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A BLOOD-BASED BIOMARKER STUDY

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A BLOOD-BASED BIOMARKER STUDY

BY
BENJAMIN EMIL STUBBE

PhD Thesis 2024

AALBORG UNIVERSITY
DENMARK

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AALBORG UNIVERSITY
DENMARK

PhD Thesis 2024

Submitted: March 2024

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STUDIES

Study I

Stubbe BE, Henriksen SD, Madsen PH, Larsen AC, Krarup HB, Pedersen IS, et al. Validation of SFRP1 Promoter Hypermethylation in Plasma as a Prognostic Marker for Survival and Gemcitabine Effectiveness in Patients with Stage IV Pancreatic Adenocarcinoma. *Cancers 2021;13(22):1–15*.

Study II

Stubbe BE, Madsen PH, Larsen AC, Krarup HB, Pedersen IS, Hansen CP, et al. Promoter hypermethylation of SFRP1 as a prognostic and potentially predictive blood-based biomarker in patients with stage III or IV pancreatic ductal adenocarcinoma. *Pancreatology 2023 August 2023;23(5):512-521.*

Study III

Stubbe BE, Larsen AC, Madsen PH, Krarup HB, Pedersen IS, Lundbye-Christensen S, et al. Promoter hypermethylation of SFRP1 as a prognostic and potentially predictive blood-based biomarker in patients with localized pancreatic ductal adenocarcinoma. *Front Oncol. 2023 Jun 2;13:1211292.*

Paper IV

Stubbe BE, Stoico, MP., Terp, SK., Madsen, PH., Lundbye-Christensen, S, Hansen, CP, Poulsen, LØ, Rasmussen, LS, Yilmaz, MK Jensen, LH, Hansen, TF, Pfeiffer, P, Larsen, AC, Krarup, HB, Pedersen, IS, Hasselby, JP, Johansen, AZ, Chen, IM, Johansen, JS, Thorlacius-Ussing, O, Henriksen, SD. Promoter hypermethylation of SFRP1 is an allele fraction-dependent prognostic biomarker in metastatic pancreatic ductal adenocarcinoma. *Submitted to Clinical Cancer Research, March 2024.*

ENGLISH SUMMARY

In Denmark approximately 1,000 individuals receive the diagnosis pancreatic ductal adenocarcinoma (PDAC) annually. Due to its poor prognosis and increasing incidence, PDAC has been projected to be the second most common cause of cancer-related death globally by 2026. The poor prognosis of PDAC is caused by several factors. Early diagnosis is difficult, resulting in most patients being diagnosed after the disease has metastasized. Furthermore, PDAC is an incredibly aggressive disease that quickly develops resistance to chemotherapy. This has resulted in PDAC being the only cancer with an increasing mortality rate in Denmark, emphasizing the need for further research.

There is a need for better markers to help evaluate prognosis in individual PDAC patients and aid clinicians in choosing therapies. As a tumor suppressor gene, Secreted frizzled-related protein 1 (SFRP1) inhibits the Wnt/β-catenin pathway. SFRP1 is often inactivated in cancer, and inactivation in tumor tissue has been linked to shorter survival. DNA hypermethylation is a normal process used to regulate gene expression. A gene becomes silenced when its promoter region becomes hypermethylated. Unfortunately, cancer cells can also exploit this process to inactivate tumor suppressor genes, which would otherwise control cell growth and division. Promoter hypermethylation of SFRP1 (phSFRP1) is the most common method of silencing.

Study I investigated the value of phSFRP1 in determining prognosis among patients with metastatic PDAC. In total 40 patients were included into the discovery cohort, 25 treated with gemcitabine and 15 with best supportive care (BSC). phSFRP1 was detected in 53% of patients. The median overall survival (mOS) was substantially shorter in patients with phSFRP1 compared to in patients with unmethylated SFRP1 (umSFRP1) (4.4 months vs. 11.6 months). There was no difference in survival according to SFRP1 methylation among BSC-treated patients (2 vs. 1.5 months). The findings were validated in an external cohort including 58 gemcitabine-treated patients. phSFRP1 was detected in 50% of patients. Likewise, the mOS was substantially shorter in patients with phSFRP1 compared to patients with umSFRP1 (3.2 months vs. 6.3 months).

Study II examined whether phSFRP1 affected the prognosis in FOLFIRINOX-treated patients with metastatic disease. The study included 52 patients with metastatic PDAC. phSFRP1 was detected in 54% of patients. The mOS was substantially shorter in patients with phSFRP1 compared to patients with umSFRP1 (6.8 months vs. 15.7 months). In addition, the study indicated that phSFRP1 provides additional prognostic information compared to known prognostic biomarkers.

Study III examined whether phSFRP1 affected the survival of patients with localized PDAC. In total 211 patients were included in study III. phSFRP1 was detected in 20%

of patients. phSFRP1 was associated with a shorter mOS compared to umSFRP1 (13.1 months vs. 19.6 months). Patients with phSFRP1 who received curative resections had a mOS 4.5 month longer than patients with phSFRP1 who did not receive curative resections. Comparatively, patients with umSFRP1 who received curative resections had a mOS 10.9 months longer than patients with umSFRP1 who did not receive curative resections.

Study IV was a larger-scale validation of phSFRP1 with an optimized methodology. The optimized methodology allowed us to detect the allele fraction (AF) of phSFRP1 and split it into three levels: high phSFRP1 AF, low phSFRP1 AF, and umSFRP1. The study included 354 patients with metastasized phSFRP1. phSFRP1 was detected in 61% of patients, where 40% had a high phSFRP1 AF and 21% had a low phSFRP1 AF. The mOS was shortest in patients with a high phSFRP1 AF, longer in patients with a low phSFRP1 AF, but the best in patients with umSFRP1 (3.0 months vs. 6.9 months vs. 8.7 months).

DANSK RESUME

Omkring 1000 mennesker bliver hvert år diagnosticeret med kræft i bugspytkirtlen (PDAC) i Danmark. På verdensplan forventes PDAC at blive den anden hyppigste kræftrelaterede dødsårsag inden 2030. Mange faktorer bidrager til den utroligt dårlige overlevelse. Diagnosen er svær at stille tidligt, så en stor del af patienterne diagnosticeres først efter kræften har spredt sig. Derudover er sygdommen utroligt aggressiv og udvikler hurtigt resistens overfor kemoterapeutiske behandlinger. Samlet betyder dette at PDAC er den eneste kræftform med en stigende dødelighed i Danmark. Dette understreger det enorme behov for forskning på området.

Der er mangel på gode prognostiske markører, som kan hjælpe med at forudsige overlevelsen og assistere klinikeren i at vælge den bedste behandling for patienten. Secreted Frizzled Related Protein 1 (SFRP1) er et tumorsuppressorgen, som er inaktiveret i mange kræftformer. En inaktivering af SFRP1 i tumorvæv er koblet til kortere overlevelse. DNA hypermethylering er en normal process, der bruges til at regulere aktiviteten af gener. En hypermethylering af et gens promoter region fører til en inaktivering. Desværre kan kræftceller også udnytte denne proces til at inaktivere gener, som ellers regulerer cellevækst og celledeling. En promoter hypermethylering af SFRP1 (phSFRP1) er den hyppigste måde, SFRP1 bliver inaktiveret på.

Det overordnede mål med de fire studier i denne afhandling var, at afklare hvorvidt en blodbaseret analyse af dette gens methylering kan være en klinisk nyttig prognostisk biomarkør for patienter med kræft i bugspytkirtlen.

Studie I undersøgte den prognostiske effekt af markøren i patienter med metastaseret PDAC. Studiets første kohorte inkluderede 40 patienter, hvoraf 25 var behandlet med kemoterapien gemcitabin og 15 med bedste understøttende behandling (BSC). phSFRP1 blev fundet i blodet hos 53% af patienterne. Median overlevelsen (mOS) blandt patienter med phSFRP1 var 11.6 mdr., og 4.4 mdr. for dem uden målbar methylering af SFRP1 (umSFRP1). Der var ingen forskel i mOS afhængigt af SFRP1 methylerings blandt patienter behandlet med BSC (2 vs. 1.5 mdr.). Studiet validerede resultaterne i en ekstern kohorte som inkluderede 58 patienter behandlet med gemcitabin. phSFRP1 var målbart i 50%. Blandt patienter med phSFRP1 var mOS 3.2 mdr., og 6.3 mdr. i patienter med umSFRP1.

Studie II undersøgte om phSFRP1 også påvirkede prognosen ved behandling med den kraftige kombinationskemoterapi FOLFIRINOX. Studiet inkluderede 52 patienter med metastaser, behandlet med FOLFIRINOX. phSFRP1 var målbart i 54%. mOS var 6.8 mdr. for patienter med phSFRP1, og 15.7 mdr. for patienter med umSFRP1. Derudover tydede studiet på, at markøren bidrager med ekstra information sammenlignet med kendte prognostiske markører.

Studie III undersøgte om phSFRP1 påvirkede prognosen i patienter med lokaliseret PDAC. Studiet inkluderede 211 patienter med stadie I-II PDAC. phSFRP1 var målbart i 20% af patienterne. Patienter med phSFRP1 havde en mOS på 13.1 mdr., sammenlignet med 19.6 mdr. ved umSFRP1. Patienter med phSFRP1 der blev kurativt opereret havde en mOS 4.5 mdr. længere end de der ikke blev opereret. Derimod havde patienter med umSFRP1 der blev kurativt opereret en mOS 10.9 mdr. længere end patienter der ikke blev opereret.

Formålet med det studie IV var at foretage en større validering af markøren med en ny og forbedret metode. Med den optimerede analysemetode var det muligt at finde allelfrekvensen (AF) af phSFRP1, og inddele den i flere grader; høj phSFRP1 AF, lav phSFRP1 AF og umSFRP1. I alt blev 354 patienter med metastaseret kræft i bugspytkirtlen inkluderet. phSFRP1 var målbart i 61%, hvoraf 40% havde en høj phSFRP1 AF og 21% en lav phSFRP1 AF. For patienter med en høj phSFRP1 AF var mOS var 3.0 mdr., sammenlignet med 6.9 mdr. for patienter med en lav phSFRP1 AF og 8.7 mdr. for patienter med umSFRP1.

ABBREVIATIONS

AF	Allele fraction
ARD	Absolute risk difference
AUC	Area under the ROC curve
BSC	Best supportive care
CA 19-9	Carbohydrate 19-9
CDK	Cyclin-dependent kinase
cfDNA	Cell-free DNA
CP	Chronic pancreatitis
CT	Computed tomography
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
GSTT1	Glutathione S-transferase theta 1
IPMN	Intraductal papillary mucinous neoplasm
IQR	Interquartile range
miRNA	MicroRNA
MMR	Mismatch repair
mOS	Median overall survival
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
phSFRP1	Promoter-hypermethylated SFRP1
phSFRP1high	Detectable phSFRP1, with a phSFRP1 allele fraction above 0.53%
phSFRP1low	Detectable phSFRP1, with a phSFRP1 allele fraction below 0.53%
PS	ECOG performance status
RMST	Restricted mean survival time
ROC	Receiver operating characteristic
RR	Relative risk
SFRP1	Secreted frizzled-related protein 1
TSG	Tumor suppressor gene
umSFRP1	Unmethylated SFRP1

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CHAPTER 1. INTRODUCTION

1.1. ANATOMY AND FUNCTION OF THE PANCREAS

The pancreas is a lobulated organ with length of approximately 14-18 cm, a weight of approximately 85-100 grams, located retroperitoneally (Figure 1) (1). The pancreas is closely related to several organs: the head is closely related to the duodenum and the vena cava while the neck is directly in front the superior mesenteric artery and the aorta, and lastly the tail of the pancreas extends to the spleen (1). The organ can be divided into four regions, namely, the tail, body, neck, and head, with the head being subdivided into the head proper and uncinate process (1). The pancreas has both exocrine and endocrine functions. The lobulated appearance of the organ is caused by the layer of connective tissue which encapsulates it. This capsule penetrates the gland as septa (2). Each lobule contains several acini, which are responsible for the exocrine function of the pancreas and comprise approximately 80% of the organ. The acini empty their secretions of proenzymes such as amylase, lipase, and trypsinogen via the pancreatic ducts (2).

In contrast, pancreatic hormones are secreted directly into the bloodstream by endocrine cells. The endocrine cells are dispersed between the acini and form small clusters – which are labelled islets of Langerhans (3). The islets are comprised of five cell types, each responsible for releasing hormones directly into the bloodstream: α-cells (which produce glucagon), β-cells (that procude C-peptide and insulin), γ-cells (that produce pancreatic polypeptide), δ-cells (which produce somatostatin) and lastly ε-cells (that produce ghrelin) (3).

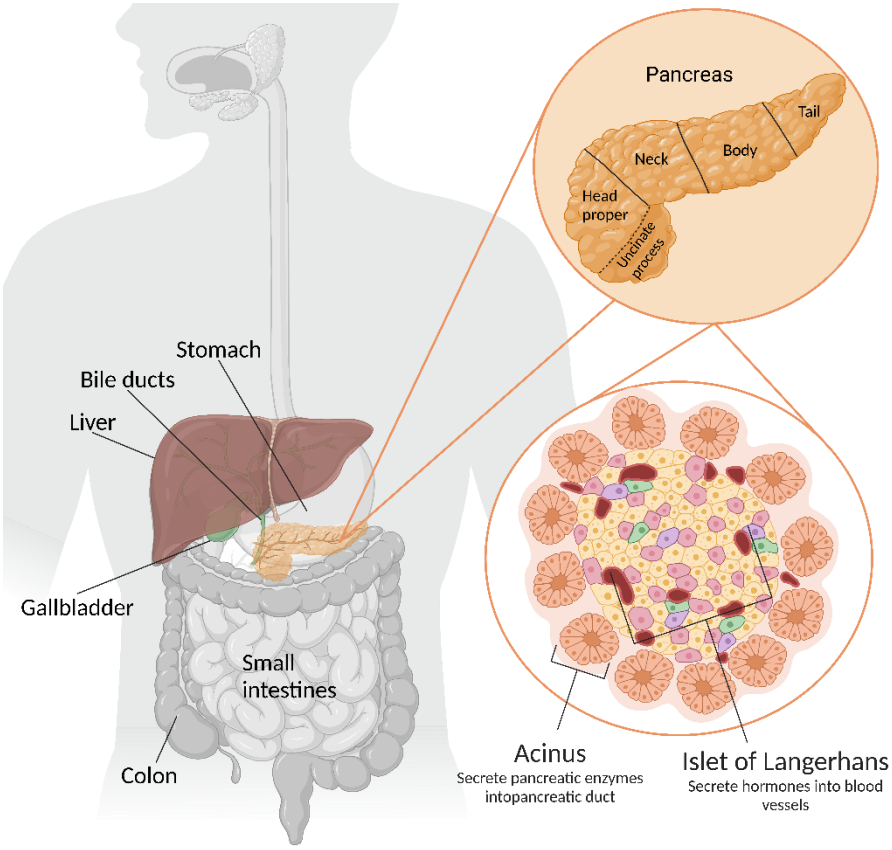

Figure 1. Anatomy of the pancreas. Created with BioRender.com.

1.2. PANCREATIC CANCER

1.2.1. PATHOLOGY OF PANCREATIC CANCER

Pancreatic cancers arise from either endocrine or exocrine cells in the pancreas. Most pancreatic cancers arise from exocrine cells, while endocrine tumors account for a discrete number of cases (< 5%). Exocrine tumors can be categorized into pancreatic ductal adenocarcinoma (PDAC) or mucinous tumors. PDAC makes up almost all cases of pancreatic cancers (approximately 95%) (4). This thesis exclusively focuses on PDAC, the most common pancreatic malignancy.

1.2.2. SYMPTOMS AND DIAGNOSIS

The diagnosis of PDAC remains an enormous unmet challenge, as most patients are diagnosed too late to be eligible for curative treatment. The most frequent symptoms of PDAC include asthenia, pain, jaundice, nausea, and weight loss(5). However, these symptoms are often vague or completely absent. Vague symptoms combined with a capacity to disseminate early and the fact that tumors are inaccessible to palpation render early diagnosis difficult (6).

Currently, no viable screening option is available for PDAC (7). Indeed, due to the relatively low incidence, a population-wide screening program is likely not feasible (8). A screening program for high-risk patients, such as specific subgroups of patients with diabetes, may be feasible, but it is currently not implemented.

The most essential part of PDAC diagnosis is clinical suspicion. Upon clinical suspicion, a multiphase computed tomography (CT) scan is considered the most reliable tool for diagnosing and staging PDAC (9,10). This is usually followed by a biopsy to verify the diagnosis. However, this is not always performed in clear-cut cases where the tumor is upfront resectable (11). Fairly frequently, the suspicion of PDAC arises incidentally as part of a less specific diagnostic workup involving CT or positron emission tomography (PET)-CT. In Denmark, all PDAC cases are discussed at multidisciplinary team conferences to determine the diagnosis, stage, and treatment plan. Additionally, all cases of borderline resectable PDAC are discussed at national multidisciplinary team conferences to prevent regional differences in treatment.

1.2.3. STAGING

PDAC is staged according to primary tumor size, involvement of lymph nodes, and whether distant metastases are present (TNM) system. Correct staging is paramount, as this is the primary tool for determining which patients are eligible for curative surgery. Unfortunately, less than 20% have resectable disease at diagnosis (12). The remaining patients present with disease that is either borderline resectable, locally advanced into nearby tissue, or with manifest distant metastases. Treatment with chemotherapy can convert a percentage of patients with either borderline resectable disease or locally advanced disease into a resectable stage (13).

1.2.4. INCIDENCE

PDAC accounts for roughly 2.5% of all adult cancer cases, and thus is far from the most common cancer (14). However, it is incredibly lethal, which results in the death of almost all patients diagnosed with the disease. Notably, the incidence has been increasing by approximately 1% yearly for since the 1990s (15). In 2020, approximately 500,000 patients were diagnosed worldwide, and every year, approximately 1000 patients are diagnosed in Denmark (16,17). The high mortality

and increasing incidence has led to PDAC being projected to become the second most common cause of cancer-related death worldwide by 2026 (18).

1.2.5. RISK FACTORS FOR PANCREATIC CANCER

1.2.5.1 Preventative risk factors

An estimated one fourth of PDAC cases can be attributed to preventable risk factors (19).

Cigarette smoking

Cigarette smoking substantially increases the risk of PDAC (20). Roughly 10-32% of deaths from PDAC are attributable to tobacco smoking (19,21). The increased risk is proportional to the quantity of consumed tobacco (22). Individuals with homozygous deletions of the gene glutathione S-transferase theta 1 (GSTT1) may be at particularly high risk when exposed to cigarette smoke (23). The relative risk (RR) of developing PDAC is at least 1.5 when comparing smokers nonsmokers (22,24). Fortunately, the excess risk appears to decrease following smoking cessation and may even reach the levels of never-smokers 5-20 years after quitting smoking (22,24).

Obesity and physical inactivity

Excess body weight is attributable to approximately 17% of PDAC cases (19). A BMI of more than 30 has been linked to an increased risk of PDAC compared to having a BMI of less than 23 (25). Compared to people of a healthy weight, overweight individuals have 10% increased risk of developing PDAC, rising to 20% in obese individuals (21). Some data suggest that individuals with a highly active lifestyle are significantly less likely to develop pancreatic cancer (25). However, a highly active lifestyle could also be a proxy measure for individuals more likely to have a healthier lifestyle overall.

Alcohol

Some evidence suggests a link between alcohol and developing PDAC. Heavy alcohol consumption appears to be associated with increased risk of developing PDAC, however moderate consumption does not appear to increase risk of developing PDAC (26). Thus, alcohol use is attributable to only 2-5% of PDAC cases (26). However, alcohol consumption is also a core contributing factor to the development of chronic pancreatitis, which also increased the risk of developing PDAC (27).

1.2.5.2 Nonpreventative risk factors

Age

Like most other tumors in adults, PDAC is heavily age-dependent (28). Only approximately 5-10% of cases occur before age 50. Cases of PDAC that develop this early are often associated with the presence of predisposing genetic factors (29). The

risk of death from PDAC rises from <2 deaths per 100,000 person-years in those aged 35-39 to more than 90 among individuals older than 80 (30).

Sex

At a global level, the incidence of PDAC is higher among men compared to women, with a ratio of 1.3:1. However, higher tobacco consumption in men may explain at least some of this. In Denmark, however, the incidence has been approximately equal in recent years.

Chronic pancreatitis

Chronic pancreatitis (CP) is characterized by progressive, irreversible fibrotic changes to the pancreatic parenchyma (27). The etiology of CP is heterogeneous but often includes alcohol abuse, smoking, autoimmune diseases, and genetic risk factors (27). Patients with CP have up to a 13 times higher risk of developing PDAC compared to the background population, and is potentially even higher in those with hereditary pancreatitis (31–33). Some evidence suggests the risk of developing PDAC increased with CP duration (32). The cumulative risk is approximately 1.8% after 10 years with CP and 4% after 20 years with CP (34).

Diabetes

Likewise, individuals with diabetes also have an increased risk of developing PDAC. A meta-analysis estimated the RR to be approximately 2.1 compared to nondiabetic individuals (35). Diabetes has also been associated with poorer prognosis of PDAC (36). The RR of developing PDAC after diabetes onset depends heavily on the duration of diabetes, being 6.7 during the first year and decreasing to 1.36 after 10 years (35). This could suggest that diabetes diagnosed in close proximity to PDAC is not a cause of PDAC but rather a consequence of it (37).

Race

There are some racial differences in PDAC incidence and prognosis, as African Americans are at a higher risk of PDAC than Caucasians (38,39). Furthermore, they are less likely to undergo surgical resection and have a higher mortality (39). The cause is likely multifactorial, including poverty, health care access, lifestyle, environmental factors, and genetics (38,39).

Genetic risk

While most PDAC cases are spontaneous, a familial accumulation of the disease can be found in 5-10% of cases (4). Several genes are associated with an increased risk of developing PDAC, as summarized in

Table 1. High-penetrance genes associated with PDAC are SPINK1, PALB2, PRSS1, BRCA2, STK11, and mismatch repair genes (40).

Table 1. Inherited Syndromes and Susceptibility Genes Associated with an Increased Risk of Pancreatic Cancer.

Inherited Syndrome	**Susceptibility Gene/Chromosomal Mutation Region**	**Increased Risk of Pancreatic Cancer**
Hereditary pancreatitis	*PRSS1* (7q35)	50- to 80-fold ↑ PDAC risk
Hereditary nonpolyposis colorectal cancer	*h* MSH2, *h* MSH1, *h* PMS2, *h* MSH3, *h* P MS1, *h* MSH6/GTBP(2,3)	Undefined ↑ PDAC risk
Hereditary breast and ovarian cancer	*BRCA2* (13q12-q13), BRCA1	3.5- to 10-fold ↑ PDAC risk
Familial atypical multiple mole melanoma syndrome (FAMMM)	*p16* (9p21)	20- to 34-fold ↑ PDAC risk
Peutz–Jeghers syndrome	*STK11/LKB1* (19p13)	75- to 132-fold ↑ PDAC risk
Ataxia-telangiectasia	*ATM* (11q22-23)	Undefined ↑ risk
Familial adenomatous polyposis	*DP 2.5 (5q12-21)*	Undefined ↑ risk
Familial pancreatic cancer	*BRCA2 (4q32-34)*	5- to 10-fold ↑ PDAC risk
Von Hippel–Lindau syndrome	*VHL (3p25)*	↑ risk of neuroendocrine tumors
Cystic fibrosis	*CFTR (7q31)*	↑ PDAC and GI cancer risk
Li-Fraumeni syndrome	*p53* (17p13.1)	↑ PDAC risk
Fanconi anemia	*FANC or FANCG* (3p22-26, 9p13, 9q22.3,16q24.3)	Slightly ↑ PDAC risk
ABO blood group	*rs9543324* (13q22)	20- to 26-fold ↑ PDAC risk
	rs401681 (5p.33)	
Undefined familial PDAC	*PALB2*	Undefined ↑ risk
Table reproduced from Yeo 2015 with permission. DOI: 10.1053/j.seminoncol.2014.12.002.		

1.2.6. TREATMENT AND PROGNOSIS OF PANCREATIC CANCER

1.2.6.1 Prognosis and treatment

Currently, worldwide, PDAC is the fourth leading cause of cancer-related death and is associated with a particularly insidious prognosis (41). While the 5-year survival has improved from 3% in 1975-1977 to 12% in 2012-2018, this is still among the worst cancer survival rates (41). The only curative treatment of PDAC is surgical removal of the entire tumor burden. The Whipple procedure, also known the pancreatoduodenectomy, and the pylorus-preserving pancreatoduodenectomy are the most commonly performed surgical procedures (42). Unfortunately, approximately 80% of diagnosed with PDAC ineligible for surgical resection at presentation (41).

Additionally, there is a high recurrence rate among patients who undergo curative resection, further worsening the prognosis (43). The poor survival of these supposedly curatively treated patients has led to the hypothesis to consider PDAC a systemic disease, even at early stages, which has led to increasingly intensified adjuvant treatment of resected patients (41,44). With this approach, the 5-year survival rate among resectable patients has improved from 29% to 42% (41,44). This supports the notion that the disease should be considered systemic even in early stages.

Nevertheless, for patients with metastatic PDAC the 5-year survival rate has remained static at 3% (41,44). Treatment options for patients ineligible for surgery are limited to palliative chemotherapy, of which the achievable median overall survival (mOS) is 4-11 months depending on treatment (45,46).

Thus, three distinct challenges have arisen: 1) How do we diagnose more patients earlier - to increase the number eligible for curative resection? This problem has proven complex, as a substantial portion of patients are asymptomatic until the disease has disseminated. Only when more patients can be curatively treated is it likely that survival can be improved substantially. 2) How do we improve survival in patients who undergo curative resection? While intensified adjuvant treatment has improved survival in these patients, several pieces of the puzzle are missing. Until we can detect micrometastatic disease in patients, we can never truly know which patients are cured or provide targeted treatment. 3) How do we improve survival and quality of life in patients diagnosed too late for curative resection? Treatment in these patients is limited to palliative chemotherapy, of which improvements have remained elusive for many years. Furthermore, targeted treatments and improved prognostic and predictive biomarkers are sorely needed to aid patients and clinicians in choosing the optimal treatment option.

1.3. MOLECULAR PATHOGENESIS OF PANCREATIC CANCER

1.3.1. CLASSIFICATION

PDAC arises from epithelial cells which line the pancreatic duct and thus has a gland-like appearance (47). PDACs are preceded by one of two forms of precancerous hyperplastic lesions: pancreatic intraepithelial neoplasias (PanINs), the most common, or intraductal papillary mucinous neoplasms (IPMNs) (48). Precursor PanIN lesions are classified into stages (I-III) based on the observed severity of nuclear abnormalities and architectural disorganization in tumor tissue (48). Low-grade PanINs are relatively common and pose relatively little risk of malignant progression (49). Conversely, higher-grade PanINs are rare and considered carcinoma in situ (49). While PDAC is molecularly a very heterogeneous malignancy, there are four common alterations sequentially acquired during the progression of PanIN lesions, Figure 2.

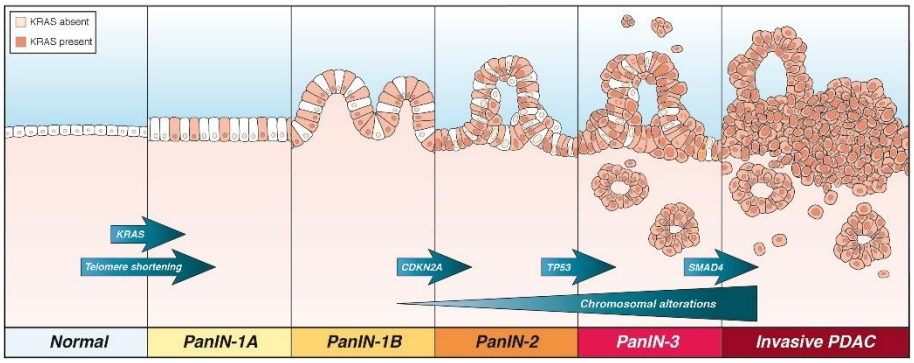

Figure 2. PanIN progression model. Reprinted with permission from Wood et al., 2019. DOI: 10.1053/j.gastro.2018.12.039.

1.3.2. KRAS

The most defining molecular characteristic of progression to PDAC, found in approximately 90% of cases, is a KRAS mutation (50). KRAS encodes a GTPase which regulates not only how cells proliferate, but also how they differentiate, and migrate (51). KRAS mutations often occur as point mutations, resulting in KRAS activation through impairment of its GTPase activity (51). Mutations in KRAS are essential in the early development of PDAC, as evidenced by their presence in almost all (90%) low-grade PanIN lesions (52). Mouse studies have demonstrated that KRAS mutations directly cause PanINs but that further malignant transformation requires subsequent inactivation of several tumor suppressor genes (48,52,53). Interestingly, some studies suggest that KRAS mutation may become dispensable in advanced PDAC, which poses challenges to developing effective targeted therapies (54,55).

1.3.3. TUMOR SUPPRESSOR GENES

Tumor suppressor genes (TSGs) act as inhibitors of oncogenesis by either inducing apoptosis, arresting the cell cycle, or inducing senescence in response to oncogenic driver mutations, thus restricting the proliferation of cells.

1.2.3.1. CDKN2A

CDKN2A, or the cyclin-dependent kinase inhibitor 2A, functions to encode for two proteins (p16 and INK4A) which blocks the cells ability to enter the S phase of the cell cycle (56). Approximately 95% of PDAC cases lose the function of CDKN2A (57,58). As p16 induces senescence after the introduction of a KRAS mutation, loss of CDKN2A is critical in PDAC pathogenesis (59). Thus, inactivation of CDKN2A follows directly after activating KRAS mutations.

1.3.4. TP53

TP53 encodes the transcription factor p53, the function of which is lost in approximately 75% of PDAC cases (57,60). Amino acid substitutions impair the function of p53 as a transcription factor by impairing the ability to bind DNA. Thus, mutant p53 loses the ability to induce the expression of tumor suppressor genes (61). Inactivation of p53 most often follows a loss of CDKN2A in advanced PanIN lesions (59). Mutations in TP53 might contribute to the highly metastatic nature of PDAC (62).

1.3.5. SMAD4

SMAD4 is a TSG which primarily regulated growth and differentiation of cells through the TGF-β signaling pathway (53). Mutations of SMAD4 are present in approximately half of PDAC cases (63). SMAD4 deficiency is associated to a rapid progression of pancreatic tumors when combined with KRAS mutations (53). Thus, SMAD4 mutations are often one of the final steps before tumor initiation and are typically found in advanced PanIN lesions after the loss of CDKN2A (59,64). Furthermore, loss of function of SMAD4 has been linked to poor prognosis (65).

1.4. GENETICS & EPIGENETICS

The human genome can be considered a sort of “instruction manual” for building an organism and its components. This instruction manual contains the necessary information to create the entire human body, from the pancreas to the eyeballs. In this analogy, the manual contains the sequence of approximately 3 billion base pairs of DNA, which comprise the genome (66). This is a profound amount of information, and cells are, generally, specialized. Naturally, it is impractical for every cell to have access to the entirety of the information. For example, many genes that are important for hepatocytes or acinar cells are not helpful for most other cells in the body.

Fortunately, we have evolved sophisticated chemical modification systems that allow for “annotations” of information that is not relevant to individual cells. Through one of several mechanisms, these annotations regulate gene expression by changing the conformation of DNA, making it less available for transcription. Thus, although the genome is the same for every cell in the body, different genes are active or inactive according to each cell type.

This information that exists *beyond* the genomic sequence has been coined the *epigenome*. The epigenome is defined as modifications occurring at a genomic level *without* a change in the DNA base pair sequence. Epigenetics, then, is the study of these modifications. An essential aspect of these modifications is that they exclusively alter how available DNA is for transcription. Thus, they are inherently changeable and potential therapeutic targets.

1.4.1. EPIGENETIC MODIFICATIONS

These annotations, or epigenetic modifications, are changes in chromatin dynamics. Euchromatin is a chromatin structure that both is open and lightly packed and thus linked to gene transcription and activation (Figure 3). In contrast, heterochromatin is a densely packed chromatin structure associated with the silencing of genes.

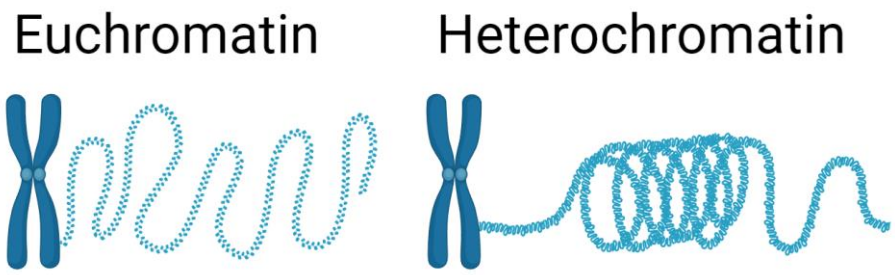

Figure 3. Chromatin structures. Created with BioRender.com.

Epigenetic modifications generally function in two ways: modifications which alter DNA-binding proteins (such as histone modifications) and modifications which directly alter DNA (DNA methylation). This thesis exclusively addresses the latter.

1.4.2. DNA METHYLATION

Through DNA methylation a methyl group (CH3) is added to the carbon at the fifth position of a cytosine base to produce 5-methylcytosine. CpG dinucleotides are a region of DNA where a cytosine is separated from a guanine by only a phosphate group. The cytosines of CpG dinucleotides can be methylated to form 5-methylcytosines. Of the nearly 30 million CpGs in the human genome, between 60-

80% are methylated (67). The majority of CpGs are located in methylated genomic sequences. However, in the promoter regions of genes CpGs tend to cluster into so-called CpG islands. In these promoter regions only 5% of CpGs are methylated in normal tissue.

The absence or presence of promoter DNA methylation functions as a transcriptional "ON" or "OFF" switch by leading to a more closed chromatin structure, making the gene less available for transcription (Figure 4) (67).

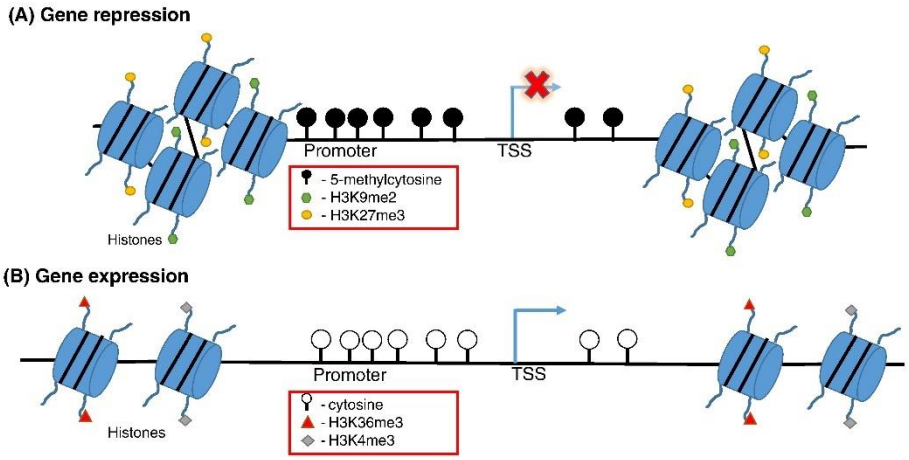

Figure 4. Promoter hypermethylation mediated gene regulation. Reprinted with permission from Paluch et al. 2016. DOI: 10.1016/j.blre.2016.02.002.

DNA methylation is important in both normal and pathological processes. While the DNA sequence is inherited intact from the parents, new methylation patterns are created in every individual during development. One example is X-chromosome inactivation. Male cells in mammals carry only one X chromosome (XY) and are thus inherently hemizygous for X-linked genes. On the other hand, female cells (XX) carry a double dose of X-linked genes, which is potentially lethal. Fortunately, a mechanism called X-chromosome inactivation has developed in female mammals to transcriptionally silence one of their two X chromosomes (68). Likewise, aberrations in methylation status occur in all cancers and are, like genetic alterations, tumor-specific. Thus, methylation markers can be used to identify the cell source of most tumors (69,70). Most cancers develop global hypomethylation early in tumorigenesis, which leads to chromosomal instability and increased tumor frequency (67,70). Promoter hypermethylations is another common alteration in cancer, which can lead to aberrant repression of TSGs (67).

1.5. CELL-FREE DNA

Cell-free DNA (cfDNA) is DNA fragments circulating freely in the bloodstream without being enclosed within cells. These fragments has a length of approximately 167 base pairs and are partially protected from DNases by proteins (71). The presence of cfDNA in the blood was originally demonstrated by Mendel and Métais in 1948 (72). However, the association with cancer was not discovered until decades later, in 1977, where significantly higher DNA concentrations in serum were demonstrated in cancer patients compared to healthy individuals (73). Similarly, a few years later, significantly higher cfDNA levels were demonstrated in patients suffering from PDAC compared to patients with acute or chronic pancreatitis (74).

Various physiological conditions such as exercise, inflammation, and surgery can substantially affect levels of cfDNA (75,76). cfDNA is quickly metabolized, with a half-life estimated to be between 16 minutes and two hours (77–79). Several mechanisms are involved in the clearance of cfDNA, including DNases in the bloodstream, and filtration in the liver and kidneys (Figure 5) (80).

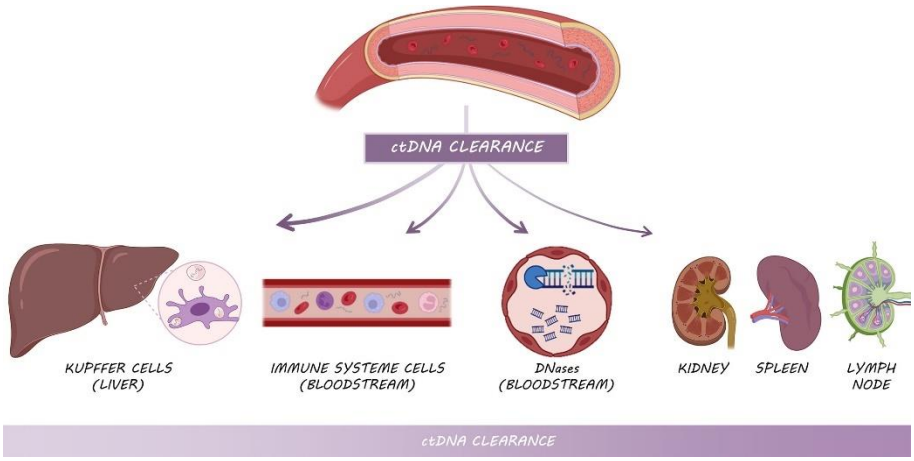

Figure 5. Mechanisms of ctDNA clearance. Reprinted with permission from Sánchez Herrero et al. 2022. DOI: 10.3389/fonc.2022.943253.

In 1989, it was discovered that tumor cells released DNA from the tumor into the bloodstream, which eventually led to the discovery of circulating tumor DNA (ctDNA) (81). In 1994, Vasioukhin et al. demonstrated RAS point mutations in ctDNA, gene alterations otherwise exclusively found in tumor cells (82). This finding implied that analyzing ctDNA could confer information on the genetic makeup of the underlying tumor. Diehl et al. further cemented the clinical relevance in 2005 by

demonstrating that ctDNA levels can reflect tumor burden and dynamics (83). Compared to cfDNA, the fragment length of ctDNA is slightly shorter at 134-144 bp (84). The frequency of detectable ctDNA varies based on tumor size, stage, and cancer type (85,86). ctDNA is detectable in almost all cases with metastasized disease but only in 40-60% of cases with localized disease (85). The quick clearance of cfDNA promotes a steady release that is detectable in the blood, evidenced by a rapid decline to undetectable values after curative surgical resection (87).

While this thesis focuses exclusively on cfDNA and ctDNA, several other tumor-derived products are released into the blood, which include circulating tumor cells, extracellular vesicles, cell-free RNA (both noncoding and mRNA), and tumor-educated platelets (88). In fact, the term "liquid biopsy" was initially coined about circulating tumor cells, but it was quickly expanded to the remaining tumor-derived products (89).

1.6. TECHNIQUES TO INVESTIGATE METHYLATION OF DNA

There are several available techniques which enable the assessment of a genes methylation status. The three most common techniques are approaches based on bisulfite conversion, approaches based on affinity enrichment assays, and lastly a restriction enzyme-based approach (90). The studies of this thesis employed a bisulfite treatment technique followed by either real-time PCR or digital droplet PCR.

Bisulfite treatment causes nonmethylated cytosines to deaminate into uracil, which is subsequently converted into thymine after PCR amplification. In contrast, methylated cytosines remain almost entirely unchanged during this process. The consequence is a translation of an epigenetic alteration (methylation status) into a genetic difference in the DNA sequence. This translation allows discrimination between methylated and unmethylated cytosines by PCR techniques (90).

There are, however, several pitfalls to be considered during this process. The first is DNA conversion: an incomplete DNA conversion will result in bias, as unconverted unmethylated cytosines will be interpreted as methylated cytosines (90). Secondly, to ensure full conversion of DNA, conversion was previously recommended to be performed over 4-18 hours at 55 °C (91). This long incubation time was unfortunately associated with another limitation: degradation of DNA. As the treatment causes breaks in single-strand DNA, this can lead to very low recovery of bisulfite-converted DNA (as low as 5%) (92). Furthermore, poor primer design or incorrect use can lead to false-positives.

The methodologies employed in the first three studies of this thesis are based on an optimized methylation-specific PCR protocol previously described by our group (93). This methodology involves deamination at higher temperatures over a shorter time to

ensure complete conversion and recovery of approximately 60% (93). The last study employs a newer commercial kit, which ensures above 80% recovery.

1.7. WNT/B-CATENIN SIGNALING

The Wnt/β-catenin pathway, which is also known as the canonical Wnt signaling pathway, is intimately involved in the regulation of several cellular processes, such as proliferation, migration, and apoptosis.

1.7.1. MECHANISMS OF THE WNT/B-CATENIN PATHWAY

This aptly named pathway involves two critical components: Wnt proteins and β-catenin. Wnt proteins are secreted signaling molecules able to bind with frizzled receptors on the surface of cells (94). β-catenin is a protein implicated in both the regulation of both cell-to-cell adhesion and, more relevant for this thesis, gene transcription (95).

This pathway is most readily understood when considered to have two states: OFF (without Wnt) and ON (with Wnt). In the absence of Wnt proteins (the OFF state), the intracellular levels (the cytoplasm and nucleus) of β-catenin are constantly being maintained at low levels (94,96). This is possible because of constant removal of β-catenin, enabled by a so-called "destruction complex". This complex is comprised of scaffolding proteins APC (Axin and adenomatous polyposis coli), CK1 (casein kinase 1), and GSK3 (glycogen synthase kinase 3) (94,96).

In the ON state (with Wnt present), a Wnt ligand will bind to the frizzled receptor and coreceptors LRP5/6. This leads to the recruitment of Dvl and subsequently the destruction complex to the membranes (94,96). When the descruction complex is recruited to the membranes, it is inactivated by Dvl polymers. This, in turn, allows β-catenin to accumulate in the cytoplasm of the cell. Lastly, when β-catenin is allowed to accumulate in the cytoplasm it can translocate into the nucleus, where it functions to upregulate the expression of Wnt target genes by interacting with various transcription factors (94,96).

Lastly, Wnt/β-catenin signaling is essential for the initiation of pancreatic carcinogenesis and is also associated with therapeutic resistance and poor prognosis (97,98).

1.8. SFRP1

Secreted frizzled-related protein 1 (SFRP1) is implicated in several biological mechanisms, including bone density regulation and myocardial repair, and is a tumor suppressor gene (99–101). This thesis mainly addresses the latter. The primary function of SFRP1 is to negatively regulate the Wnt/β-catenin pathway (96,101).

Through this role SFRP1 also helps regulate proliferation, migration, and apoptosis. SFRP1 enacts its modulatory role through one of three mechanisms: A) direct binding to the Wnt ligand, B) by binding to frizzled receptors, thus competitively inhibiting Wnt ligand from binding to the receptor, and C) by direct binding with cytoplasmic β-catenin (Figure 6) (101). The presence of SFRP1 actively contributes to maintaining the Wnt/β-catenin pathway in the OFF state. Without SFRP1 present, Wnt ligand can freely bind to the Fz receptor, leading to the inactivation of the destruction complex as well as the accumulation and subsequent translocation of intracellular β-catenin.

Loss of SFRP1 expression in tumor tissue is a poor prognostic factor numerous cancers, including PDAC (102–106). Promoter hypermethylation is the primary regulatory mechanism of SFRP1 (101). Indeed, promoter hypermethylation of SFRP1 and subsequent silencing occur very early in most tumor types, including pancreatic, gastric, breast, cervical, ovarian, renal, prostate, and colon tumors, carcinomas, and hematological malignancies such as AML (101,107). Promoter hypermethylation occurs early in PDAC and in approximately 70% of tumors (105,108).

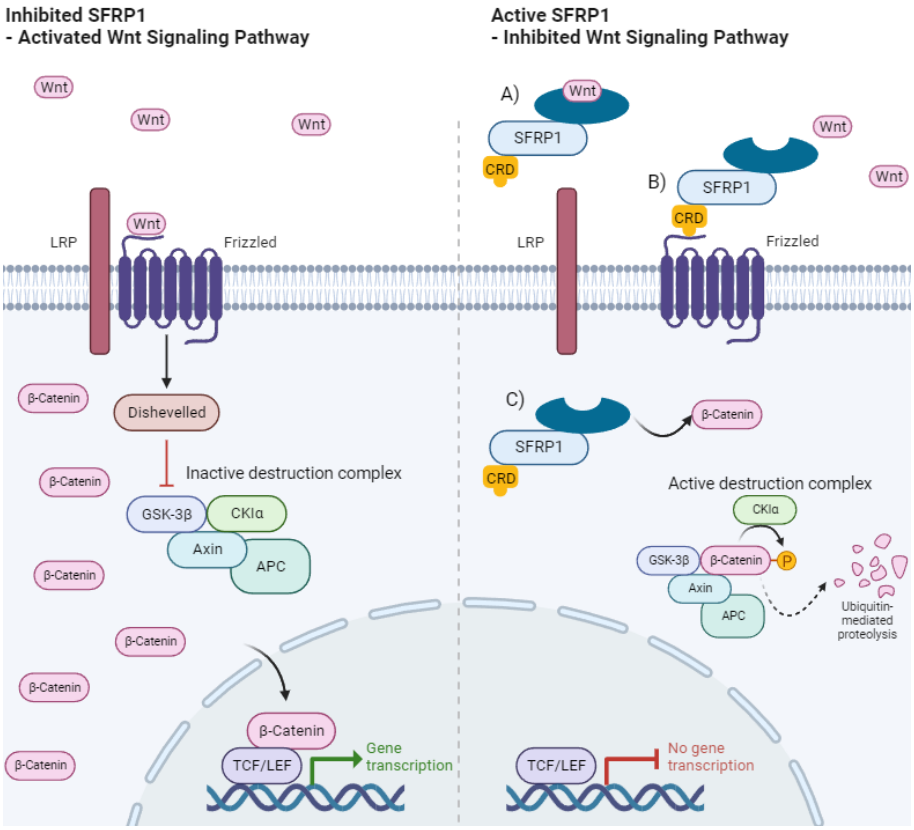

Figure 6. Interactions of SFRP1 with the Wnt signaling pathway. Created with BioRender.com. Adapted from Baharudin et al. 2020, with permission. DOI: 10.3390/cancers12020445

CHAPTER 2. AIMS AND SCOPE

2.1. STUDY I

- To determine and validate whether phSFRP1 in cfDNA is an independent prognostic biomarker in patients with metastatic gemcitabine-treated PDAC.

2.2. STUDY II

- To determine whether the prognostic effects of phSFRP1 in cfDNA extend to patients with metastatic FOLFIRINOX-treated PDAC as well as patients with stage III PDAC.

2.3. STUDY III

- To examine whether the prognostic effects of phSFRP1 in cfDNA extend to patients with stage I-II PDAC.

2.4. STUDY IV

- To determine whether a high allele fraction of phSFRP1 in cfDNA impacts the prognosis of patients with stage IV PDAC more than a low or absent allele fraction of phSFRP1.

CHAPTER 3. MATERIALS AND METHODS

3.1. PATIENTS

All patients were included into one of two Danish biobank studies, either the BIOPAC study (ClinicalTrials.gov registration number NCT03311776, BIOmarkers in patients with PAncreatic cancer; www.herlevhospital.dk/BIOPAC/) study, or the GIVTE study (ClinicalTrials.gov ID: NCT00660205, "Venous Thromboembolism and Haemostatic Disturbances in Patients with Upper Gastrointestinal Cancer"). The BIOPAC study included patients upon referral to the oncological department of one of the seven Danish University hospitals participating in the BIOPAC study after diagnosis of PDAC. The diagnosis was confirmed by histopathological analysis. Patients in the BIOPAC study were included between July 2008 and October 2020. In the GIVTE study, patients were included at time of suspicion of cancer in the upper gastrointestinal tract when referred to the Department of Gastrointestinal Surgery, Aalborg University Hospital. The GIVTE study included patients from February 2008 to February 2011. All data were entered into a database prospectively.

All patients were included prospectively and had not received previous chemotherapy when included. After inclusion, patients received treatment afterr the Danish guidelines at the time. Patients included were all more than 18 years old at time of inclusion and signed an informed consent form prior to their inclusion in the respective studies. No clinical data were received before methylation analysis was completed.

3.2. BLOOD SAMPLES

Blood samples in all studies were collected prior to any treatment. Either EDTA plasma samples or serum samples were used, according to the study. Samples taken for the GIVTE study at Aalborg University Hospital were centrifuged at 4,000 rpm and 4°C for 20 minutes, while samples from the BIOPAC study were centrifuged at 2,300 × g and 4°C for 10 minutes. In all studies samples were processed and subsequently frozen at -80°C in under two hours from sampling time.

3.3. ANALYTICAL METHODS

3.3.1. STUDIES I-III

Studies I-III used methylation-specific real-time PCR and a rapid bisulfite protocol to identify hypermethylated cytosines. The protocol was first described by Pedersen et

al. in 2012 (93). All methylation analyses were performed in a blinded manner. In both preamplification and second-round PCR the hemi-methylated MESTv1 gene was employed as a reference gene.

3.3.1.1 Sample preparation

3.3.2. PREPARATION OF SAMPLES

Following thawing of samples, extraction of cfDNA was performed with the easyMAG platform (NucliSens®, bioMérieux) using instructions from the manufacturer and approximately 1 ml of sample material (either EDTA plasma or serum). The extracted DNA from the above procedure was subsequently eluted in 35 µl of elution buffer (NucliSens®, bioMérieux).

Afterwards 50 µl of deamination solution was combined with the extracted DNA and heated to 90℃ for 10 min to deaminate. The solution was then allowed to cool to room temperature. Finally, the solution was purified using the easyMAG platform (NucliSens®, bioMérieux) and eluted in 25 µl mM KOH (93).

3.3.3. PREAMPLIFICATION

An initial preamplification was performed using the outer primers to expand the small amounts of methylated DNA. SFRP1 was analyzed as part of a panel with 27 additional genes as part of a previous PhD study (109).

A reaction buffer was mixed from the following, comprising 25 µl in total for each sample: PCR buffer, 250 nM of all outer primers (28 genes in total), 0.3 U Cod Uracil-DNA Glycosylase (Cod UNG ArcticZymes®), 0.6 mM dNTP, 13 µM $MgCl_2$, and finally 1.5 U Taq Polymerase (MyTaq™ Bioline®). The reaction buffer was subsequently dispersed to 200 µl PCR tubes. This was followed by incubation of the PCR tubes for five minutes at 37°C, after which they were heated to 95°C for five minutes, and then allowed to cool to room temperature.

Finally, 25 µl of purified deamination solution was added to every tube, followed by 20 rounds of preamplification PCR performed as follows: denaturing at 92°C for 15 seconds, annealing at 55°C for 30 seconds, and extending at 72°C for 30 seconds.

3.3.4. SECOND-ROUND PCR

Subsequently, real-time PCR analysis of the preamplified product was carried out in individual wells for each gene. To prepare each reaction, a buffer mix of 10 µl with 0.4 µM inner probes and primers was dispensed across 30 separate wells in of a PCR plate containing 96-wells. A reaction mix of 710 µl was prepared with PCR buffer, 15 U Taq polymerase (BIOTAQ™, Bioline), 10 µM $MgCl_2$, and finally 250 µM dNTPs.

Afterwards 10 µl of the preamplification product was added to the reaction mix. Subsequently, 20 µl of the reaction mix was distributed to every individual well with probes and primers. Finally, real-time PCR was performed for 45 cycles as follows: denaturing at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, and extending at 72 °C for 30 seconds.

3.3.5. STUDY IV

3.3.5.1 Sample preparation

Dual-strand digital PCR assays were designed using Beacon Designer 8.21 for the previously examined SFRP1 promoter (110–112). A non-CpG-containing region of EPHA3 was used as a reference (113). Primers and probes were manufactured by TAG Copenhagen.

3.3.5.2 DNA isolation and bisulfite treatment

After thawing, the plasma was centrifuged at 12,000 × g for 10 minutes at 4°C. cfDNA was extracted from available plasma (0.5-2 ml plasma) on the QIAsymphony (Qiagen) using the DSP Circulating DNA Kit (Qiagen). Afterward, the isolated cfDNA was eluted using 60 µl of elution buffer followed by storing at -20°C. Samples were stored at -20°C for up to two weeks awaiting further processing. DNA was then evaporated to 20 µl by low-temperature vacuum centrifugation using the SAVANT DNA120 SpeedVac Concentrator. DNA was then bisulfate converted with the EZ-96 DNA Methylation-Direct™ MagPrep kit (Zymo Research) using instructions from the manufacturer. Three different controls were included when performing the bisulfite conversion: 1) a nontemplate control (water), 2) a methylation-positive control (Zymo Research, commercially available in vitro methylated DNA), and 3) two different methylation negative controls (Zymo Research, commercially available in vitro nonmethylated DNA). Subsequently, the converted cfDNA was either analyzed immediately using ddPCR or stored at -20°C for up to two weeks before final analysis.

3.3.5.3 Digital droplet PCR

Next, we analyzed the promoter methylation status of SFRP1 was analyzed with the QX200™ Droplet Digital™ PCR System (ddPCR, Bio-Rad). A ddPCR mixture with a volume of 22 µl was created as follows: 1x ddPCR Supermix for Probes (Bio-Rad), 227 nM of each SFRP1 probe, 1.36 µM of each SFRP1 primer, 284 nM EPHA3 probe, 909 nM of EPHA3 primer (as a reference gene), and bisulfite-converted DNA. Subsequently the AutoDG system (Bio-Rad) was used to create droplets. Afterwards 50 rounds of PCR was performed as follows using the C1000 Touch Thermal Cycler (Bio-Rad): initial heating to 95 °C for 10 minutes followed by 50 cycles of heating to

94 °C for 1 min, cooling to 55°C for 2 min, and heating to 98 °C for 10 min with a ramp rate of 1 °C/s.

3.3.5.4 Data analysis

Fluorescence data for individual droplets was analyzed with the QX Manager software version 1.2 (Bio-Rad). A minimum threshold of 10,000 accepted droplets was set for wells to be analyzed.

The number of copies of methylated target and unmethylated control DNA per well (concentration) was calculated. Naturally, the target gene contained signals from both sense and antisense strands, while the control only included a single strand. A normalized allele fraction was obtained by dividing the SFRP1 concentration by twice the concentration of the EPHA3 (control gene).

3.3.6. ETHICS

The North Denmark Regional Research Ethics Committee approved the current study (N-20130037, ClinicalTrials.gov ID: NCT02079363). All patients provided informed oral and written consent before inclusion in the study. All patients were older than 18 years upon time of inclusion.

3.3.7. STATISTICS

The methylation status was dichotomized as follows in studies I-III: A detectable cycle threshold value in the second round of PCR (within 45 cycles) was interpreted as promoter-hypermethylated SFRP1 (phSFRP1). If there was no detectable cycle threshold within 45 cycles, the methylation status of SFRP1 was interpreted as unmethylated (umSFRP1). In study IV, samples completely without a phSFRP1 allele fraction were interpreted as umSFRP1. Samples with any detectable phSFRP1 allele fraction were interpreted as promoter-hypermethylated SFRP1. An optimal cutoff for samples with a detectable phSFRP1 allele fraction was determined using maximally selected rank statistics (114).

Baseline characteristics are presented as the median and interquartile range (IQR), mean and range, or number and percentages. Differences in baseline characteristics were investigated with either the Kruskal–Wallis test for continuous variables or the Pearson chi-square test for categorical variables.

Survival time was calculated as the time from the pretreatment blood sampling until death from any cause or the end of follow-up. Disease-free survival was calculated as the time from R0 resection until disease recurrence, end of follow-up, or death from any cause. Lastly, the progression-free survival was calculated as the time from the

pretreatment blood sampling until either disease progression, end of follow-up, or death from any cause.

Kaplan–Meier curves were used to visualize survival, supplemented with log-rank tests. In study I, Cox proportional hazards regression was employed to assess survival. In studies II-IV, the proportional hazards assumption was violated; thus, Cox regression was inappropriate. Hence, the pseudo-observation approach was taken using one of two other established association measures: restricted mean survival time (RMST) or absolute risk differences (ARD) (115–117). As the event rate among these patients was so high (due to the short survival time), analysis of differences between groups at various time points from 3-24 months was possible.

The abovementioned analyses quantifies the risk factors and their associations with mortality. These analyses were followed by an evaluation of the predictive performance. Studies II and IV included ROC analysis to evaluate the performance of the models for predicting mortality at different time points. Predictive mortality is measured by the area under the ROC curve (AUC), where an area of 0.5 corresponds to no better than flipping a coin, and an area of 1.0 represents perfect discrimination.

The potential risk factors of age, ECOG performance status (PS), sex, and treatment were included in all studies. CA 19-9 was included in studies II-IV. Additionally, the stage of disease was included in study III.

Initially, univariable models were fitted for SFRP1 as well as the chosen covariates to evaluate the prognostic impact of individual variables. This was followed by analysis in multivariable models, including all predetermined potential covariates.

Statistical tests were considered statistically significant with p values of < 0.05. Furthermore, 95% confidence intervals were calculated where applicable. All statistical analysis was performed in Stata (v. 16 or 17), StataCorp, LLC, TA, USA, or R version 4.2.2., R Foundation for Statistical Computing, Vienna, Austria.

CHAPTER 4. SUMMARY OF RESULTS

Here, the results of the papers or proposed papers are briefly summarized. Please refer to the papers or proposed papers for a complete overview of the results (112,118,119). The results not included in the papers are presented in full.

4.1. PAPER I

4.1.1. DISCOVERY COHORT

4.1.1.1 Baseline characteristics

The discovery cohort included 40 patients in total, 25 of whom were treated with gemcitabine and 15 of whom were treated with BSC. The proportion of patients with phSFRP1 was 48% (19/40).

Patients treated with BSC had a significantly worse performance status. The remaining baseline characteristics are presented in the published article (112).

4.1.1.2 Survival rates

Patients treated with gemcitabine had a longer mOS than those treated with BSC (6.2 months vs. 2.0 months). The mOS of gemcitabine-treated patients with phSFRP1 was substantially shorter than gemcitabine-treated patients with umSFRP1 (4.4 months vs. 11.6 months) (Figure 7). In contrast, the difference in mOS according to SFRP1 methylation status was negligible among patients treated BSC (2.0 months vs. 1.5 months.

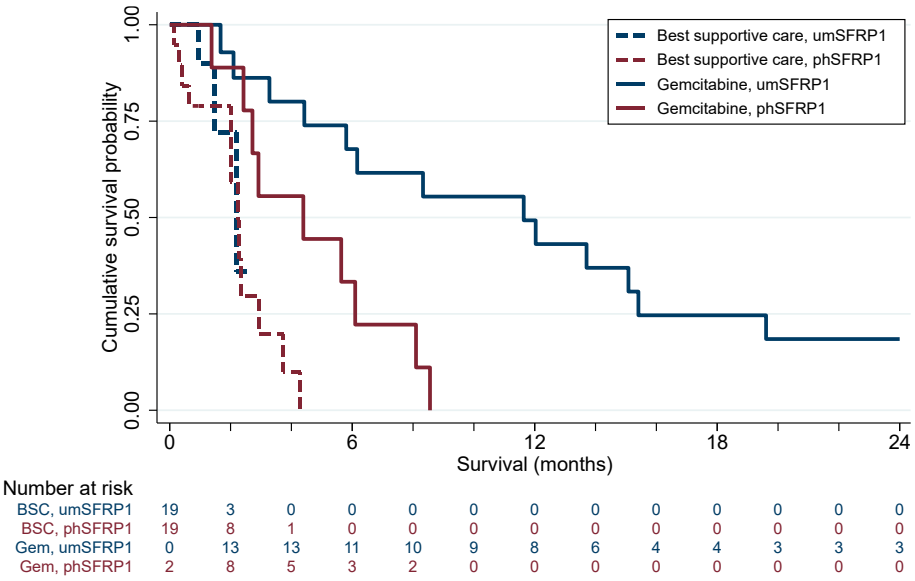

Figure 7. The Kaplan–Meier survival curves from the discovery cohort of study I. Reprinted with permission from Stubbe et al. 2021. DOI: 10.3390/cancers13225717.

4.1.1.3 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), treatment (BSC or gemcitabine), age (age > 65 years or age < 65 years), performance status (0, 1 or 2), and sex (female or male). The following variables were significantly associated with shorter survival in multivariable analysis:

- SFRP1 methylation status
  - phSFRP1 vs. umSFRP1: HR 3.48 (1.39-8.7)
- Treatment
  - Gemcitabine vs. BSC: HR 0.29 (95% CI: 0.09-0.92)
- Performance status
  - PS 1 vs. 0: HR 4.67 (95% CI: 1.80-12.13)
  - PS 2 vs. 0: HR 1.80 (95% CI: 0.65-5.01)
- Sex
  - Female vs. male: HR 2.16 (95% CI: 1.02-4.56)

4.1.2. VALIDATION COHORT

4.1.2.1 Baseline characteristics

The validation cohort included 58 patients with stage IV PDAC treated with gemcitabine. The proportion of patients with phSFRP1 was 50% (28/56).

Patients with phSFRP1 were significantly more likely to have liver metastasis than patients with umSFRP1. The remaining baseline characteristics are presented in the published article (112).

4.1.2.2 Survival rates

The mOS of patients with phSFRP1 was shorter than patients with umSFRP1 (3.2 months vs. 6.3 months) (Figure 8).

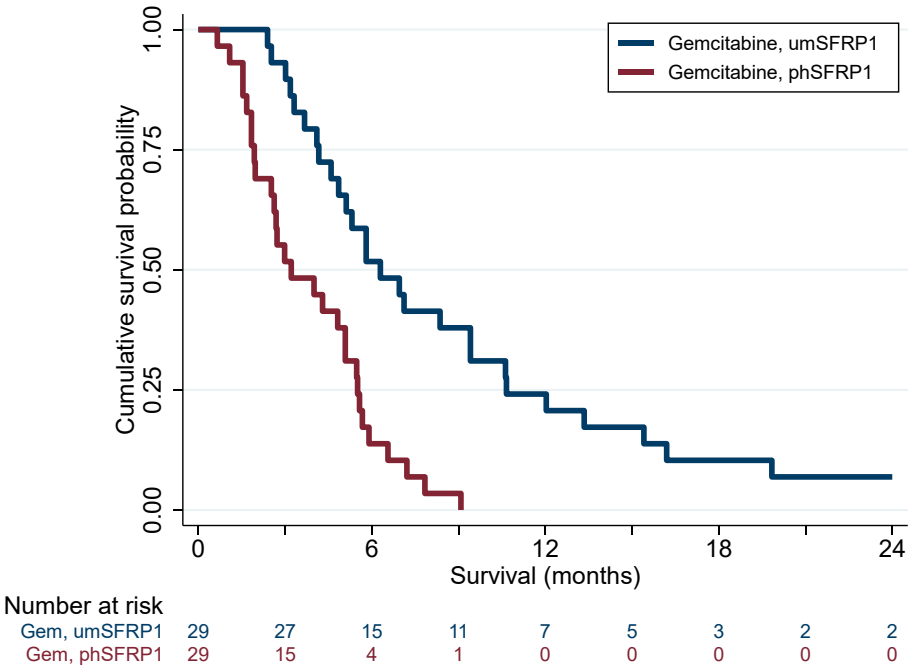

Figure 8. The Kaplan–Meier survival curves for the validation cohort of study I. Reprinted with permission from Stubbe et al. 2021. DOI: 10.3390/cancers13225717.

4.1.2.3 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), age (age > 65 years or age < 65 years), performance status (0, 1 or 2), and sex (female or male). The following variables were significantly associated with shorter survival in multivariable analysis:

- SFRP1 methylation status
    - phSFRP1 vs. umSFRP1: HR of 3.53 (95% CI: 1.85-6.74)

4.2. STUDY II

4.2.1. STAGE IV PATIENTS

4.2.1.1 Baseline characteristics

Fifty-two FOLFIRINOX-treated patients were included in study II. The proportion of patients with phSFRP1 was 44% (23/52).

There were no significant differences in the distribution of baseline characteristics (118).

4.2.1.2 Survival

Patients with phSFRP1 had a shorter mOS compared to patients with umSFRP1 (6.8 months vs. 15.7 months) (Figure 9A). Additionally, the 2-year survival among patients with phSFRP1 was only 4%, compared to 24% among patients with umSFRP1.

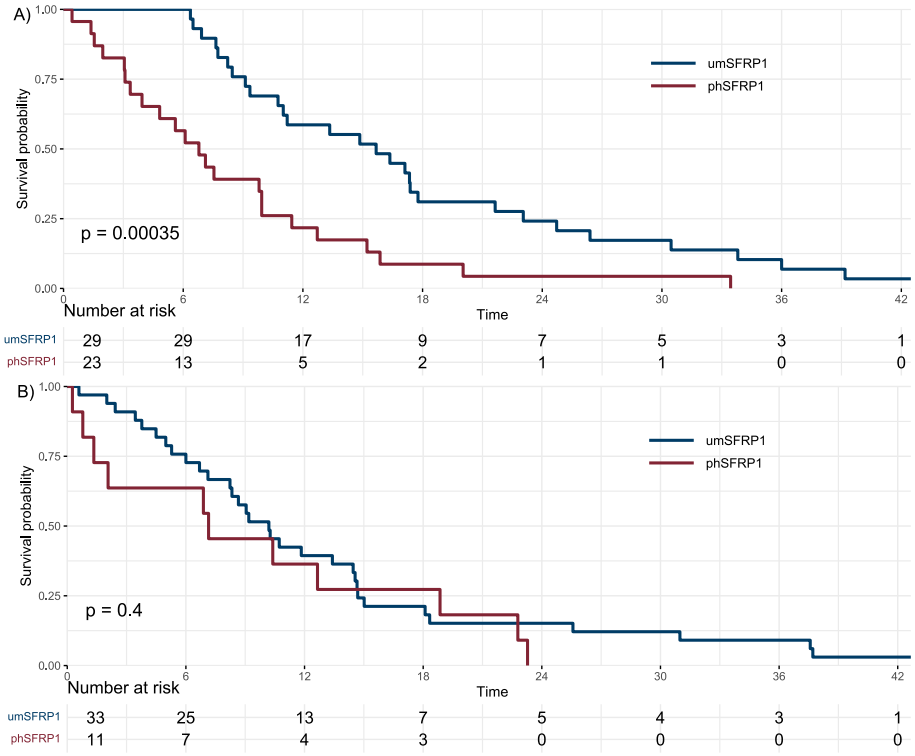

Figure 9. The Kaplan–Meier survival curves from study II. A) PDAC patients of stage IV treated with FOLFIRINOX. B) PDAC patients of stage III. Reprinted with permission from Stubbe et al. 2023, DOI: 10.1016/j.pan.2023.05.003.

4.2.1.3 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), age (age > 65 years or age < 65 years), performance status (0 or 1), sex (female or male), and CA 19-9 (above 860 or below 860). In the multivariable analysis, the following variables were associated with an increased absolute risk of death:

At 12 months:

- SFRP1 methylation status
    - phSFRP1 vs. umSFRP1: Absolute risk difference (ARD) of 42.3 (95% CI: 18.1-66.6)

At 24 months

- SFRP1 methylation status

- phSFRP1 vs. umSFRP1: ARD of 18.2 (95% CI: 0.5-35.8)

4.2.1.4 Predictive analysis

Adding SFRP1 methylation status to models containing the clinical variables age, ECOG PS, and sex led to substantial increases in predictive performance (AUC: 0.77-0.78) compared to the addition of CA 19-9 (0.66-0.73).

4.2.2. STAGE III PATIENTS

4.2.2.1 Baseline characteristics

Study II included 44 patients with locally advanced PDAC. The proportion of patients with phSFRP1 was 25% (11/44). Patients with phSFRP1 were more likely to receive only best supportive care. No statistically significant differences were detected between the remaining characteristics (118).

4.2.2.2 Survival

The mOS was not significantly shorter among patients with phSFRP1 compared to umSFRP1 (7.4 months vs. 10.2 months) (Figure 9B).

4.2.2.3 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), treatment (BSC, gemcitabine, or FOLFIRINOX), age (age > 65 years or age < 65 years), performance status (0, 1 or 2), sex (female or male), and CA 19-9 (above or below 860). In the multivariable analysis, the following variables were significantly associated with an increased absolute risk of death:

At 12 months:

- Treatment
  - Gemcitabine vs. BSC: ARD of -44.8% (95% CI: -73.9, -15.7)
  - FOLFIRINOX vs. BSC: ARD of -69.7% (95% CI: -108.8, -30.7)

At 24 months:

No significant associations were detected.

4.3. STUDY III

4.3.1. BASELINE CHARACTERISTICS

Study III included 211 patients with localized PDAC (stage I or II). The proportion of patients with phSFRP1 was 20% (43/211).

There was a significant difference in the following variables:

- Stage
- Type of chemotherapy

4.3.2. SURVIVAL

The mOS was significantly shorter among patients with phSFRP1 compared to patients with umSFRP1 (13.1 months vs. 19.6 months) (Figure 10).

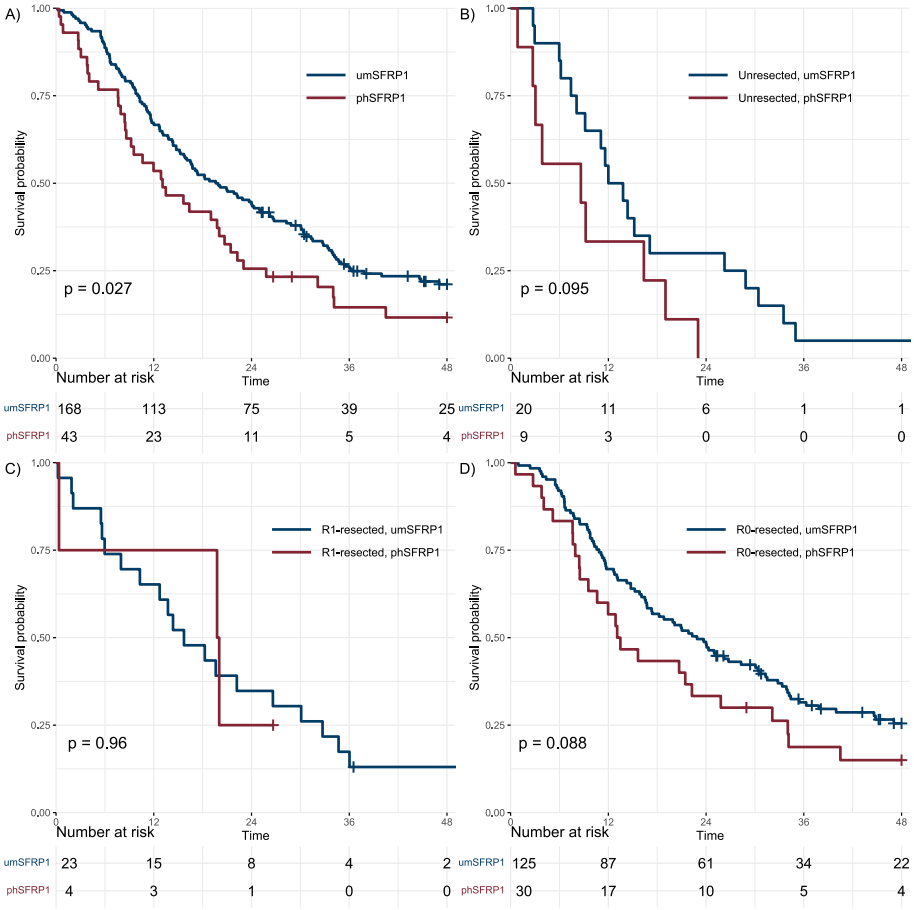

Figure 10. The Kaplan–Meier survival curves from study III. Stage I-II PDAC patients. Reprinted with permission from Stubbe et al. 2023, DOI: 10.3389/fonc.2023.1211292

4.3.2.1 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), treatment (adjuvant chemotherapy or not), resection status (R0-resected, R1-resected or unresected), performance status (PS > 1 or PS ≤ 1), stage (stage I or stage II), age (age > 65 years or age < 65 years), sex (female or male), and CA 19-9 (above 167 or below 167). The following variables were significantly associated with reduced restricted mean survival time (RMST) in the multivariable models:

At 12 months

- SFRP1 methylation status

    - phSFRP1 vs. umSFRP1: RMST -1.2 months (95% CI: -2.1, -0.2)
- Treatment
    - Adjuvant chemotherapy vs. no chemotherapy: RMST 2.5 months (95% CI: 1.2, 3.8)
- Performance status
    - PS > 1 vs. PS ≤ 1: RMST -2.3 months (95% CI: -3.9, -0.7)
- CA 19-9
    - CA 19-9 > 167 vs. CA 19-9 < 167: RMST -1.1 months (95% CI: -1.8, -0.3)

At 24 months

- SFRP1 methylation status
    - phSFRP1 vs. umSFRP1: RMST 2.7 months (95% CI: -5.0, -0.5)
- Treatment
    - Adjuvant chemotherapy vs. no chemotherapy: RMST 4.9 months (95% CI: 2.2, 7.6)
- Resection status
    - R0-resection vs. no resection: RMST 3.7 months (95% CI: 0.8, 6.6)
- Performance status
    - PS > 1 vs. PS ≤ 1: RMST -5.8 months (95% CI: -9.2, -2.5)
- Stage
    - Stage II vs. stage I: RMST -3.3 months (95% CI: -6.3, -0.3)
- Ca 19-9
    - CA 19-9 > 167 vs. CA 19-9 < 167: RMST -4.0 months (95% CI: -6.0, -2.1)

4.3.2.2 Serum vs. plasma

The results from the first two studies indicated that the target material in serum was at sufficient levels to be detectable. Indeed, in Study I, the proportion of patients with detectable phSFRP1 was approximately the same in the discovery (plasma samples) and validation (serum samples) cohorts. Likewise, the hazard ratio was approximately equal in the multivariable analysis. However, in retrospect, this may be different in patients of lower stages, as cell death in cancer tissue is less frequent. Thus, the dilution associated with serum samples may cause more sampling issues than in stage IV patients, where ctDNA is abundant. As such, here follows additional analyses.

In patients with serum samples (n = 171), the proportion of patients with phSFRP1 was 16% (27/171). Comparatively, in patients with plasma samples (n = 40), the proportion of patients with phSFRP1 was 40% (16/40).

Likewise, performing univariate regression analyses exclusively on the 171 patients with serum samples revealed a loss of life of 0.7 (-2.17, 0.75) months at 12 months

and 2.4 (-5.67, 0.87) months at 24 months. In contrast, in the 40 patients with plasma samples, phSFRP1 was associated with a loss of life of 3.2 (-5.29, -1.06) months at 12 months and 5.3 (-10.35, -0.28) months at 24 months.

4.3.3. DISEASE-FREE SURVIVAL

There were no significant differences in median disease-free survival in patients with phSFRP1 compared to umSFRP1 (12.2 months vs. 12.9 months).

4.3.3.1 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), treatment (adjuvant chemotherapy or not), performance status (PS > 1 or PS ≤ 1), stage (stage I or stage II), age (age > 65 years or age < 65 years), sex (female or male), and CA 19-9 (above 167 or below 167). The following variables were significantly associated with reduced RMST in multivariable models:

At 12 months

- Stage of disease
  - Stage II vs. I: RMST -1.5 months (95% CI: -2.4, -0.6)
- CA 19-9
  - CA 19-9 > 167 vs. CA 19-9 < 167: RMST -3.0 months (95% CI: -4.0, -2.0)

At 24 months

- Stage of disease
  - Stage II vs. I: RMST -7.1 months (95% CI: -9.6, -4.6)
- CA 19-9
  - CA 19-9 > 167 vs. CA 19-9 < 167: RMST -6.7 months (95% CI: -9.2, -4.2)

4.3.4. PROGRESSION-FREE SURVIVAL

There were no significant differences in median PFS among patients with phSFRP1 compared to umSFRP1 (3.9 months vs. 9.0 months).

4.3.4.1 Multivariable regression analysis

The multivariable model included the variables SFRP1 methylation status (phSFRP1 or umSFRP1), treatment (adjuvant chemotherapy or not), resection status (R0-resected, R1-resected or unresected), performance status (PS > 1 or PS ≤ 1), stage

(stage I or stage II), age (age > 65 years or age < 65 years), sex (female or male), and CA 19-9 (above 167 or below 167).

No variables were significantly associated with reduced RMST in multivariable models at either 12 or 24 months.

4.4. STUDY IV

4.4.1. BASELINE CHARACTERISTICS

Study IV included 354 patients with stage IV PDAC. phSFRP1 was detectable in 217 patients, and undetectable in 137 patients (umSFRP1). Conversely, 143 patients had a high phSFRP1 allele fraction (phSFRP1^{high}), and 74 had a low phSFRP1 allele fraction (phSFRP1^{low}).

Significant differences were found between the following variables:

- Age
    - Patients with phSFRP1^{high} were significantly younger (median 66 years) than patients with umSFRP1 or phSFRP1^{low} (both median 71 years).
- CA 19-9
    - Patients with phSFRP1^{high} had significantly higher levels of CA 19-9 (median 8170) than patients with umSFRP1 or phSFRP1^{low} (median 695 and 2700, respectively).
- Performance status
    - Patients with phSFRP1^{high} had a significantly worse performance status compared to patients with umSFRP1 or phSFRP1^{low}.
- Location of the primary tumor
    - Patients with phSFRP1^{high} tumors were more likely to have a caudally located tumor than patients with umSFRP1 or phSFRP1^{low} tumors.
- Location of metastasis
    - Patients with phSFRP1 were significantly more likely to have liver metastasis than patients with umSFRP1 or phSFRP1^{low}.

4.4.2. SURVIVAL

The mOS of patients with any detectable phSFRP1 allele fraction was significantly shorter compared to patients with umSFRP1 (4.2 months vs 9.1 months) (Figure 11A). Stratifying patients with detectable phSFRP1 into low or high allele fraction revealed the mOS of patients with phSFRP1^{high} to be 3.4 months and 7.2 months in patients in phSFRP1^{low}. The 3-month mortality risk was approximately equal between patients with umSFRP1 and phSFRP1^{low}.

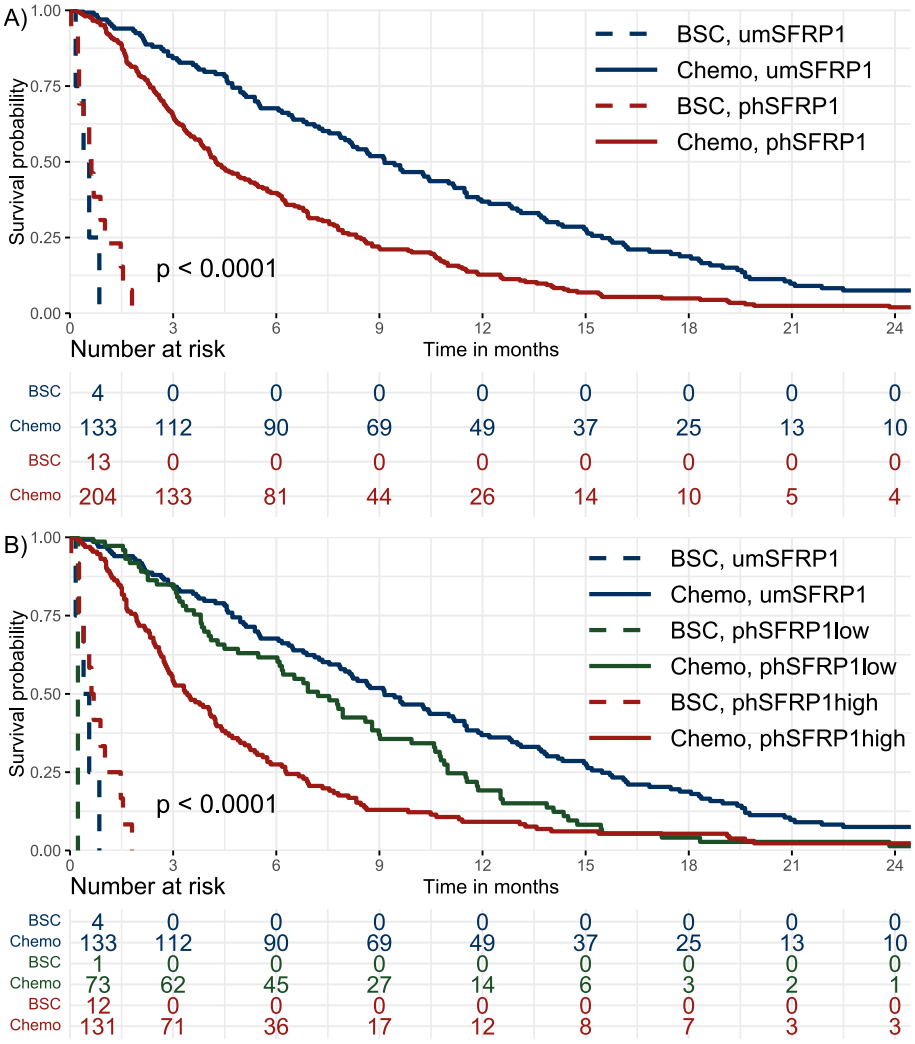

Figure 11. The Kaplan–Meier survival curves from study IV. Patients with stage IV PDAC were grouped according to the SFRP1 methylation status. A) Kaplan–Meier curve with a dichotomized analysis of SFRP1 methylation status. umSFRP1, unmethylated SFRP1; phSFRP1, any detectable phSFRP1 allele fraction. B) Kaplan–Meier curve with SFRP1 methylation status grouped into three levels according to phSFRP1 allele fraction. $phSFRP1^{low}$, phSFRP1 allele fraction < 0.53%; $phSFRP1^{high}$, phSFRP1 allele fraction > 0.53%.

4.4.2.1 Multivariable regression analysis

The multivariable models includes all risk factors. The following variables were associated with an increased absolute risk of death. A complete overview of the models is presented in Figure 12.

3 months

- SFRP1 methylation status
  - phSFRP1^{high} vs. umSFRP1: ARD 26.4% (95% CI: 15.6, 37.2)
- Performance status
  - PS > 1 vs. PS 0-1: ARD 23.4% (95% CI: 9.2, 37.5)
- Age
  - Age > 65 vs. age < 65: ARD 11.1% (95% CI: 1.5, 20.7)
- Treatment
  - BSC vs. gemcitabine: ARD 50.9 (95% CI: 36.4, 65.4)
  - Gem/Cap vs. gemcitabine: ARD -18.8% (95% CI: -38.9, -6.3)

6 months

- SFRP1 methylation status
  - phSFRP1^{high} vs. umSFRP1: ARD 37.7% (95% CI: 26.5, 49.0)
- Performance status
  - PS > 1 vs. PS 0-1: ARD 18.0% (95% CI: 5.3, 30.6)
- Age
  - Age > 65 vs. age < 65: ARD 15.5% (95% CI: 4.8, 26.1)
- Treatment
  - BSC vs. gemcitabine: ARD 21.2 (95% CI: 6.5, 36.0)
  - Gem/Nab vs. gemcitabine: ARD -18.4% (95% CI: -30.1, -6.7)
  - Gem/Nab/Toci vs. gemcitabine: ARD -26.7% (95% CI: -45.0, -8.5)

12 months

- SFRP1 methylation status
  - phSFRP1^{high} vs. umSFRP1: ARD 22.6% (95% CI: 13.0, 32.3)
  - phSFRP1^{low} vs. umSFRP1: ARD 13.2% (95% CI: 1.5, 24.9)
- Performance status
  - PS > 1 vs. PS 0-1: ARD 10.6% (95% CI: 3.3, 17.9)
- CA 19-9
  - CA 19-9 > 860 vs. CA 19-9 < 860: ARD 10.3% (95% CI: 1.1, 19.5)
- Treatment
  - Gem/Nab/Toci vs. gemcitabine: ARD -24.0% (95% CI: -45.5, -2.6)
  - FOLFIRINOX vs. gemcitabine: ARD -19.2% (95% CI: -35.0, -3.5)

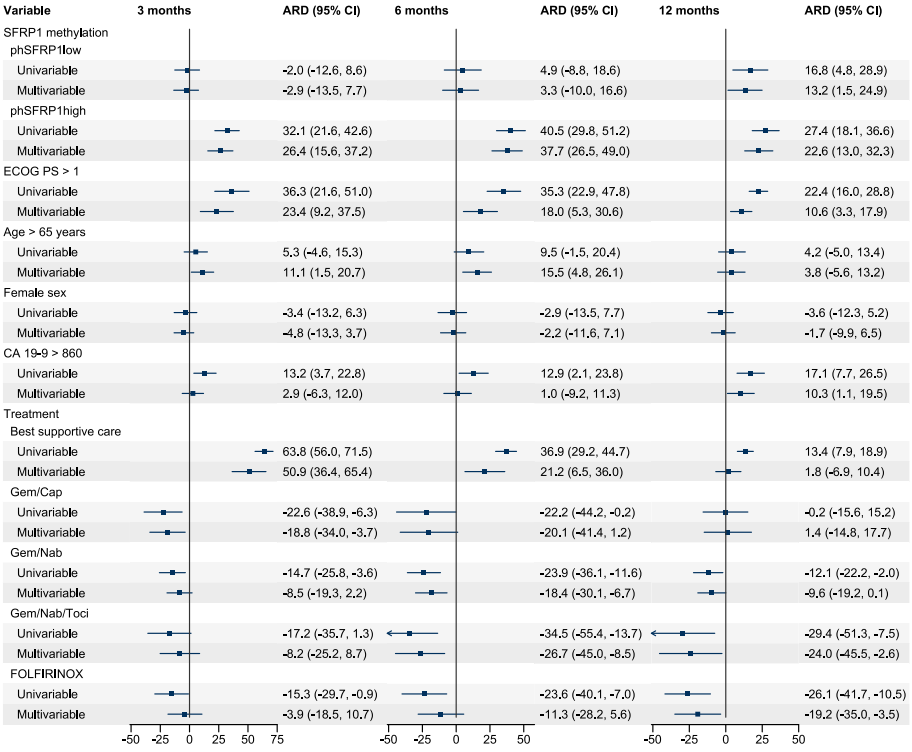

Figure 12. Crude and adjusted absolute risk differences for patients in study IV. Patients with stage IV PDAC were treated with BSC or gemcitabine, gemcitabine and capecitabine (Gem/Cap), gemcitabine and nab-paclitaxel (Gem/Nab), FOLFIRINOX or gemcitabine, nab-paclitaxel, and tocilizumab. Analyses were performed based on both univariable and multivariable models containing all variables at 3, 6, and 12 months. phSFRP1^{low}, phSFRP1 AF below 0.53%; phSFRP1^{high}, phSFRP1 AF > 0.53%.

4.4.2.2 Predictive analysis

ROC curves were computed with 10-fold cross-validation to evaluate whether the addition of SFRP1 methylation status improved predictive performance (Figure 13).

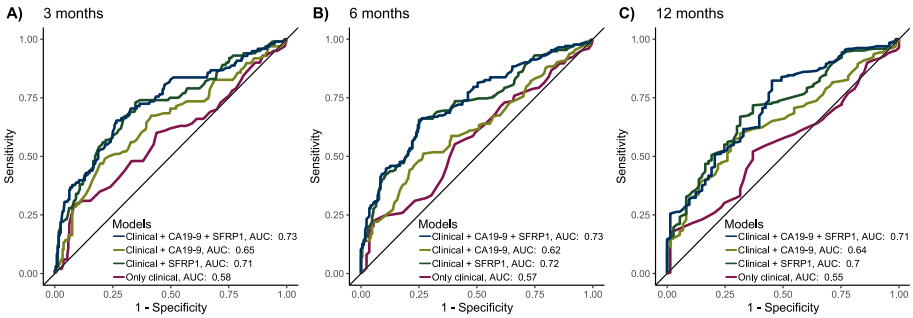

Figure 13. Performance of models in predicting 3-, 6-, and 12-month mortality. The models included the following variables: ***Only clinical****, the clinical variables age > 65, sex and ECOG PS;* ***Clinical + CA19-9****, the clinical variables as well as CA19-9 > 860;* ***Clinical + SFRP1****, the clinical variables as well as the SFRP1 methylation status (umSFRP1, phSFRP1^{low} or phSFRP1^{high});* ***Clinical + CA19-9 + SFRP1****, clinical variables as well as CA19-9 and the SFRP1 methylation status.*

Adding the SFRP1 methylation status to the model including only clinical variables significantly improved the predictive performance at all time points (AUC 0.7-0.72). The addition of CA19-9 to clinical variables increased the predictive power moderately at 3 and 12 months but had little effect at the 6-month point (AUC: 0.62-0.65).

The models that included the SFRP1 methylation status, CA19-9, and clinical variables had the highest predictive power at all time points (AUC: 0.71-0.73).

CHAPTER 5. DISCUSSION

5.1. MAIN FINDINGS

Throughout this thesis, we evaluated the prognostic performance of a cfDNA-based analysis of promoter hypermethylation of SFRP1 in patients with PDAC. Survival among patients with PDAC is extremely poor, with relatively modest improvements over the past decades. There is a need for improved prognostic tools, as the current prognostic tools are heavily limited. Here, we demonstrated that in patients with metastatic PDAC, phSFRP1 confers a substantially poorer prognosis than umSFRP1. In these patients, phSFRP1 either rivals or is a stronger prognostic factor than the best current prognostic factors (PS, CA 19-9).

Furthermore, this biomarker is present in more than half of the population with metastatic PDAC. Together, these findings indicate that the analysis of phSFRP1 could have a great degree of clinical utility if routinely used as an additional prognostic biomarker in patients with metastatic PDAC. As this biomarker only requires a blood sample and no specialized equipment, it could be implemented relatively quickly and cheaply in clinical practice. The results were less conclusive in patients with localized or locally advanced disease. While phSFRP1 was linked to shorter survival compared to umSFRP1 in patients with stage I-II disease, the effect sizes were substantially smaller than those in metastatic disease. Further research is required to ascertain whether the prognostic impact is strong enough to warrant a systematic analysis of the SFRP1 methylation status in lower-stage patients.

The following sections discuss the studies, their methodologies, and their results in more detail.

5.2. LIMITATIONS

While the results presented in this thesis are promising, the studies have limitations. First, the studies were performed retrospectively, which generally imposes a risk of selection bias. However, registration of all patients and data was performed prospectively. Furthermore, all samples were analyzed blinded to clinical data. The only selection was due to analytical malfunctions or patients who were not chemotherapy-naïve. In combination, these factors limit the risk of selection bias.

A second limitation is the inherent reliance of liquid biomarkers on the release of a sufficient amount of DNA from the tumor to be detectable. While the sensitivity of the analysis methods is high, they require successful sampling of DNA fragments for detection. Sampling is unlikely an issue in metastatic disease, as most metastatic tumors release ctDNA in sufficient quantities to be reliably detectable with relatively

sparse amounts of material. However, lower-stage tumors are associated with substantially less shedding of DNA. In studies I-IV, 1 ml of either plasma or serum was analyzed. Based on the results from this thesis, this appears sufficient to ensure detection for metastatic disease. Data from study III indicate that phSFRP1 is also a poor prognostic factor when detected in lower-stage PDAC. However, the detection frequency of phSFRP1 was much lower than what was witnessed in metastatic disease. When considering that phSFRP1 is likely an early event in PDAC, the low detected frequency in lower-stage disease may not reliably reflect the tumor landscape. This could indicate that 1 ml of material is insufficient to ensure detection in lower stages, but it may be solved by analyzing more material. Other studies have analyzed 4 ml or even as much as 8 ml (120).

A third possible limitation is the methylation analysis. Both primers in the nested qPCR (studies I-III) were designed to be methylation-specific. This approach was based on several considerations but involves some risk of introducing a bias through isolated amplification. Another approach could be to amplify independently from the methylation status of the target. However, the amount of unmethylated cfDNA in the blood is much greater than that of methylated cfDNA. The dual methylation-specific primers were chosen based on a concern that amplifying all DNA would completely drown out the few copies of methylated cfDNA. A further limitation of the qPCR-based methodology is that the nested PCR setup likely impairs some of the quantitative aspects of the technique. The two primary goals of moving to the ddPCR-based approach were to 1) be able to forgo the preamplification step to obtain a complete quantification and remove the risk of introducing bias and 2) be able to receive an allele fraction of phSFRP1. Other sequencing techniques were also considered. For example, pyrosequencing could confer information regarding individual CpG sites. However, pyrosequencing is associated with a significantly higher limit of detection of approximately 5%, compared to 0.005% for ddPCR (121,122).

A further limitation was the available material, as only serum was available in some cases. While ctDNA is detectable in both plasma and serum, plasma is generally considered to be the best choice (80). In serum samples, wild-type cfDNA can be released by leukocytes during the clotting process. This release can lead to a dilution of plasma ctDNA in serum (123,124). Additionally, there is evidence of higher interpatient variation in serum samples (125). Unfortunately, only serum was available in the validation cohort of study I, the majority of patients in study II, and in the subgroup analysis of study III. In both the discovery cohort (plasma) and the validation cohort (serum) of study I, phSFRP1 had a significant and approximately equal effect on survival.

Furthermore, the proportion of patients with detectable phSFRP1 was approximately equal (53% vs. 50%), indicating that sufficient DNA was present in the serum of metastatic PDAC to detect phSFRP1 reliably. However, the use of serum may limit

detection in patients with lower-stage disease, which is prone releaseing of less ctDNA. This could lead to a bias toward no difference.

Furthermore, the Danish follow-up program limited the estimation of disease-free and progression-free survival. During the study period, patients received CT scans only upon when recurrence was suspected and not as part of regular screening. Regular screening may have caught recurrence slightly earlier, causing slight changes in PFS and DFS.

In general, misspecification of outcomes could lead to bias, but in the present studies, all outcomes (being times of death) were correctly registered within the timeframes of the studies.

5.3. STRENGTHS

A primary strength of the studies in this thesis is the well-defined cohorts. All patients were consecutively and prospectively included into the respective studies, which minimizes the risk of selection bias and increases the generalizability of the findings, as all eligible patients were included. Furthermore, follow-up was carried out consistently, and all clinical data were entered prospectively, ensuring accurate and reliable data. The follow-up period was long, and there was zero censoring at the time points of interest in the studies. This increases the validity of survival analyses and reduces the risk of bias, as almost all patients' outcomes were accurately observed and accounted for in statistical analyses. Additionally, all methylation analyses were performed blinded to clinical data and patient identity, reducing the risk of unintentional bias.

Moreover, liquid biopsy has several advantages in contrast with the more traditional tissue biopsy. Liquid biopsies reduce the risks of complications and discomfort by being only minimally invasive. Furthermore, this could facilitate more frequent monitoring of patients at no additional risk.

Prognostic biomarkers for PDAC are currently heavily limited. In the studies of this thesis, we proposed and thoroughly validated a promising new blood-based prognostic biomarker in external cohorts. Furthermore, results of study IV indicate that the biomarker depends on the level of methylated DNA. This stratification could substantially improve the prognostic accuracy.

5.4. ANALYTICAL CONSIDERATIONS

In studies I-III the methylation analysis was based on an optimized qPCR-based methodology, which allowed for high recovery of methylated cfDNA for its time. The

methylation analysis in study IV was based on the improved ddPCR methodology. However, the implementation of the ddPCR analysis was not entirely straightforward. Several considerations were made for the combination of probes for ddPCR analysis. At the time of sample analysis, the laboratory was limited to a PCR machine with two colors.

Several combinations of probes were tested and considered:

- Methylated sense SFRP1 | unmethylated sense SFRP1
    - Optimally, an unmethylated SFRP1 probe would function as a control. This would allow for a precise and reliable determination of the phSFRP1 allele fraction. Unfortunately, the unmethylated SFRP1 probe proved unreliable. Therefore, this combination was discarded.
- Methylated sense SFRP1 + methylated antisense SFRP1 | unmethylated sense SFRP1 + unmethylated antisense SFRP1
    - This approach was to run both sense and antisense probes in the same color, effectively doubling the captured DNA. However, although the sensitivity was improved, the unmethylated probe was still unreliable. Thus, this approach was discarded for a third approach.
- Methylated sense SFRP1 + methylated antisense SFRP1 | EPHA3
    - This approach was also applied to optimize the capture of methylated target DNA by running both sense and antisense probes in the same color. However, here, a known unmethylated gene (EPHA3) was chosen as a reference gene. EPHA3 was known in the laboratory to be reliable and previously demonstrated in the literature to be a stable reference gene. This combination was chosen for the analysis.

The addition of a phSFRP1 allele fraction was a substantial benefit of the ddPCR methodology, allowing for further risk stratification of patients. However, while there was an additional benefit to stratifying patients based on phSFRP1 AF, the prognostic impact of a dichotomized analysis was still strong. Thus, a dichotomized analysis using qPCR is still a valid option, which might be considered in poorer regions where ddPCR may be unavailable. As a consequence, the analysis can also be performed in lower-income countries.

5.5. STATISTICAL CONSIDERATIONS

Analysis of survival with the Cox model is an often-used statistical approach for investigating the association between survival and one or more variables within a prespecified timeframe. Supplemented with illustrations of Kaplan–Meier curves, this constitutes the most common way to perform survival analysis. The Cox model

allows for the consideration of multiple predictors and handles censoring well. However, the hazard ratio is inherently counterintuitive.

Additionally, the Cox model requires several assumptions to be met. The first is proportional hazards. Specifically, this requires the HR to be constant during the timeframe of interest. However, the effect of exposure often wears off over time, violating the proportional hazards assumption, which was the case in studies II-IV. As further discussed by Hernán, the Cox model also has several other disadvantages (126).

The second assumption is independent censoring, which is also assumed by the Kaplan–Meier plot. Specifically, this means that censored patients should have the same hazard as those who remain. In all four studies, censoring was absent during the timeframe of interest, i.e., the status of all patients was known upon their exit from the study.

While methodologies exist to accommodate the Cox model to these violations, quantifying comparisons between groups becomes cumbersome and increasingly counterintuitive. Alternatively, other measures of association have been introduced, building upon so-called pseudo-observations (115–117). These are the absolute risk difference (ARD) in mortality and restricted mean survival time (RMST). Pseudo-observations only assume independent censoring. In our datasets, this assumption was not violated. ARD has the advantage of being easy to interpret, directly comparing absolute mortality at different time points. However, it requires time points to be prespecified. By choosing 3, 6, 12, and 24 months, we gained insight into when the association is the strongest and when it wears off.

RMST is defined as the average survival time from time 0 to a prespecified time point. Additionally, while less well known, RMST is also easy to interpret, as it provides an absolute difference in survival time according to the predictors. Like ARD, RMST also requires a prespecified time point, which can influence the results. Both methods are suitable and well-established methods for survival analysis, and their use depends on preference.

5.6. COMPARISON TO LITERATURE

Several clinical, histological, and molecular factors are prognostically relevant for patients with PDAC (127). While the results of this thesis are promising, SFRP1 is not the only prognostic factor to consider. The current results indicate that phSFRP1 provides additional information to that of known prognostic factors, such as age, PS, and CA 19-9. The proposed biomarker could be used in conjunction with current known prognostic factors, providing additional value. However, there may be further value in combining it with other biomarkers.

5.6.1. CLINICAL FACTORS

5.6.1.1 Age

Age in geis a significant prognostic factor in life, cancer in general, and, unsurprisingly, also PDAC (128). The papers of this thesis have included age in statistical analyses with a cutoff of 65 years. This particular cutoff is often used in clinical and epidemiological studies. However, it is not obvious whether it is the optimal choice in the current scenario. The median age of onset of PDAC is relatively old at approximately 70 years of age. Thus, an argument could be made for considering other cutoffs, potentially the average age of onset. Other possibilities could be to include age as either a continuous variable or use multiple age categories (e.g., >50, 50-64, 65-79, 80+). This would improve risk stratification and reduce residual confounding in multivariable analysis but substantially impact the ease of interpretation.

Although age is a known prognostic factor, it was not the main focus of these studies. Raising the cutoff would naturally move more "elderly" and potentially frail patients into the "younger" group. We considered it at least as interesting to determine whether the prognosis is *better* in the youngest patients rather than worse in the eldest population. For this reason, it was deemed an appropriate middle ground to keep the cutoff at 65 years.

However, while not shown, the different approaches outlined above have all been examined statistically and demonstrated no discernible differences in the effects of age as a prognostic factor or on the effects of phSFRP1 in multivariable models.

Overall, across studies I-IV, age had only sparse impact on survival. Interestingly however, in study IV patients with phSFRP1^{high} had a median age five years lower than those with phSFRP1^{low} or umSFRP1. Despite their younger age, they had a substantially worse prognosis, which could indicate a more aggressive tumor subtype among the younger patients.

5.6.1.2 Performance status

The ECOG performance status (PS) is a tool for estimating a patient’s ability to perform certain activities in their daily life without assistance. It is a widely used prognostic tool and one of the most essential for choosing potential treatments (129,130). Generally, patients of good PS (generally PS 0-1) can benefit from combination chemotherapy, while patients with scores > 2 or with numerous comorbidities can only tolerate single-agent chemotherapy. Patients of poor PS do not appear to benefit of combination chemotherapy (131). Poor PS is often a major factor in deciding the optimal treatment for the patient, or indeed, an opting-out of treatment.

The survival among patients who receive only best supportive care is 1.1-2.9 months (45,112,132–134).

In some subgroups of studies I-IV, patients with phSFRP1 had significantly worse PS or a tendency toward worse PS. For example, in study IV, the patients with phSFRP1^{high} had a significantly worse PS in contrast to patients with umSFRP1 or phSFRP1^{low} despite being significantly younger. A critical hypothesis to rule out is whether the effects of phSFRP1 could be explained simply by their poorer PS. However, this does not appear to be the case. Across all four studies, the effect size and significance of phSFRP1 on survival was relatively unimpacted when adjusting for PS in multivariable analysis.

Additionally, phSFRP1 significantly improved performance in predicting mortality compared to models with only clinical factors. This indicates that the effects of phSFRP1 cannot merely be explained as a side effect of having a worse performance status. In contrast, this could indicate that phSFRP1 tumors tend to be more aggressive, thus inferring a worse prognosis.

5.6.1.3 Sex

Some literature suggests differences in the mortality of PDAC according to sex (135). For this reason, the variable was included in our multivariable analysis. In our results, sex did not appear to significantly impact survival.

5.6.2. BIOMARKERS

5.6.2.1 CA 19-9

The most commonly used biomarker for PDAC is serum carbohydrate 19-9 (CA 19-9). Higher levels of CA 19-9 are linked to a worse prognosis. Likewise, decreases in CA19-9 levels following chemotherapy or surgery correlate with a better prognosis (136). However, the utility is limited by the fact that a proportion of the Caucasian population (5-10%) completely lack expression (137). Furthermore, the marker is not cancer-specific. A value higher than 37 is considered elevated in healthy individuals, and a value larger than 37 in resected patients is a poor prognostic factor. However, CA 19-9 in metastatic disease is almost universally elevated to much higher degrees. Thus, several cutoffs for clinical relevance have been suggested, but currently, a consensus has yet to be reached. Thus, cutoffs based on the median were established in studies II and III according to either metastatic or localized disease.

5.6.2.2 KRAS Mutations

KRAS mutations are the most common alteration in PDAC, present in almost all cases (~90%) (50). However, in contrast to its almost ubiquitous presence in tissue, the

presence of mutant KRAS in cfDNA varies greatly (26%-73% of cases) (138). The presence of mutant KRAS in cfDNA has been linked to shorter survival compared to patients without (139). Additionally, detection of mutant KRAS in cfDNA both before and after surgery has been linked to increased risk of recurrence as well as a shorter overall survival (140).

5.6.2.3 SMAD4

Similar to KRAS, the SMAD4 signal transduction protein is also a commonly inactivated TSG in PDAC (63). SMAD4 deletion is associated with an increased mortality in patients with PDAC (141). Additionally, loss of SMAD4 has been linked to resistance to radiotherapy (142). Interestingly, however, a loss of SMAD4 has also been associated with higher sensitivity to chemotherapies targeting the cell cycle (143).

5.6.2.4 microRNAs

MicroRNAs (miRNAs) are pieces of noncoding RNAs which act as gene regulators by interacting with mRNAs (144). miRNAs are measurable in most body fluids, including serum, urine, breast milk, peritoneal fluid, and saliva (144,145). PDAC tissue has been shown to have a distinctive miRNA expression profile compared to normal pancreatic tissue or chronic pancreatitis tissue (146). miR-196a-2 has been linked to higher mortality in PDAC patients (146). Additionally, a combination of six miRNAs was able to differentiate patients with lymph node metastatic PDAC into either long-term survivors or short-term survivors (146).

5.6.2.5 Homologous recombination deficiency

A few subgroups of PDAC respond better to certain therapeutics. This includes PDAC with mutations in either of the genes responsible for homologous recombination (PALB2, BRCA1, or BRCA2). Approximately 6% of PDAC cases have this type of deficiency (147). These genes are responsible for repairing double-stranded breaks of DNA, and thus, mutations in these genes results in an increased sensitivity to therapies that induce them, such as platinum-based therapies (147). A progression-free survival benefit has been reported in patients with deficient homologous repair (10.1 months vs. 6.9 months in controls) (147).

Furthermore, the development of poly ADP-ribose polymerase (PARP) inhibitors has indicated that the repair defect is exploitable in other ways. These PARP inhibitors also cause DNA lesions which are repaired by homologous recombination under normal conditions (148). PARP inhibitors have been associated with longer progression-free survival in BRCA1- or 2-positive patients with metastatic PDAC compared to placebo (7.4 months vs. 3.8 months) (149). However, no impact on overall survival was detected.

5.6.2.6 Mismatch Repair Genes and Microsatellite Instability

The function of the mismatch repair proteins (MMRs) (MLH1, MSH2, MSH6, and PMS2) is to identify errors in DNA base insertion or deletion and subsequently repair them (150). A deficiency in MMRs leads to the repetition of microsatellite sequences. Microsatellites are sequences of approximately 6 base pairs present in repetitive patterns throughout genomic DNA. A tumor with widespread deficient MMR in the genome is termed MSI-high, which is the case in roughly 2% of PDAC tumors (151). MSI-high tumors have higher expression of mutation-associated neoantigens. This promotes the immune system to recognize cancer cells, leading to a higher sensitivity to immune checkpoint inhibitors (152). Some research has linked microsatellite instability in PDAC to a substantially longer survival compared to patients without mismatch repair deficiency (62 months vs. 10 months) (153). Additionally, the survival of patients with metastatic PDAC with mismatch repair gene mutations appears promising, with an mOS of up to 16.5 months (154). However, some other research has indicated a less impressive effect, reporting overall survival rates of 35.1 months vs. 29.2 months and median disease-free survival rates of 21.4 months vs. 15.6 months (155). Furthermore, an analysis of 78 PDAC tumors indicated that the MMRs of MLH1, MSH2, MSH6, and PMS2 expression were not significantly associated with shorter survival (156).

5.6.2.7 Osteopontin

The phosphoprotein osteopontin is typically synthesized by macrophages, osteoblasts, endothelial cells, and smooth vascular muscle cells. It is associated with the extracellular matrix and is mainly found in bodily secretions (157,158). High levels of osteopontin has been demonstrated in PDAC patients compared with healthy controls, which may help in detecting PDAC (157). Furthermore, high values of osteopontin (> 150 ng/ml) in patients with PDAC has been linked to higher mortality (159).

5.6.2.8 Immune response and inflammatory markers

The inflammatory response is integral to PDAC carcinogenesis and the antitumor response. PDAC is associated with changes in clinical laboratory values. Three examples are the neutrophil-lymphocyte ratio, the levels of C-reactive protein, and the platelet-lymphocyte ratio, all of which have been linked to shorter survival in PDAC patients (160–162). Interestingly, a the neutrophil-lymphocyte ratio and platelet-lymphocyte ratio have also been linked to R0 resectability (163).

5.6.2.9 Circulating tumor cells

Circulating tumor cells (CTCs) are tumor cells released into free circulation in the bloodstream. CTCs have been identified in the blood of patients with both localized,

locally advanced, and metastatic PDAC (164). The detection of CTCs in blood has been linked to a substantially worse prognosis in PDAC patients compared to the absence of CTCs (165). Furthermore, CTCs could have implications as a diagnostic tool, as a specificity of 83% and sensitivity of 74% have been shown (166). However, the isolation of CTCs is challenging, and methodologies are highly varied, limiting their applicability (164).

5.7. TUMOR-AGNOSTIC OR TUMOR-INFORMED

There are two main methodologies to examining ctDNA – the tumor-informed approach and the tumor-agnostic approach; this thesis mainly addresses the latter. Both approaches have several strengths and limitations, to be discussed in the following. The former approach necessitates an upfront genomic analysis of tumor tissue to identify alterations specific to the individual tumor. Based on the detected alterations, an assay can then be tailored to identify these variants in the blood (120,167–169). This has become an intense area of study, with potentially practice-changing implications to detect minimal residual disease. It has been shown that colon cancers recur quickly, often within a year, if ctDNA is not entirely cleared after curative surgery (120). Furthermore, this methodology could detect recurrence earlier, potentially more than 8 months before radiologic imaging (120). ctDNA-guided treatment is no worse than the current standard of care in predicting whether patients require additional chemotherapy to clear their remaining cancer or whether it might be possible to deescalate and avoid chemotherapy (170).

However, there are also some limitations to this approach. The main strength of the methodology in analyzing tumor tissue is also a limitation. Tissue samples of poor quality or low tumor cellularity or samples that yield low amounts of DNA may render sequencing impossible. In a study examining more than 11,000 samples, this was the case in approximately 9% of cases (171). Furthermore, many variants detected by sequencing are novel, and thus, their prognostic impact is unknown (171).

Additionally, tissue availability is a limitation, which may continually worsen. Considering the increasing use of neoadjuvant chemotherapy in many cancers, acquiring sufficient for molecular analysis may become a challenge. This problem is particularly pronounced in PDAC, which often consists of small, difficult-to-access tumors with substantial stroma. Furthermore, in patients with metastatic disease, the available tissue is often limited to only biopsy, further limiting available tissue for molecular profiling. The tumor-informed approach may also be limited by the intratumoral heterogeneity in metastatic disease (172). This could be partially alleviated by biopsy of multiple locations and repeated biopsy of the primary tumor site, but this is impractical and associated with substantial risks for the patient.

In contrast, the tumor-agnostic approach, or liquid biopsies, is easily repeatable and minimally invasive, which could enable dynamic monitoring of the cancer

environment. Additionally, since ctDNA is shed from all cancer sites throughout the body, it could confer a more complete snapshot of the malignancy (173,174). Reports regarding concordance between tissue and liquid biopsy vary. Some report concordance to be high, being mostly concordant but with 6-25% of variants found exclusively in one test (175,176). Additionally, the timing of samples is essential, as one study reported a concordance of 75-78% between paired tissue and liquid biopsy samples when tests were less than 7 days apart, decreasing to 50-52% when tests were more than 365 days apart (176). However, another study demonstrated that clinically relevant alterations are detectable in ctDNA in almost 80% of cases where ctDNA was absent in the matched postprogression tumor biopsy (177). This could indicate that the liquid biopsy approach has several advantages compared to traditional tissue biopsy in the metastatic setting.

However, there are of course limitations associated to liquid biopsies. The main limitation of liquid biopsies is that low concentrations of ctDNA, or dilution of ctDNA by abnormally high cfDNA values, can limit detection (75,178). This is a substantial limitation in early detection but less so in metastatic disease, where ctDNA is often present in high quantities (85).

Another limitation of liquid biopsies is clonal hematopoiesis, a normal age-associated process where somatic mutations occur in hematopoietic stem cells. An accumulation of these mutations in stem cells can confer the mutations to blood cells (179). The genes DNMT3A, JAK2, ASXL1, and TET2 are commonly mutated in this manner (180). This could cause false-positive results of a liquid biopsy.

Finally, being exclusively reliant on the blood emphasizes the importance of a consistent pipeline for collecting, storing, and processing samples. Minor protocol deviations can cause massive differences in results (181).

5.8. SFRP1 AND CHEMORESISTANCE

The exact mechanisms of how phSFRP1 leads to a poor prognosis are unclear. As mentioned in this thesis, reduced SFRP1 expression in tumor tissue has been linked to poor prognosis in several cancers. A more aggressive tumor subtype associated with phSFRP1 could explain the poor prognosis. However, another explanation could be based on a higher degree chemoresistance conferred by phSFRP1 in these patients. Both activation of the Wnt/ß-catenin pathway and phSFRP1 has previously been linked to chemoresistance (182–184). The results from studies I-III indicate that phSFRP1 does not appear to impact prognosis among patients treated with only BSC. This could support the hypothesis that the observed poor prognosis is at least partly explained by a reduced sensitivity to chemotherapy. If true, this could be an indication that phSFRP1 could potentially be a predictive biomarker, as explored in studies II-III. However, it is possible that the patients treated with BSC are simply in such a poor

physical condition that prognostic markers are ineffective in assessing prognosis in these patients appropriately.

Regardless of whether phSFRP1 is prognostic, predictive, or both, these patients have an abysmal prognosis. Further research is required to ascertain the mechanisms of action and determine which way to progress.

5.9. POTENTIAL CLINICAL UTILITY

5.9.1. AS A PROGNOSTIC BIOMARKER

As shown throughout this thesis, phSFRP1 is a robust prognostic biomarker. Furthermore, studies II and IV indicate that this biomarker substantially increases performance in predicting mortality compared to usual clinical factors and CA 19-9.

Based on the results presented here, the prognosis of PDAC patients with phSFRP1 is incredibly poor. Specifically, study IV suggests the mOS of patients with phSFRP1^{high} to be approximately 3 months. While these patients had a worse PS than patients with umSFRP1 or phSFRP1^{low}, they were also significantly younger. Comparatively, the survival of PDAC patients treated with only BSC is very short. Recent studies estimate the mOS to be between 1.1 and 2.6 months (45,132–134). Of course, these patients are heavily selected, as patients of good PS and without comorbidities are generally treated with single or combination chemotherapies. Naturally, patients treated with only BSC are generally unfit for chemotherapy, being older, frailer, and having substantial comorbidities. Historical data suggest that before effective chemotherapies for PDAC, the mOS of patients treated with BSC is approximately 3 months (12,185,186).

With that in mind, the survival of chemotherapy-treated patients with phSFRP1^{high} may only be marginally longer than those treated with only BSC. The short survival among these patients raises the question of how much this subgroup of patients truly benefits from chemotherapy.

However, this discussion is not straightforward. In addition to an expected improvement in survival, treatment with chemotherapy is also associated with both stabilization of health-related quality of life and improved management of pain (187,188). Thus, treatment with chemotherapy could be indicated, even if it only results in marginal improvements in survival. However, achieving pain management and stable quality of life depends on disease control and is closely correlated with improvements in survival (187). Thus, it remains to be seen how well quality of life and pain would be managed in this subgroup of patients where treatment with chemotherapy does not markedly improve survival.

Based on this, some patients could be better off with early referral to a palliative team and intensive palliative care to optimize their quality of life in their remaining time.

The blood-based analysis of phSFRP1 is fast, inexpensive, and relatively easy to implement. Thus, it could quickly become an additional clinically useful prognostic tool. However, this decision cannot be made exclusively based on this biomarker. This biomarker could be a tool to aid the clinician and the patient in making a joint decision about what treatment option is the best choice for the individual.

5.9.2. AS A PREDICTIVE BIOMARKER AND TREATMENT TARGET

Several aspects of phSFRP1 are potentially exploitable, making it a prime choice for developing targeted treatment options. Additionally, alterations in SFRP1 expression could confer sensitivity to certain types of treatment.

One example is EGFR2-negative, HR-positive metastatic breast cancer with alterations in SFRP1. A recent study have linked treatment with the combination of cyclin-dependent kinase (CDK) 4/6 inhibitors and endocrine therapy to improved overall survival in this disease (189). Interestingly, the potential utility of CDK 4/6 inhibitors for PDAC is also being investigated in several clinical trials (190). Current research indicates that CDK4/6 inhibitors could have clinical benefits in PDAC as maintenance therapy after initial chemotherapy or as part of a combination treatment (190). A recent study established that patients with SFRP1 alterations in ctDNA had increased sensitivity to the CDK4/6 inhibitor ribociclib (191). Thus, SFRP1 could be a guide for personalized treatment with CDK4/6 inhibitors.

Additionally, as mentioned previously in this thesis, promoter hypermethylation of a gene is an epigenetic alteration. These are, by definition, inherently changeable and thus potentially reversible. Hypomethylating treatments already exist, although they are primarily used in hematological cancers (192). However, several clinical trials have examined a possible role for these treatments in PDAC (193). Reactivation of SFRP1 has been linked to resensitization to certain chemotherapies in lung cancer and acute myeloid leukemia (183,194). Thus, a phSFRP1-guided approach to epigenetic therapies could be feasible.

However, most demethylating drugs are currently globally demethylating, which can be cause for concern, as they could also demethylate latent oncogenes and worsen prognosis (192,195). Additionally, this approach may be limited by resistance to hypomethylating treatments of certain promoter regions (196).

Another approach is a recent concept of tumor “mimetics” (197). As the function of SFRP1 is through a secreted protein, a drug or molecule could be identified to replicate its functions. Thus, these findings could be replicated in patients with nonfunctioning SFRP1. This concept was explored for SFRP1 in a recent study by

Dahl. et al. (197). They screened almost 200,000 compounds to identify mimetic leads which inhibited the Wnt/β-catenin pathway. They successfully identified a mimetic lead that downregulates the phosphorylated LRP6 receptor (197). While this is still a new concept, it remains very promising.

CHAPTER 6. CONCLUSION

In summary, the collective findings of the four studies highlight the potential role of phSFRP1 as both a prognostic and potentially also predictive biomarker for PDAC. While distinct in the populations and treatment regimens, the studies combined elucidated the clinical significance of phSFRP1 in PDAC prognosis and therapeutic response. Furthermore, these studies implicate SFRP1 as a prime target for targeted treatments.

The initial study was a combined discovery study with an external validation cohort, examining and validating the prognostic utility of phSFRP1 in cfDNA in stage IV PDAC patients receiving gemcitabine. The subsequent two studies expanded the horizon to include more diverse patient cohorts and treatment regimens. Finally, study IV used newer, optimized methodologies in a larger cohort of patients. This study further validated the findings and determined the prognostic effects of phSFRP1 to be dependent on allele fraction.

These investigations underscore the robustness of the observed associations between phSFRP1 and patient outcomes in stage IV patients regardless of chemotherapy treatment. Notably, the findings also indicate that the survival of patients with phSFRP1 who receive chemotherapy is only marginally improved in contrast with patients receiving only BSC. This could indicate that patients with phSFRP1 are more resistant to chemotherapy.

The results were less conclusive in patients with lower stages. While a significant effect on survival was observed in patients with localized disease, the frequency of phSFRP1 detection was only approximately 20%. This was relatively low compared to the 45-65% seen in stage IV and approximately 70% of PDAC tumors. It is uncertain whether lower-stage tumors shed less phSFRP1, do not contain phSFRP1, or analysis of more material is required. Additional investigations are required to determine this.

In summary, the studies of this thesis underscore the importance of phSFRP1 as a promising biomarker for the personalized management of PDAC. The knowledge gained from these investigations could impact treatment decisions and the pursuit of novel therapeutic avenues.

CHAPTER 7. PERSPECTIVES

PDAC is notorious for its late diagnosis, limited treatment options, and limited efficacy of said treatments. Furthermore, while the survival of patients with PDAC has improved moderately over past decades, the it remains among the worst of all cancer types. Combined with the increasing incidence, PDAC has been estimated to become the second most common cause of cancer related death on a global scale within a few years.

Fortunately, with the advent of personalized medicine, the field of cancer research has and is undergoing a transformation. As novel targeted therapeutic treatments are gradually being developed, the traditional one-size-fits-all treatment approach is slowly receding. This is already benefitting a few (unfortunately rather small) subgroups of patients with PDAC. Hopefully, the continuation of these efforts will gradually increase the number of patients with PDAC for whom better, targeted treatments for their disease are available.

The field of epigenetics is a critical part of this puzzle. The stereotypical mutations in KRAS, TP53, SMAD4, and CDKN2A have long been known associated with the development of PDAC. However, these genetic alterations do not explain the complex heterogeneity of PDAC disease progression or the response to treatment. It is becoming increasingly clear that epigenetic alterations play a role in not only the progression of cancer, but also the response to treatment, and its capability for metastasis (193). Furthermore, the blood-based assessment has substantial advantages compared to traditional tissue biopsy.

Our studies have shown that phSFRP1 is potentially a clinically relevant prognostic biomarker. The value is further increased as results point towards an additional prognostic effect when combined with known prognostic markers. The discovery and thorough research of specific epigenetic alterations are of the utmost importance as they are reversible, unlike genetic alterations. This provides possible treatment targets for various treatments, either seeking to reverse the methylation or mimic the effects of downregulated proteins. Additional investigation is required to establish the clinical benefit of this biomarker.

CHAPTER 8. LITERATURE LIST

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