 10^4 cells was added into each chamber, and the entire plate was incubated for 24 hours. Following the incubation period, the chambers were emptied, and any remaining cells in the interior of the chambers were removed using a cotton stick. The cells located on the exterior bottom of the chambers were fixated with 100% methanol and subsequently stained with crystal violet. After staining the cells for 20 minutes, any remaining colour was washed off, and the chambers were left to dry at room temperature after which the bottom membrane of the chamber was removed. To make counting and therefore quantification possible, the membrane of the chamber was cut out, placed onto a slide and mounted with Vecta Mount Mounting Medium. The assay was performed in duplicates and repeated three times.

3.2.6.2. Proliferation assay

PaNC-1 cells (transfected with siRNA and control siRNA)
MDEM + 10% FBS + 0.1% Streptomycin/Penicillin
MTT Reagent
Isopropanol

Cell proliferation was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MTT assay determines cell viability and

proliferation through the measurement of tetrazolium salts within the cells and allows a comparison between different experimental groups. Before the experiment, two groups were established: AsPC-1 cells which had been transfected with siRNA and AsPC-1 cells which had been transfected with negative control siRNA. The cells were then seeded in triplicates into a 96-well plate containing 100 µl of culture medium and 5000 cells. After an incubation period of 24 hours, ten µl of MTT reagent (50 µg/well) was added to each well, and the cells were incubated for another 4 hours. The purple Formazan crystals, being a product of intracellular MTT metabolization inactive cells, were then solubilised using 100 µl of Isopropanol and absorbance was measured with a multimode reader at 560 nm wavelength. The measurements of the 24-hour timepoint were set as day 0 (d0). The MTT assay was repeated for 48h, 72h and 96h as described above, rendering d1, d2 and d3.

3.2.6.3. ELISA

Human Protein FAM3C ELISA Kit

The enzyme-linked immunosorbent assay serves to detect and quantify a substance of interest such as proteins in a given sample by binding of antibodies and the following colour reaction which can be detected by a photometer. In this case, the Human Protein FAM3C ELISA Kit by Cusabio was used according to the manufacturer's instructions. The serum samples used in the assay were collected preoperatively from PDAC patients, aliquoted and stored at -80°C. Before the experiment, all serum samples were diluted in a ratio of 1:2 using the provided sample diluent. In addition, a volume of 1 ml of sample diluent was added to the FAM3C antigen standard rendering a stock solution with a concentration of 4000 pg/ml which was then further diluted into a series of 8 standards with gradually decreasing concentrations (4000, 2000, 1000, 500, 250, 125, 62.5, 0 pg/ml). Before starting the assay, all components were prepared and brought to room temperature. As a first step, a volume of 100 µl of each standard and patient sample was added into a well of a provided 96-well plate. After incubating the plate for 2 hours at 37°C, the liquid was discarded, and 100 µl of 1x Biotin-antibody was added to each well. The plate was then incubated for 1 hour at 37°C after which the liquid was removed, and each well was washed three times with a volume of 200 µl of a provided washing buffer. The plate was inverted and blotted against a paper towel to make sure that all of the washing buffers were removed after which 100 µl of 1x HRP-avidin was added to each well, and the plate was incubated for 1 hour at 37°C.

Next, the washing process was repeated five times, and 90 µl of TMB Substrate was added to each well. After incubating the plate for 25 minutes at 37°C, the colour reaction was stopped by adding 50 µl of stop solution to each well. The plate was gently shaken to ensure proper mixing after which the optical density of each well was measured using a microplate reader at a wavelength of 450 nm. The assay was performed in duplicates.

Tissue type Plasminogen Activator Human ELISA Kit

The Tissue type Plasminogen Activator Human ELISA Kit by Abcam was used according to the manufacturer's instructions. The serum samples used in the assay were collected preoperatively from PDAC patients, aliquoted and stored at -80°C. Before the experiment, all serum samples were diluted in a ratio of 1:20 using the provided sample diluent. Furthermore, a stock solution with a concentration of 2000 pg/ml was prepared and gradually diluted into 8 tubes containing decreasing concentrations (1000, 500, 250, 125, 62.5, 31.3, 15.6, o pg/ml). Before starting the assay, all components were prepared and brought to room temperature. As the first step of the assay, each well of a provided 96-well plate was washed twice with 400 µl of a provided washing buffer. After excess washing buffer was removed, the standard solutions as well as the diluted serum samples were added into each well. After that 50 µl of HRP-Conjugated Antibody was added to each well and the plate was incubated at room temperature for 2 hours. After the incubation period, the wells were emptied and washed for a total of 6 times with washing buffer after which excess washing buffer was removed and 100 µl of TMB Substrate was added. The plate was incubated for 10 minutes and the color reaction was stopped by adding 100 µl of Stop Solution into each well. The plate was gently shaken to ensure proper mixing after which the optical density was measured using a microplate reader at a wavelength of 450 nm. The assay was performed in duplicates.

3.2.7. Statistics

3.2.7.1. Statistical analysis

To perform a statistical analysis of the collected data, the GraphPad Prism 6 software was used. Analysis of patient survival status was carried out using the Kaplan-Meier estimator and the Log-Rank (Mantel-Cox) test. To further evaluate a correlation between different data sets the Chi-Square test was utilised. Statistical significance was defined as $p < 0.05$.

4. Results

4.1. ALDH1A3 is an unfavorable prognostic factor in PDAC

To further confirm the correlation between ALDH1A3-positivity in the tissue and shorter postoperative survival that was described by Kong et al., the patient cohort was enlarged from 50 to 162 patients. A pathologist previously confirmed the diagnosis of PDAC. Patients that died within three months after surgery were excluded in order to balance out possible deaths due to the invasive surgery. The remaining inclusion criteria were defined rather broad as an essential characteristic of a clinically relevant biomarker is its applicability to a large patient cohort rather than a small pre-selected subgroup. The immunohistochemical staining of the tissue showed cytoplasmic localisation of the target protein. Positive staining for ALDH1A3 was seen in cancer as well as islet cells. The cut-off for the ALDH1A3-positive patient cohort was set to <10% stained cancer cells. The analysis showed that 36% of the samples were positive for ALDH1A3 whereas 64% of the samples were negative for ALDH1A3 (Figure 4).
Survival analysis showed that the ALDH1A3-positive patient cohort had a significantly shorter postoperative survival time (Figure 5). We were thus able to show that ALDH1A3 expression in resected cancer tissue and postoperative survival of the patients significantly correlated in a cohort of 162 patients.

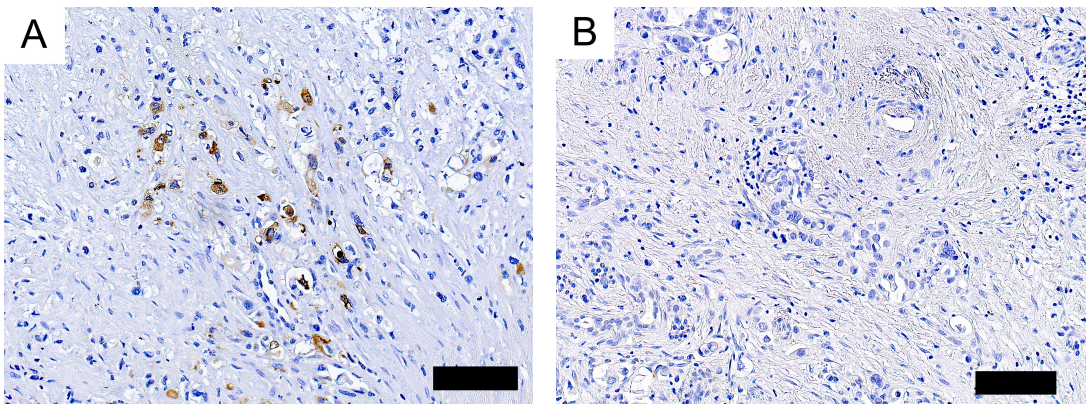

Figure 4: Representative IHC pictures shows staining of ALDH1A3 in human PDAC. (A) ALDH1A3-positive cancer cells. (B) ALDH1A3-negative cancer cells, scale bar: 100 µm.

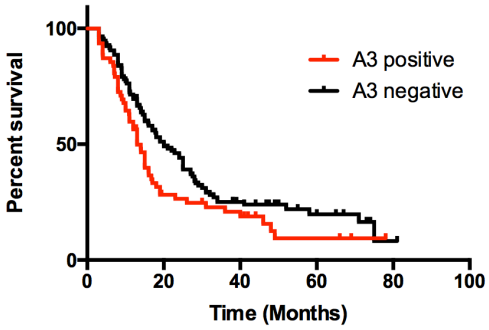

Figure 5: Kaplan-Meier survival analysis shows PDAC patients postoperative survival time; survival time of ALDH1A3-positive patients (median survival: 13 months; n=59) is significantly shorter than the survival time of ALDH1A3-negative patients (median survival: 21 months; n=103); p = 0,012

4.2. The bioinformatical analysis shows a positive correlation between ALDH1A3 and eight other genes in three different datasets

To identify genes that have a correlating expression with ALDH1A3, a bioinformatical analysis was performed using publicly available datasets. The gene correlations were further validated using a cohort of patients that underwent resection surgery because of pancreatic cancer at the Department of Surgery of the Technical University Munich. Immunohistochemistry previously determined the ALDH1A3 expression of these samples.

Analysis revealed that the ALDH1A3-positive subtype that was identified in PDAC tissue also exists in human PDAC cell lines as well as patient derived xenograft (PDX) models. Representative markers were searched on a genome-wide scale and were validated by applying strict statistical tools including the 2-means cluster algorithm for vector quantisation. We applied the 2-means clustering algorithm on three microarray datasets generated from the GEO database to define ALDH1A3-positive and ALDH1A3-negative samples (Figure 6).

Thus, we identified twelve genes that showed a correlated expression in three independent microarray datasets for micro-dissected cancer tissues, cancer cell lines and PDX's (Table 3). Eight of the twelve genes showed significant positive correlation with ALDH1A3 in all three datasets: ALDH1A3, EMP1 (epithelial membrane protein 1), FAM3C (family with sequence similarity 3, member C), IRS2 (insulin receptor substrate 2), MAML2 (mastermind-like transcriptional coactivator 2), MCC (mutated in colorectal cancers), PMEPA1 (prostate transmembrane protein, androgen induced 1) and SP100 (SP100 nuclear antigen) (Table 4). As FAM3C is a secreted protein and is consequently detectable in the serum, it was chosen for further analysis. This work

was contributed by Prof. Maggie Haitian Wang (Centre for Clinical Research and Biostatistics (CCRB), Chinese University of Hong Kong)

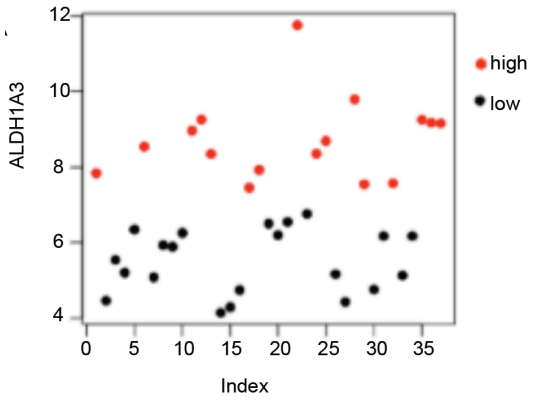

Figure 6: 2-Mean Clustering using the target gene ALDH1A3 shows clear separation. The algorithm serves to divide a given number of data points in a coordinate system into two clusters. As a first step, two centroids are randomly placed at different locations of the coordinate system. After that, every data point is assigned to the centroid that it is closest to. The centroid is reassigned to a new location at which it is closest to all data points that were previously assigned to it. As a next step, all data points are once again assigned to the centroid to which they are closest to. This algorithm of reassigning data points and moving the centroids is repeated until the two centroid points reach a final location which does not change anymore, rendering a given number of data points separated into two clusters. In our analysis, the 2-mean clustering yielded two separate groups: high ALDH1A3 and low ALDH1A3.

Sample type	PDXs	Cell lines	Micro-dissected tissue
Data	GSE51798	GSE17891 GSE21654	GSE17891
Sample size	35	22 + 20 = 42	27
Platform	Affymetrix U133 Plus 2.0	Affymetrix U133 Plus 2.0	Affymetrix U133 Plus 2.0
ALDH1A3 (p-value)	1.49E-10	5.26E-13	1.03E-7
Alpha	9.14E-7	9.14E-7	9.14E-7
# of gene p< 0.01	1144	2348	692
Overlap	12		

Table 3: List of genes that show a correlated expression in PDAC cell lines, micro-dissected tissue and PDXs.

		PDX		Cancer cell lines		Microdissected tissues	
Number	Gene	P value	Corr	P value	Corr	P value	Corr
1	ALDH1A3	1.49E-10	1	5.26E-13	1	5.26E-13	1
2	EMP1	1.37E-03	0.53	6.40E-03	0.50	1.10E-03	0.75
3	FAM3C	2.72E-03	0.36	3.93E-04	0.59	5.56E-03	0.81
4	IRS2	3.38E-04	0.53	8.85E-05	0.45	4.74E-03	0.33
5	MAML2	2.68E-03	0.51	2.40E-05	0.67	1.48E-03	0.78
6	MCC	2.55E-03	0.55	5.65E-03	0.42	8.41E-03	0.47
7	PMEPA1	7.57E-04	0.65	5.97E-05	0.72	1.09E-03	0.71
8	SP100	1.24E-03	0.38	4.46E-04	0.64	4.30E-03	0.53

Table 4: List of genes correlating with ALDH1A3 expression.

4.3. ALDH1A3 and FAM3C show a correlated expression in human PDAC cell lines

To test a correlated expression in human PDAC cell lines, ALDH1A3 and FAM3C expression were measured by western blot in four pancreatic cancer cell lines: AsPC-1, T3M4, Panc1 and MiaPaCa-2. Results showed that both proteins had a correlated expression in these cell lines (Figure 7). Interestingly, both proteins showed the highest expression in the AsPC-1 cell line which was then chosen for further analyses. The AsPC-1 cell line was first established in 1982 with cells taken from the ascites puncture of a 62-year old Caucasian woman with a metastasised pancreatic ductal adenocarcinoma of the pancreatic head [103]. The tumor cells were moderately differentiated and produced mucin as well as pancreas cancer-associated antigen (DU-PAN-2) and carcinoembryonic antigen (CEA)[103].

To further investigate the in vitro relation of ALDH1A3 and FAM3C in human PDAC cell lines, siRNA transfection for FAM3C was performed on AsPC-1 cells. To achieve the maximal reduction of FAM3C expression in the AsPC-1 cells, different siRNA's, as well as different siRNA concentrations, were tested as a first step of this experiment. The siRNA, which showed the highest reduction of FAM3C expression in western blot analysis was chosen for the experiment (Figure 8A). Different concentrations of siRNA were tested to determine the concentration at which cell growth was impaired the least while achieving the most effective reduction of FAM3C expression at the same time. The western blot analysis showed that the transfection for FAM3C and consecutive reduction of gene expression led to a significantly reduced expression of ALDH1A3 (Figure 8B).

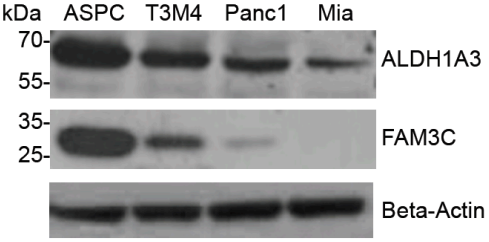

Figure 7: Western blot analysis demonstrates a correlated expression of ALDH1A3 and FAM3C in AsPC-1, T3M4, Panc1 and MiaPaCa-2 (Mia) cell lines.

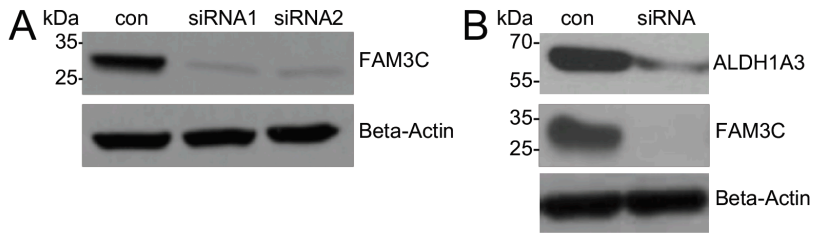

Figure 8: (A) Western blot shows the expression of FAM3C in AsPC-1 cells after siRNA transfection for FAM3C. siRNA1 was used for further experiments (B) Western blot analysis shows the expression of ALDH1A3 and FAM3C in AsPC-1 cells after siRNA transfection for FAM3C.

4.4. ALDH1A3 is expressed in pre-neoplastic lesions in mouse models of pancreatic cancer

To further investigate the importance of ALDH1A3 in early stages of the disease, pre-neoplastic lesions in mouse models were immunohistochemically stained for ALDH1A3. Chronic pancreatitis was induced using three different models: Caerulein injection, pancreatic duct ligation (PDL) or a combination of injection and PDL. Microscopically, the pancreatic tissue showed signs of inflammatory infiltration and proliferation of fibrotic tissue. Also, the number of acinar cells was reduced, and the glandular structure that is typically seen in healthy pancreatic tissue disappeared. Healthy pancreatic tissue was replaced by ductal structures that were dilated and often had metaplasia of the epithelial cells.

Furthermore, lower grade PanIN's were present, as characterised by epithelial lesions and mucin-producing columnar cells[16]. Staining of this remodelled tissue revealed that ALDH1A3 was present in all three models of early carcinogenesis and was located in PanIN lesions as well as areas of acinar-to-ductal metaplasia (Figure 9), indicating that ALDH1A3 is indeed involved in early pancreatic carcinogenesis. Other structures except islet cells were ALDH1A3 negative.

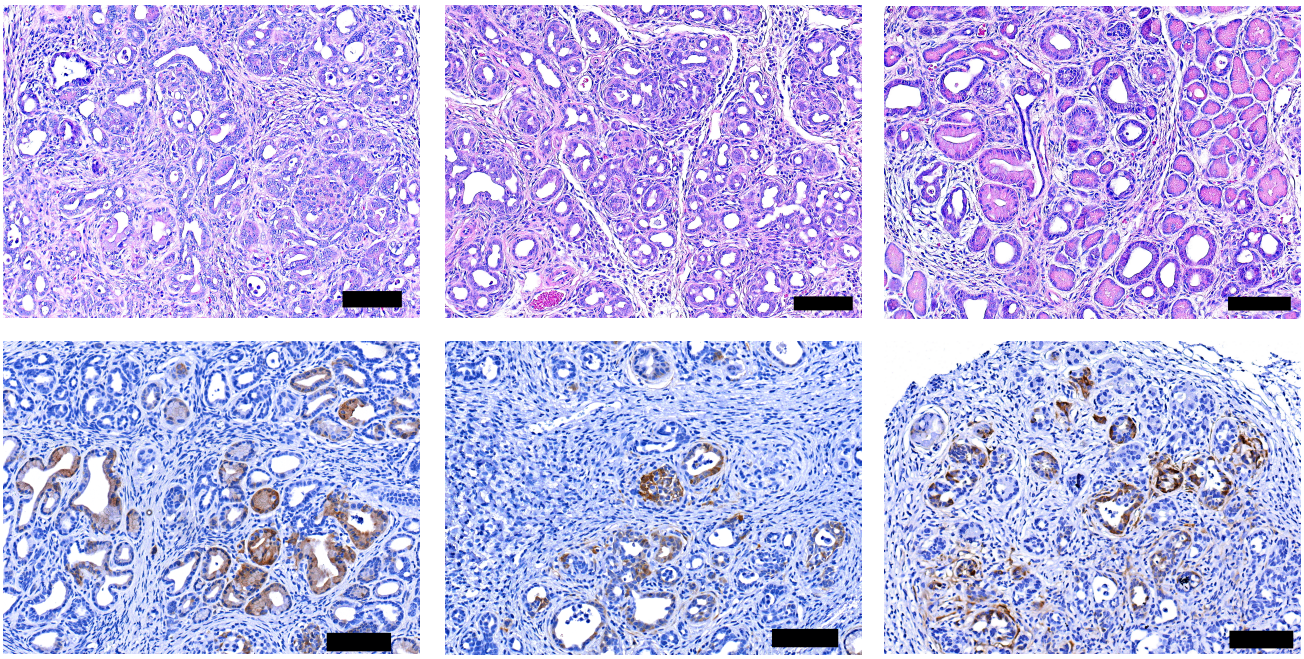

Figure 9: Caerulein injections and surgical ligation of the pancreatic duct in the splenic lobe trigger an inflammatory response. In KrasG12D mice, the inflammation results in processes of early carcinogenesis. Mice were subjected to either caerulein injection (left), surgical duct ligation (middle) or a combination of the above (right). All mice were sacrificed after two weeks, and the pancreata were harvested. Representative IHC of ALDH1A3 shows staining in the inflamed sections of the pancreas, scale bar: 100 µm (bottom). Representative H&E stained sections of the inflamed pancreas showing signs of early carcinogenesis, scale bar: 100 µm (top).

4.5. FAM3C doesn't affect cell proliferation, invasion or EMT in AsPC-1 cells

To examine the role of FAM3C in the cell proliferation and invasion, an MTT assay and an invasion assay were performed. The assays were carried out using AsPC-1 cells that had previously been transfected with FAM3C siRNA as well as cells which had been transfected with negative control siRNA. The measurement of cell proliferation at multiple time points showed no difference between the two groups (Figure 10A). The invasion assay revealed that the cells' ability to invade the matrigel through enzyme secretion and chemotaxis was not impaired by a reduced expression of FAM3C (Figure 10B).

Furthermore, the expression of the EMT marker E-cadherin was investigated in cells transfected with the target siRNA and the negative control siRNA. Although FAM3C has previously been shown to play a role in EMT, reduced expression of FAM3C had no impact on epithelial marker expression (Figure 10C).

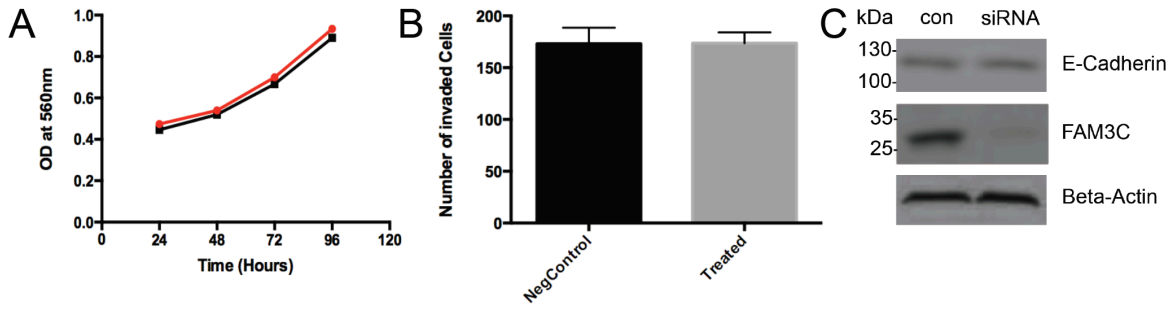

Figure 10: (A) MTT assay shows no difference between the control group (red) and the experimental group (black). Optical density was measured at 560 nm at four different time points (24h, 48h, 72h, 96h) (B) Invasion assay shows no significant difference in cell count between the experimental and the control group after a 24h incubation period. (C) Western blot analysis shows no difference in E-cadherin expression.

4.6. ALDH1A3 and FAM3C expression don't correlate in human PDAC tissue

To examine a potentially correlating expression of ALDH1A3 and FAM3C in tissue samples of human PDAC, tissue sections (n=106) were immunohistochemically stained for FAM3C. These patients were part of the cohort that had previously been stained for ALDH1A3 and had the pathologically confirmed diagnosis of PDAC. The stained structures had an intracellular localisation and appeared to have a granular staining pattern (Figure 11A). Cancer cells and tissue sections that underwent malignant transformation were strongly stained in 41% of patient samples. FAM3C-

positivity was not restricted to cancer cells as the surrounding tissue that appeared to be healthy showed some staining as well, although to a smaller extent. A recent study about FAM3C expression showed that the staining pattern of FAM3C in breast cancer cells correlated with survival[94]. The authors showed that a granular staining pattern, compared to more diffuse staining of the cytosol, significantly correlated with a poor prognosis[94]. After examination of the stained pancreatic tissue together with a pathologist from the Pathology Department of the TU Munich, the different staining patterns could not be seen in the pancreatic cancer cells.

Chi-square analysis showed that ALDH1A3 and FAM3C had no correlating expression in the tissue samples of human PDAC (Table 5). As a next step, FAM3C-positivity in resected PDAC tissue was examined as an independent prognostic factor for postoperative survival. Kaplan-Meier analysis showed that FAM3C-positivity in the tissue sample had no significant correlation with postoperative survival in PDAC patients (Figure 12). However, the tendency that FAM3C positive patients have a shorter median survival (14,5 months vs 20 months) when compared to FAM3C negative patients could be observed.

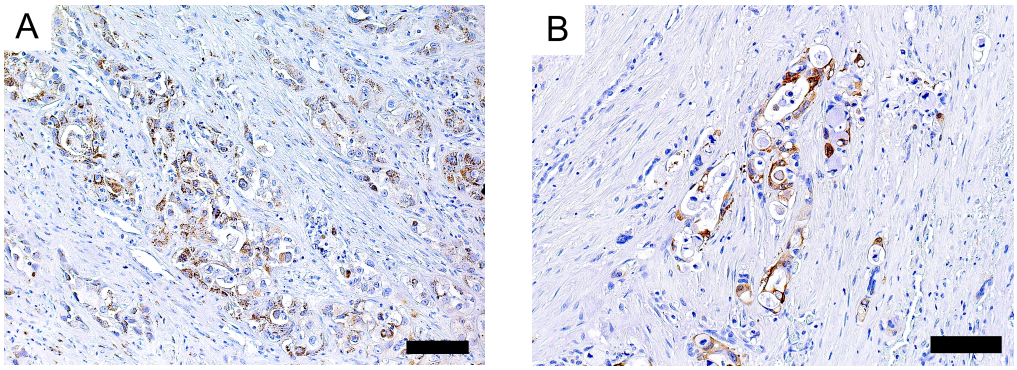

Figure 11: (A) Representative IHC staining shows staining of FAM3C in human PDAC.
(B) Representative IHC staining shows staining of ALDH1A3 in human PDAC, scale bar: 100 µm.

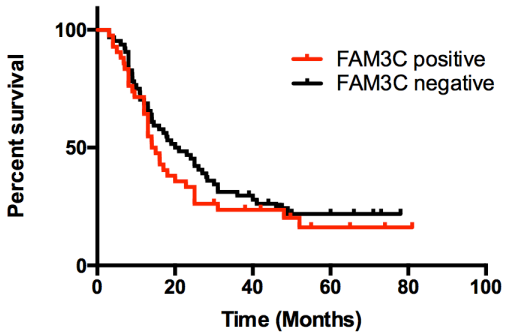

Figure 12: Kaplan-Meier survival analysis shows PDAC patients' postoperative survival time; survival time of FAM3C-positive patients (median survival: 14,5 months; n=43) is not significantly different from the survival time of ALDH1A3-negative patients (median survival: 20 months; n=63); p = 0,28

	ALDH1A3 positive	ALDH1A3 negative	Total
FAM3C positive	19	43	43
FAM3C negative	20	43	63
Total	39	67	106
p-value		0,19	

Table 5: Chi-Square analysis of the correlation between ALDH1A3- and FAM3C-expression in human PDAC tissue shows no correlation.

4.7. Preoperative FAM3C serum levels show no correlation with ALDH1A3 expression in human PDAC tissue samples

As the analysis of ALDH1A3 and FAM3C in tissue samples showed no correlation, we proceeded to further examine a possible correlation between ALDH1A3-positivity in the tissue and levels of FAM3C in preoperative serum samples. As such, serum samples (n=62) were analysed using an Enzyme-linked Immunosorbent Assay (ELISA).

Chi-square analysis showed no correlation between FAM3C concentration in serum samples and ALDH1A3-expression in the tissue (Table 6). Furthermore, Kaplan-Meier analysis showed that FAM3C levels in serum did not have a significant effect on postoperative survival of PDAC patients (Figure 13). Nevertheless, a tendency of 11 months of postoperative survival in patients with high FAM3C levels compared to 19 months in patients with low FAM3C levels was seen.

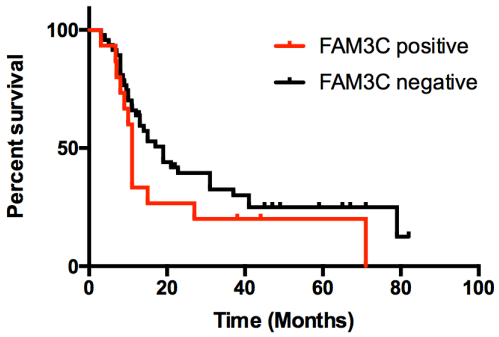

Figure 13: ELISA measured serum FAM3C levels Kaplan-Meier survival analysis shows PDAC patients' postoperative survival time; survival time of FAM3C-positive patients (median survival: 11 months; n=15) and FAM3C-negative patients (median survival: 19 months; n=47) is not significantly different; p = 0,156

	ALDH1A3 positive	ALDH1A3 negative	Total
FAM3C positive	3	17	20
FAM3C negative	11	19	30
Total	14	36	50
p-value		0,095	

Table 6: Chi-square analysis of the correlation between ALDH1A3-expression in the tissue and FAM3C-expression in the serum show no correlation. The number of patients (n=50) is lower than the original number (n=62) of analyzed serum samples as some serum samples had no matching tissue sample available.

4.8. Expression of PLAT in the serum doesn't correlate with ALDH1A3 expression in human PDAC tissue

The tissue-type Plasminogen Activator (PLAT/t-PA) is an enzyme that has a very similar function to the urokinase Plasminogen Activator (uPA) which plays an important role in the activation of FAM3C[94]. As PLAT is a protease that activates Plasmin and thus interacts with the activating mechanism of FAM3C, its expression could correlate with the expression of FAM3C and potentially ALDH1A3. Furthermore, PLAT is a protein that is secreted into the bloodstream and hence constitutes a potential serum biomarker[104]. ELISA measured PLAT concentration in the sera of 22 patients. All patients were diagnosed with PDAC and sera were taken before surgical resection of the pancreas. Results showed that serum levels of PLAT did not correlate with ALDH1A3 expression in the resected tumor tissue (Table 7).

As a next step, the role of PLAT as an independent prognostic biomarker for the postoperative survival of PDAC patients was further investigated.

Although there was an apparent tendency of patients with lower PLAT serum levels surviving longer after surgery, the results were ultimately not statistically significant (Figure 14).

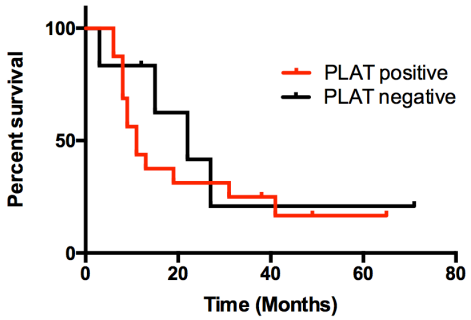

Figure 14: ELISA measured serum PLAT levels. Kaplan-Meier survival analysis shows PDAC patients' postoperative survival time: survival time of PLAT-positive patients (median survival: 11 months; n=16) and PLAT-negative patients (median survival: 22 months; n=6) is not significantly different; p = 0,413.

	ALDH1A3 positive	ALDH1A3 negative	Total
PLAT positive	2	14	16
PLAT negative	3	3	6
Total	5	17	22
p-value		0,19	

Table 7: Chi-square analysis of the correlation between ALDH1A3-expression in the tissue and PLAT-expression in the serum shows no correlation.

4.9. The potential correlation between SP100, MCC and IRS2 and ALDH1A3 in human PDAC tissue

Some of the proteins that significantly correlated with ALDH1A3 in the bioinformatical analysis were analysed regarding their ability to further subclassify the ALDH1A3-positive subtype. Although these genes do not encode for secreted proteins and thus do not have a potential role as a serum biomarker, characterisation of different PDAC's could lead to a better understanding of the disease and yield potential therapeutic targets. The patient cohort that was chosen for this evaluation consisted of 80 patients of which 40 were ALDH1A3-positive, and 40 were ALDH1A3-negative. The three genes that were chosen for immunohistochemical staining of the patients' tissue were IRS2, MCC and SP100. Insulin receptor substrate 2 (IRS2) is a protein that plays an essential role in the intracellular signalling pathway of insulin and has previously been associated with cancer[105,106]. The MCC gene encodes for the colorectal mutant cancer protein which has been shown to be altered in patients with colorectal cancer[107]. The SP100 gene encodes for a protein which is part of a complex inside the nucleus which plays a role in the regulation of the cell cycle and thus has a tumor suppressing function in a cell[108].

Unfortunately, all three genes were expressed in more than 90% of the stained cancer tissue and did not correlate with ALDH1A3 expression. Immunohistochemical staining for IRS2 and MCC showed a cytoplasmic localisation of the target protein (Figure 15). Staining for SP100, however, revealed a nuclear as well as a cytoplasmic subcellular localisation of the target structure (Figure 15).

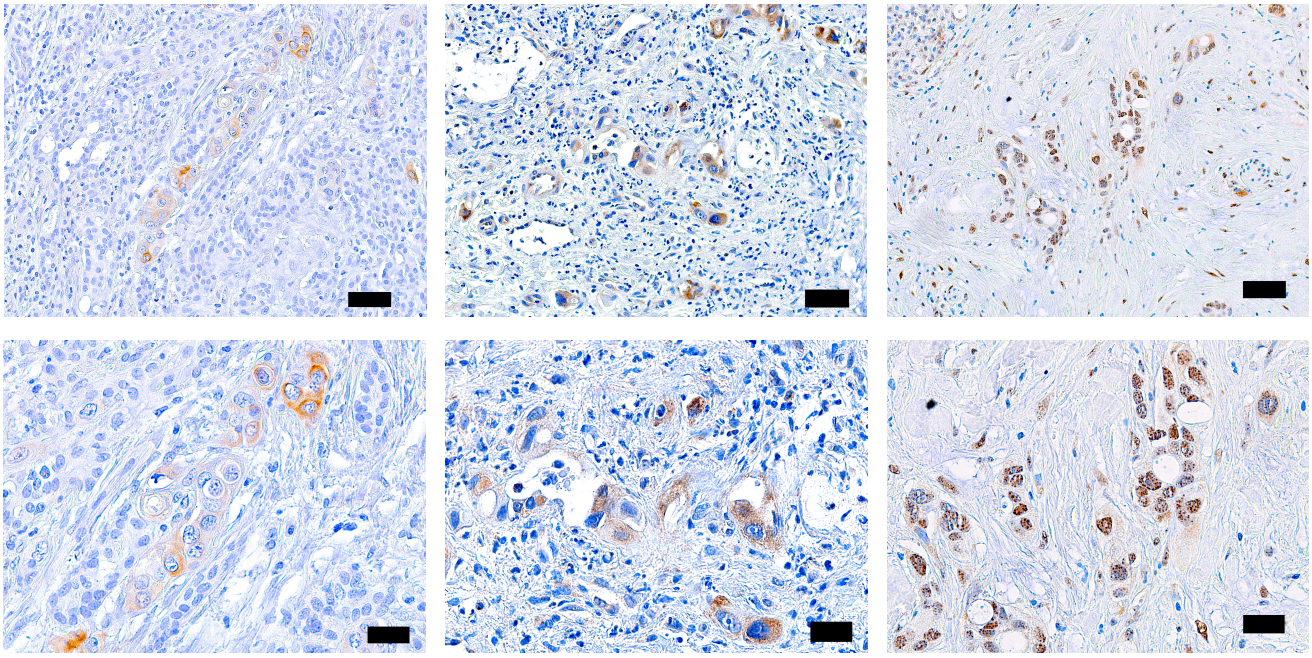

Figure 15: Representative IHC staining shows staining of IRS2 (left), MCC (middle) and SP100 (right) in human PDAC. Scale bar: 50 µm (above); Scale bar: 20 µm (below).

4.10. ALDH1A3 doesn't correlate with KRT81, a novel marker for an aggressive PDAC subtype

A study which was published in 2016 identified Keratin 81 (KRT81) as a tissue marker for an aggressive subtype of PDAC[109]. KRT81 is detectable in human pancreatic cancer tissue by IHC and labels the QM-PDA (quasi-mesenchymal) subtype of PDAC that has previously been described to have an especially poor prognosis[110]. In order to investigate a potentially correlating expression of ALDH1A3 and KRT81 in tissue samples of human PDAC, a group of 106 patients was immunohistochemically stained for KRT81. All tissue samples were part of the larger patient cohort of 162 patients that had previously been stained for ALDH1A3. The staining pattern showed a cytoplasmic location of the target structure (Figure 16A&B). In some cancer cells the staining pattern appeared to be granular with small dot-like structures in the cytoplasm. KRT81 positivity in the pancreatic tissue was generally restricted to islet cells and cancer tissue although in some samples KRT81 staining could be seen in areas of acinar to ductal metaplasia.

Chi-square analysis did not show any correlation in the expression of ALDH1A3 and KRT81 had no correlated expression in the tissue of human PDAC patients (Table 8). Although there was a tendency for KRT81-positive patients to have a shorter median

survival compared to KRT81-negative patients (14,25 vs 20 months), the Kaplan-Meier analysis ultimately showed no significant difference in survival between our patient cohorts (Figure 17).

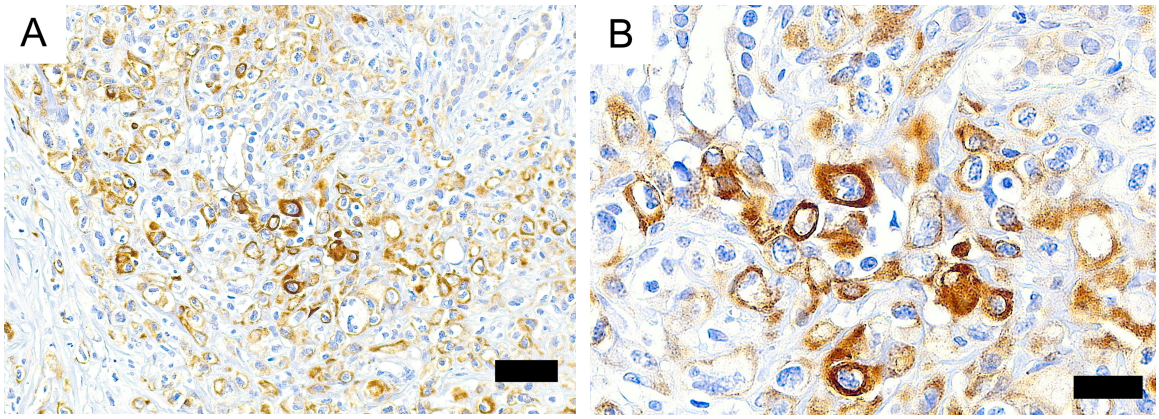

Figure 16: (A) Representative IHC staining shows staining of KRT81 in human PDAC, scale bar: 50 µm (B) Representative IHC staining shows staining of KRT81 in human PDAC, scale bar: 20 µm

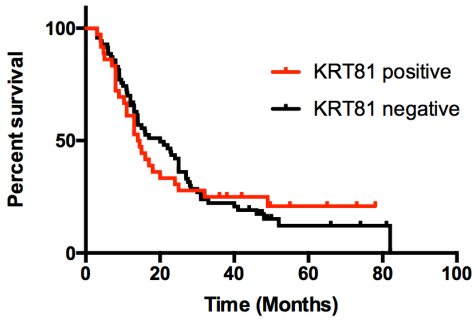

Figure 17: Kaplan-Meier survival analysis shows PDAC patients' postoperative survival time; survival time of KRT81-positive patients (median survival: 14,25 months; n=36) is not significantly different from the survival time of KRT81-negative patients (median survival: 20 months; n=70); p = 0,51

	ALDH1A3 positive	ALDH1A3 negative	Total
KRT81 positive	12	28	40
KRT81 negative	16	50	66
Total	28	78	106
p-value		0,52	

Table 8: Chi-Square analysis of the correlation between ALDH1A3- and KRT81-expression in human PDAC tissue shows no correlation.

5. Discussion

5.1. Introduction

The present study aimed to identify a serum biomarker that correlates with ALDH1A3 expression in the resected PDAC tissue and to get a better understanding of the ALDH1A3-positive PDAC subtype. We were able to show that ALDH1A3 expression in pancreatic cancer tissue correlates with postoperative survival in a patient cohort that was enlarged from the original cohort in the publication by Kong et al. As a first step, we identified eight genes that had a correlated expression with ALDH1A3 in three different datasets. A selection of these genes was then checked regarding correlation with the ALDH1A3 expression in the cancer tissue with a particular focus on FAM3C. A possible correlated expression was checked in preoperatively collected serum samples as well as resected pancreatic cancer tissue using different methods including immunohistochemistry, immunoblotting, ELISA and cell function experiments with siRNA-mediated reduction of gene expression. We could confirm that ALDH1A3 and FAM3C expression correlate in pancreatic cancer cell lines and that a reduced FAM3C expression leads to a reduction of ALDH1A3 expression. Nonetheless, we ultimately could not identify a protein that correlated with ALDH1A3 expression levels, neither in patients' tissue nor sera. These results have to be thoroughly analysed, discussed and put into the context of current research in order to draw conclusions regarding a different approach and the focus of future research.

5.2. Enlargement of the patient cohort

The enlargement of the patient cohort from an original 50 patients in the 2016 publication from Kong et al. to 162 patients was able to underline the prognostic significance of ALDH1A3 expression in PDAC tissue. The percentages of ALDH1A3-positive and negative patients in the original publication (41% positive vs 59% negative) were similar to the ones in the enlarged cohort (36% positive vs 64% negative). This similarity could also be seen in the postoperative survival time of PDAC patients in the original publication (14 months vs 22,8 months) and this study (13 months vs 21 months). Nevertheless, when looking at the survival curves, one can see that the separation of the curves in the original publication from Kong et al. is much clearer. This difference is not explicable, as both patient cohorts had similar inclusion and exclusion criteria. Also, the patients of both studies were operated in the same

hospital, and their tissue, as well as their serum, was taken and handled under very similar conditions in the same laboratory. However, both studies ultimately show that ALDH1A3-positivity in resected PDAC tissue significantly (p = 0,012) correlated with poor postoperative prognosis. These findings are in line with studies of ALDH1A3 as a marker for poor prognosis in other tumor entities such as breast and gallbladder cancer as mentioned in the introduction[89,90].

5.3. The relationship between ALDH1A3 and FAM3C

The Western blot analysis of four different pancreatic cancer cell lines showed that ALDH1A3 and FAM3C expression appears to correlate on the protein level. Both proteins have previously been associated with poor prognosis and metastasis in different tumor entities[89,90,93,94,95]. The role of FAM3C as an inducer of EMT has accurately been described and characterized[93].

The ALDH1A3-positive PDAC subtype described by Kong et al. as well as the activation of FAM3C in cancer cells depend on the same intracellular molecular signaling mechanisms, evolving around oncogenic Ras-mediated Mek/Erk hyperactivation and defining their carcinogenic potential[73,93]. Also, FAM3C and ALDH1A3 have both been connected to changed intracellular glucose metabolism. Kong et al. have identified the glucose metabolism to be altered in the ALDH1A3-positive PDAC subtype although this alteration has not been specifically associated with ALDH1A3 itself. A study about diabetes in mice has shown that FAM3C represses gluconeogenesis in the liver through Akt phosphorylation and nuclear exclusion of transcription factor FOXO1 and hence dysregulates glucose metabolism[111].

Western blot analysis showed that FAM3C and ALDH1A3 expression correlate in all pancreatic cancer cell lines investigated while the highest levels were seen in AsPC-1 cells. The FAM3C and ALDH1A3 levels of normal non-neoplastic cells should have been measured as well to get a reference and to be able to make a comparison. This was done by a study that showed that Panc-1 cells have a 4,2-fold upregulated expression of FAM3C compared to non-neoplastic pancreatic duct cells[96].

The role of FAM3C has been extensively studied in breast cancer[93,94]. Subcellular localisation and upregulated FAM3C secretion have been associated with poor prognosis and metastasis[93,94]. A study showed that FAM3C secretion and expression is upregulated in metastatic breast cancer cell lines MDA-MB-468 and MDA-MB-231, whereas its expression and secretion is downregulated in nonmetastatic breast cancer

cell lines T47D and MCF7[94] (Table 9). Increased ALDH and more specifically ALDH1A3 expression have been associated with metastasis in breast cancer as well[89]. ALDH activity in different breast cancer cell lines was measured by the aldefluor assay and showed that the T47D, MCF7 and MDA-MB-231 cell lines had microscopic aldefluor-positive cells (0%-1%).

In contrast, the MDA-MB-468 cell line had the highest aldefluor activity[89] (Table 9). Quantitative polymerase chain reaction (qPCR) showed that high mRNA levels of ALDH1A3 highly correlated with high aldefluor activity in breast cancer cells and identified the ALDH1A3 isotype to be responsible for aldefluor activity[89]. These results show that FAM3C and ALDH1A3 expression correlate in three out of four of the breast cancer cell lines that were investigated except the MDA-MB-231 cell line.

Although no studies have made a connection between ALDH1A3 and FAM3C, they both play a role in similar metabolic pathways and are correlatingly expressed in different breast cancer cell lines. Furthermore, this study shows that ALDH1A3 and FAM3C expression correlate in all pancreatic cancer cell lines investigated. Additionally, a more direct connection between the two proteins was shown as a siRNA mediated transient reduction of FAM3C expression in AsPC-1 cells led to a decreased ALDH1A3 expression. In the future, it would be interesting to know more about the relationship between FAM3C and ALDH1A3 and whether similar intracellular mechanisms control the expression of the two proteins. As mentioned above, the expression of both proteins depends on the Mek/Erk pathway. Interestingly, both proteins seem to be connected to the STAT3 pathway as activation of STAT3 promotes ALDH1A3 expression in non-small cell lung cancer (NSCLC) and FAM3C activates STAT3 through upregulation of the PDGF-receptor in hepatocellular carcinoma (HCC)[112, 113]. This connection should be further investigated in the context of pancreatic cancer.

Another approach to get a better understanding of whether or not the two proteins interact could be a yeast two-hybrid assay. This assay has first been developed in 1989 and examines the in vivo protein-protein interaction of two given proteins[114]. This is made possible by the synthesis of two recombinant proteins which consist of 1.) one of the genes in question and 2.) part of a transcription factor. If the two proteins interact with each other, the transcription factor is completed, and the transcription of a reporter protein is activated which can then be detected.

Regarding further investigation of the relation between ALDH1A3 and FAM3C within the cell, an approach using immunofluorescence microscopy could lead to a better understanding. It would be interesting to see whether ALDH1A3 and FAM3C are expressed in the same areas within the cancer tissue and malignant cells. As this study shows, ALDH1A3 is expressed in stages of early carcinogenesis in mouse tissue and thus allows the hypothesis that it plays a role in the malignant transformation of the tissue. Additional staining for FAM3C of these tissue samples could lead to a better understanding of whether or not FAM3C plays a role in early carcinogenesis or if it is a protein which plays a role in later stages of malignant transformation.

Breast cancer cell lines	FAM3C expression	Aldefluor activity
T47D	-	-
MCF7	-	-
MDA-MB-468	+	+
MDA-MB-231	+	-

Table 9: Table shows the FAM3C expression and aldefluor activity of breast cancer cell lines T47D, MCF7, MDA-MB-468, MDA-MB-231

5.4. Tissue

5.4.1. Aldehyde dehydrogenase family 1 member A3

In this study, we show that ALDH1A3 is expressed in different mouse models of early stages of pancreatic carcinogenesis such as PanIN's and areas of acinar-to-ductal metaplasia. Interestingly, the surrounding normal pancreatic tissue was not ALDH1A3-positive. The fact that positive staining for ALDH1A3 is only seen in morphologically abnormal and ultimately neoplastic cells of the exocrine pancreas is something we were able to confirm in tissue samples of late stages of pancreatic cancer in patient samples as well. Thus, increased expression of ALDH1A3 in pancreatic exocrine tissue seems to be characteristic of cells that undergo malignant transformation and can be detected in the early stages of the transformation process. This further underlines the value of ALDH1A3 as a prognostic marker in PDAC as ALDH1A3 expression seems to correlate with morphological changes of pancreatic cells. Even though we were not able to find a protein that has a correlating expression with ALDH1A3 in either tissue or serum samples, this approach might still be interesting for future research.

5.4.2. Family with sequence similarity 3 member C

5.4.2.1. Positive/Negative

The staining of pancreatic cancer tissue for FAM3C and subsequent Kaplan-Meier analysis showed no correlation between FAM3C-positivity and poor prognosis in the patient cohort of this study. Even though a clear difference in the extent of FAM3C expression in the cancer tissue could be seen, this difference did not have an effect on survival in this patient cohort. As FAM3C is a secreted protein which carries out its functions in cell signalling in the extracellular space, immunohistochemical staining of pancreatic cancer cells might not be the appropriate way of assessing FAM3C expression. Nonetheless, some studies of other cancer types have shown FAM3C-positivity in resected cancer tissue to significantly correlate with poor prognosis.

A study about FAM3C expression in colorectal cancer showed that FAM3C is overexpressed in 67.5% of tissue samples and that patients with a high FAM3C expression in the resected tissue have a significantly lower 5-year survival rate[115]. Interestingly, this was shown in human hepatocellular carcinoma as well, in which strong cytoplasmic staining compared to weak cytoplasmic staining significantly correlated with poor or good prognosis, respectively[113]. A different approach was chosen in a study about FAM3C expression in oesophagal squamous cell carcinoma (ESCC) that determined mRNA of resected ESCC samples by qRT-PCR and showed that high expression of FAM3C significantly correlated with poor prognosis[116]. Measurement of mRNA levels by qRT-PCR in the resected tissue of the patient cohort of our study could make a more quantifiable assessment of FAM3C expression possible. Also, the studies about colorectal cancer and ESCC compared FAM3C expression in the cancer tissue to normal healthy tissue[115,116]. The authors were able to show that FAM3C expression was increased in the cancer tissue compared to the adjacent healthy tissue. These findings could be confirmed in the context of pancreatic cancer in our study.

5.4.2.2. Cytoplasmic/Granular

Multiple studies have shown that altered subcellular localisation of FAM3C in resected cancer tissue correlates with survival[93,94,95,115]. An early study that investigated FAM3C expression in healthy human tissue showed that FAM3C expression could not be seen in various epithelial tissues such as bladder and stomach while FAM3C is expressed in secretory tissues such as colon, mammary gland and pancreas[93]. The study

described the staining pattern as granular[93]. Interestingly, the same study examined FAM3C expression and staining patterns in cancer tissue as well and showed that many cancer tissues including colon, mammary gland, liver and prostate showed an enhanced FAM3C expression and a cytoplasmic staining pattern[93]. Unfortunately, the authors of this study did not mention if pancreatic cancer tissue was stained and if the same pattern could be seen.

Furthermore, the study showed that altered subcellular localization and a cytoplasmic staining pattern of FAM3C in breast cancer cells significantly correlates with patients' survival[93]. This finding was further investigated and confirmed by another study about breast cancer in 2014[94]. The colorectal cancer study compared the FAM3C staining pattern of healthy tissue to cancer tissue and showed that in contrast to the non-neoplastic cells which have a granular staining pattern, the cancer cells exhibit a cytoplasmic staining pattern[115]. Unfortunately, the difference in staining pattern was not further studied within the cancer patient cohort itself. Although strong cytoplasmic staining correlated with poor prognosis compared to weak cytoplasmic staining in HCC patients, granular staining could not be shown to correlate with a good prognosis[95].

In this study, the resected pancreatic cancer tissue of 106 patients was stained for FAM3C. Similar to the studies mentioned above, FAM3C shows a granular staining pattern which, to a lower extent can be seen in the regular surrounding tissue and particularly in areas of malignant transformation. Cytoplasmic staining of cancer cells was also seen but was almost always present alongside granular staining thus making a clear distinction between cytoplasmic and granular subtype impossible in the context of pancreatic cancer. These findings might be in line with the study mentioned above which showed that granular staining of FAM3C is present in healthy pancreatic tissue but does not mention enhanced cytoplasmic staining in pancreatic cancer[93]. The stained tissue was also shown to a pathologist of the TU Munich who confirmed that a distinction between cytoplasmic and granular staining is not possible.

The authors of the study about FAM3C expression in breast cancer stained the tissue for the urokinase plasminogen activator receptor (uPAR), which was shown to play a role in the proteolytic processing of FAM3C[94]. The combined evaluation of FAM3C and uPAR expression in the tissue could be shown to correlate even more significantly with the patient cohort's survival[94]. The staining of pancreatic cancer tissue for uPAR and following combination with FAM3C for more specific subtyping could potentially identify a patient group with a particularly poor prognosis in the context of PDAC.

5.4.2.3. Cell function experiments

FAM3C is a protein which has previously been associated with EMT and tumor growth. In this study, FAM3C expression of four different pancreatic cancer cell lines was assessed by western blot analysis. Panc-1, T3M4 and MiaPaCa-2 cell lines were all established from the primary tumor site whereas the AsPC-1 cell line was initially taken from metastatic cancer cells in the ascites of a patient with pancreatic cancer[103,117,118,119]. As the AsPC-1 cells would have had to undergo an epithelial-to-mesenchymal transformation as part of the metastatic process, FAM3C expression was expected to be the highest in the AsPC-1 cells. The western blot analysis in this study proofed this hypothesis correct.

A study about the significance of the guanine nucleotide-exchange protein 100 (GEP100) for malignancy in pancreatic cancer investigated the invasive potential and EMT marker expression of different pancreatic cancer cell lines[120]. Similar to FAM3C, GEP100 had previously been associated with EMT in cancer[120]. Amongst other cell lines, the study measured the expression of GEP100 in AsPC-1 and Panc-1 cells which correlated with FAM3C expression. GEP100 expression was much higher in the AsPC-1 cells compared to the Panc-1 cells[120]. Also, the authors of the study assessed the invasiveness of 6 different pancreatic cancer cell lines by matrigel invasion assay including three cell lines from a primary tumor and three cell lines from metastatic sites. The assay showed that AsPC-1, as well as another cell line from a metastatic site (Patu8988), were much more invasive than the other cell lines[120].

Furthermore, E-cadherin expression of the six cell lines was determined by western blot and showed that AsPC-1 expressed very little E-Cadherin[120]. The authors of the GEP100 study chose the Patu8988 cell line which is similar to the AsPC-1 cell line regarding invasiveness and E-cadherin expression for further cell experiments[120]. Interestingly, a shRNA mediated reduction of GEP100 in those cells leads to a 35% decrease in invasive abilities, a 3-fold increase in E-cadherin expression while a difference in the MTT assay assessing cell viability could not be detected[120]. Because of these results, reduced expression of FAM3C in AsPC-1 cells was expected to lead to an increase in E-cadherin expression. Moreover, this was already shown to be true for FAM3C in the context of breast cancer as a shRNA mediated reduction of FAM3C expression in breast cancer cells leads to an increase in E-cadherin expression[94]. In our study, a siRNA mediated reduction of FAM3C affected neither E-cadherin

expression nor invasiveness of the AsPC-1 cell line. In the future, a siRNA transfection and progressive reduction of FAM3C expression in the Patu8988 cell line could potentially yield new insights. Although the experimental setup was different, other studies showed that a siRNA mediated reduction of the expression of specific proteins in AsPC-1 cells leads to a decrease in the number of invading cells in the matrigel assay[121,122]. One of the studies mentioned above-performed siRNA transfection and the following functional cell experiments with Panc-1 cells in addition to AsPC-1 cells which showed a similar decrease in invasiveness[121]. Performing the cell experiments with multiple pancreatic cancer cell lines instead of just the AsPC-1 cell line could potentially yield additional insights. In summary, it can be said that our study was not able to show that a reduced FAM3C expression has a significant impact on the invasiveness or E-cadherin expression of a metastatic pancreatic cancer cell line (AsPC-1).

5.5. Serum

5.5.1. Tissue-type plasminogen activator

The tissue-type plasminogen activator (PLAT/t-PA) is a protein which has previously been associated with different types of cancer including pancreatic cancer. In this study, we examined a possible correlation between postoperative levels of PLAT in the sera of pancreatic cancer patients and the postoperative prognosis as well as a possible correlation with ALDH1A3 expression in the tissue. PLAT was chosen as a candidate because of its role in the activation of FAM3C and it has previously been associated with invasiveness, proliferation and angiogenesis in human pancreatic cancer cells as well as in mouse models[123]. Interestingly, to our knowledge there is no published data about a correlation of PLAT expression in the tissue or serum of patients with pancreatic cancer and prognostic significance or overall survival. However, several studies have examined a possible correlation between PLAT expression and survival in other tumor entities and showed that a high expression could either be a positive or negative predictive marker, depending on the type of tumor investigated. Raigoso et al. published data which showed that high expression of t-PA in the cytosol of cancer cells in resected tissue samples correlated with shorter overall survival[124]. The perception of PLAT as a marker for poor patient outcome is challenged by a publication from Corte et al. which demonstrated that a high expression of t-PA in the cytosol of breast cancer cells correlated with a longer relapse-free and overall

survival in the subgroup of patients with a negative lymph node status[125]. This correlation could not be seen in the subgroup with positive lymph node status, but throughout the entire patient cohort (n=800) high levels of tPA in the cytosol of cancer cells correlated with smaller and more differentiated tumors.

In the context of our study, we were not able to show that the serum levels of PLAT correlate with either ALDH1A3 expression in the tissue or overall survival. This might be because a total of 22 patients were included in the experiment. Even though the data is not significant, a clear difference in median survival between the PLAT-positive and PLAT-negative group (11 vs 22 months) can be seen. According to this data, increased serum levels of PLAT could be interpreted as a negative factor when looking at overall survival in pancreatic cancer patients. This would go in line with other work showing that t-PA adds to the invasiveness and proliferation of pancreatic cancer cells in vitro.

5.5.2. Family with sequence similarity 3 member C

As FAM3C is a secreted protein which has been shown to play a role in EMT, it was chosen for further analysis when the bioinformatical analysis showed FAM3C to correlate with ALDH1A3 expression. It remains unclear if serum FAM3C levels constitute a significant marker for either diagnosis or prognosis in any cancer. A study by Kraya et al. investigated the potential function of FAM3C as a serum marker for autophagy activity in melanoma patients[126]. The authors demonstrated that FAM3C levels were significantly increased in melanoma cells with high autophagic activity.

In our study, we could show that there is an apparent tendency of patients with high FAM3C levels to have a shorter postoperative survival (11 vs 19 months). As the p-value is 0,156, we were not able to show this tendency to be significant, but a clear separation of the two survival curves is visible in the Kaplan-Meier analysis. An enlargement of the patient cohort and the collection of new serum samples might lead to statistically significant results in the future.

5.6. Other IHC markers

5.6.1. Keratin 81

Keratin 81 was previously identified as a tissue marker for a PDAC subtype with an, especially poor prognosis. Noll et al. were able to show that KRT81 is a specific marker for the QM-PDA subtype of pancreatic cancer which was shown to be particularly

aggressive. These findings were validated in a patient cohort of 231 patients. As Kong et al. established ALDH1A3 as a marker for an aggressive subtype of PDAC, we chose to stain parts of our patient cohort for KRT81 in order to see if KRT81 and ALDH1A3 were markers for the same subtype.

Ultimately, we could show that ALDH1A3 and KRT81 don't correlate in pancreatic cancer tissue. ALDH1A3 could be a marker for a subtype of pancreatic cancer which has not been described in the original publication by Collison et al. Collison et al. characterised the KRT81-positive PDAC subtype by specific gene expression profiles from which they drew conclusions about its malignancy. In contrast, Kong et al. chose an approach in which the ALDH1A3-positive PDAC subtype was characterized according to the intracellular signaling pathways on which it relies and the histomorphological features of the resected tissue. This could be a possible explanation as to why the expression of the two proteins doesn't correlate in human PDAC.

In our patient cohort, the difference between postoperative survival of KRT81-positive patients and KRT81-negative patients was not statistically significant even though we applied the same antibody that was used by Noll et al. Nonetheless, the staining pattern described by Noll et al. appears to be very similar to the pattern which could be seen in our patient cohort. Also, the clinical features of the KRT81-positive cohorts were very much comparable. Noll et al. showed that 35% of the patients were KRT81-positive and that these patients had a mean survival of 16,5 months. In line with these findings, we found 34% of the patients of our cohort to be KRT81-positive, having a mean survival of 14,25 months.

In a publication from 2017, researchers who were also part of the Noll et al. publication from 2015 stated that some scattered KRT81 positive tumor cells could frequently be found in otherwise negative tumors which could lead to a misclassification of a tumor as a whole. Muckenhuber et al. suggested that the cut-off would be set to more than 30% of cancer cells being positive for KRT81. This increased cut-off value leads to a significant decrease in patients that are classified as KRT81-positive and allows for a much more specific classification. Regarding KRT81 staining in our patient cohort, we might have set the cut-off value too low as well. This could be a possible explanation for the fact that the difference between mean survival is not significant in our study.

5.6.2. Other markers

Bioinformatical analysis rendered several genes that have correlating expression levels with ALDH1A3 of which we chose IRS2, SP100 and MCC for further investigation because of their role in other cancers that were described in previous publications. Immunohistochemical staining showed that none of them could be used to further subclassify PDAC as most of the PDAC samples stained were strongly positive for these proteins.

The mutated in colorectal cancers gene (MCC) has previously been identified as a tumor suppressor gene that is inactivated in 50% of sporadic colon carcinomas by promoter methylation[127]. Also, it has been shown to have a decreased expression in HCC's[128]. A recent study has shown that MCC is involved in the process of EMT by interacting with E-cadherin and that cells which had a shRNA-mediated reduction of MCC expression had a significantly decreased cell-cell junction integrity and were significantly less invasive[129]. MCC staining in our cohort showed the same staining pattern that was described in previous publications. Nonetheless, MCC does not seem to be fit to serve as a marker for a particular subtype of pancreatic cancer as it is expressed in the vast majority of pancreatic cancer samples.

The SP100 nuclear antigen has been shown to have a tumor suppressing function and to play a role in the control of cell growth[130]. In the context of cancer, a study about SP100 expression in brain tumors showed that high malignancy brain tumors have a significantly decreased expression of SP100 on the mRNA and protein level when compared to low malignancy cancer entities of the brain[131]. A study about laryngeal cancer showed that low SP100 expression correlated with a poorly differentiated tumor[132]. Both studies described a nuclear staining pattern of the SP100 protein which is very similar to the patterns seen in our cohort. Interestingly, the laryngeal cancer study showed that a nuclear staining pattern correlates with a well-differentiated tumor whereas a cytoplasmic staining pattern correlates with low differentiation. Even though some cytoplasmic staining could be seen in the samples of our cohort, no clear distinction of cytoplasmic or nuclear staining could be made in the context of PDAC.

As the insulin receptor substrate-2 (IRS2) is part of the IRS family, it plays a role in the intracellular signalling pathway of Insulin and thus can have an oncogenic effect when mutated or overexpressed[133]. A study about IRS2 expression in colorectal cancer showed that the mRNA levels, as well as the immunohistochemical staining intensity of IRS2, increased with increasing stages of colorectal cancer (normal tissue to

adenocarcinoma)[134]. The staining patterns described in this study were similar to the patterns seen in our patient cohort. This study identified a clear correlation between increased IRS2 expression and cancer progression. The pancreatic cancer tissue samples that were stained and evaluated in our study were all at an advanced stage of the disease and were IRS2-positive in more than 90% of the cases. It would be interesting to see if IRS2 expression increases with the progression of pancreatic cancer but as most of the diagnosis is made at a late stage of the disease it is hard to retrieve preneoplastic tissue. Another study showed that the percentages of positive samples in prostate cancer (31%) and endometrium cancer (41%) were much lower than in our patient cohort thus making a subclassification possible[135]. In our study, we could show that, at least at an advanced stage of pancreatic cancer, a vast majority of patients have IRS2-positive tumors which make IRS2 unfit to further subclassify the disease.

5.7. Summary and outlook

This study was designed to find a serum biomarker for the ALDH1A3-positive PDAC subtype in order to allow diagnosis and assessment of prognosis before surgery. We could show that ALDH1A3-positivity in resected PDAC tissue does correlate with postoperative survival in an enlarged patient cohort of 162 patients. Also, we were able to detect ALDH1A3 in the early stages of pancreatic carcinogenesis in mice. Hence, ALDH1A3 is a promising marker as it is not expressed in normal pancreatic cells and is detectable at an early stage of the disease. A focus of future research on the role of ALDH1A3 in pancreatic cancer and exploration of correlating protein levels in the tissue and ultimately the serum is advisable in order to identify indicators of high ALDH1A3 expression or connected pathways.

We identified FAM3C which correlates with expression levels of ALDH1A3 in the bioinformatical analysis and is a secreted protein that is detectable in the serum of patients. Also, we were able to demonstrate that patients who have an increased expression of FAM3C in resected PDAC tend to have a shorter postoperative survival time compared to patients who express low levels of FAM3C. This tendency could be confirmed by measuring the FAM3C levels in the sera of PDAC patients. Furthermore, we showed that patients who express high levels of PLAT tend to have a shorter mean survival after resective surgery when compared to patients with lower PLAT levels. The recruitment of more patients and the collection of more serum samples of PDAC patients could further underline the prognostic significance of FAM3C and PLAT expression in PDAC in the future.

6. References

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